Arrhythmogenic cardiomyopathy beyond monogenetic disease

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General introduction and outline of this thesis

GENERAL INTRODUCTION

Cardiomyopathies

The term cardiomyopathy literally translates to "heart muscle disease". The initial term was proposed to describe rare heart muscle diseases which were not due to coronary artery disease.[1] Later, the term was used to classify disorders of the heart muscle with no known cause.[2] This was adopted by the World Health Organization (WHO) and the International Society and Federation of Cardiology (ISFC). Additionally they introduced a new term "specific heart muscle disease" to indicate heart muscle diseases with a known cause.[3] This distinction was discarded in the next classification, which was based on the dominant pathophysiology, and when possible on etiological factors.[4] Subsequently heart muscle disease caused by coronary artery disease, or systemic hypertension, would also fall under the definition of cardiomyopathy, and be categorized as ischemic or hypertensive cardiomyopathy respectively.[4] In 2006, 10 years later, in the scientific statement of the American Heart Association (AHA)[5] cardiomyopathies were defined as:

"… a heterogeneous group of diseases of the myocardium associated with mechanical and/or electrical dysfunction that usually (but not invariably) exhibit inappropriate ventricular hypertrophy or dilatation and are due to a variety of causes that frequently are genetic. Cardiomyopathies either are confined to the heart or are part of generalized systemic disorders, often leading to cardiovascular death or progressive heart failure-related disability."

The first major distinction this AHA classification made were between primary cardiomyopathies, which are confined to the heart muscle itself, and heart muscle disease as part of generalized systemic disorders, which they called secondary cardiomyopathies. The primary cardiomyopathies were then subdivided in a genetic, mixed, and acquired group. With this new classification system the first steps were made in classification of cardiomyopathies according to their genetic basis. Another important notion was that heart muscle disease caused by other cardiovascular abnormalities, such as coronary artery disease, valvular disease, hypertension, and congenital heart disease should not fall under the definition of cardiomyopathy. This has also been incorporated in the 2008 position statement of the European Society of Cardiology (ESC), in which cardiomyopathy was defined as:

"*A myocardial disorder in which the heart muscle is structurally and functionally abnormal, in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease sufficient to cause the observed myocardial abnormality*"

The classification system proposed by the ESC is based on the specific morphological and functional phenotypes, as they present to the attending physician usually during cardiac

imaging (echocardiography). After this distinction, the phenotypes are classified into familial/genetic and non-familial/non-genetic phenotypes.[6] An overview of the AHA (2006) and ESC (2008) classifications systems is provided in **Figure 1**.

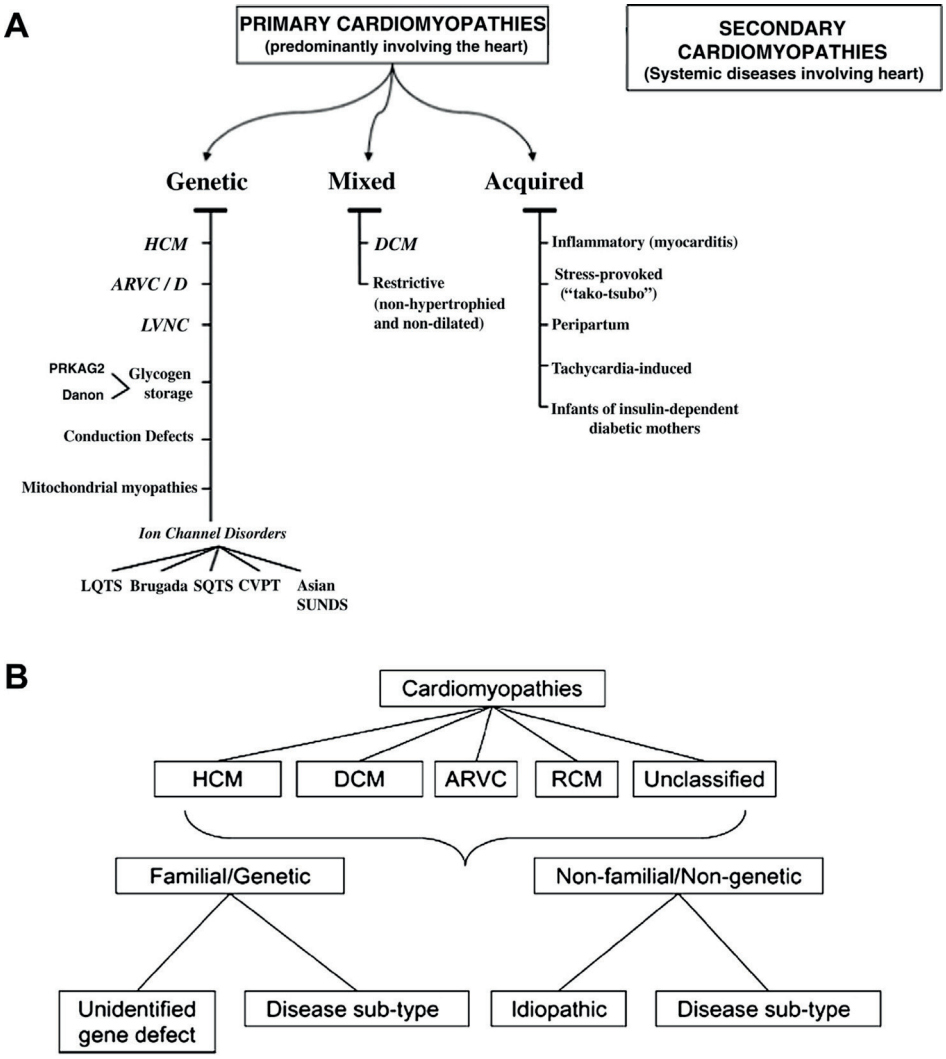

Figure 1. Overview of the (A) American Heart Association[5] and (B) European Society of Cardiology[6] classification systems. ARVC/D = arrhythmogenic right ventricular cardiomyopathy/dysplasia, DCM = dilated cardiomyopathy, HCM = hypertrophic cardiomyopathy, LVNC = left ventricular noncompaction cardiomyopathy, RCM = restricted cardiomyopathy. Adapted from Maron et al.[5] and Elliott et al.[6]

Various cardiomyopathy subtypes are recognized. The most frequently found subtypes of cardiomyopathy are hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) and arrhythmogenic cardiomyopathy (ACM), (**Figure 2**). Hypertrophic cardiomyopathy is

characterized by thickening of the heart muscle due to presence of hypertrophy or due to the intracellular accumulation or interstitial infiltration of metabolites. Dilatation and an impaired left ventriculair function are the hallmarks of dilated cardiomyopathy. In arrhythmogenic cardiomyopathy there is fibrofatty replacement of the right and/or left ventricular myocardium.[6] The term arrhythmogenic cardiomyopathy was recently posed in the 2019 Heart Rhythm Society consensus statement and encompasses different forms.[7] The form with mainly right ventricular involvement is called arrhythmogenic right ventricular cardiomyopathy (ARVC), predominant left ventricular involvement is referred to as arrhythmogenic left ventricular cardiomyopathy (ALVC) and in the case of equal affected ventricles it is termed biventricular arrhythmogenic cardiomyopathy.[7]

The familial character of cardiomyopathies is recognized in both the AHA and the ESC classification systems. Cardiomyopathies are frequently characterized by their familial occurrence. For instance systematic familial evaluation of 60 patients with dilated cardiomyopathy (DCM) of unknown origin showed that in more than half of the families there were two or more family members with DCM.[8] Familial DCM has been estimated to occur in 20% to 50% of the cases with idiopathic DCM.[9] In a large cohort of hypertrophic cardiomyopathy (HCM) probands cardiologic screening of relatives demonstrated HCM in 30% at first screening and 16% developed HCM during a follow-up of seven years.[10] With regard to arrhythmogenic cardiomyopathy (ACM), family clustering is also reported. By investigating family members of nine families in which cases of juvenile sudden cardiac death due to ACM occurred they demonstrated that in eight out of nine families at least two members also had signs of disease.[11]

The mode of inheritance in cardiomyopathies is most often autosomal dominant **(Figure 3)**. This means that there is a genetic variant in one of two copies of a gene (excluding the genes which reside on the sex chromosomes) causing the observed disease, i.e. cardiomyopathy. Such a genetic variant was identified for the first time in hypertrophic cardiomyopathy in 1990 on one of the copies of the Myosin Heavy Chain 7 (*MYH7*) gene in a large family. This gene encodes for the protein β-myosin heavy chain and is a part of a larger protein complex called type II myosin, which plays a major role in cardiac muscle contraction. The variant co-segregated with hypertrophic cardiomyopathy in an autosomal dominant pattern in a multigenerational pedigree.[12]

Currently many genes are known to be implicated in the pathophysiology of the different inherited cardiomyopathies (**Figure 4**). Notably, this relates to cardiomyopathies with adult onset of disease; concerning pediatric cardiomyopathy a larger subset of genes are putatively associated with disease. There are also genes which are implicated in more than one subtype of cardiomyopathy. This phenomenon, a single gene causing different phenotypes, is called

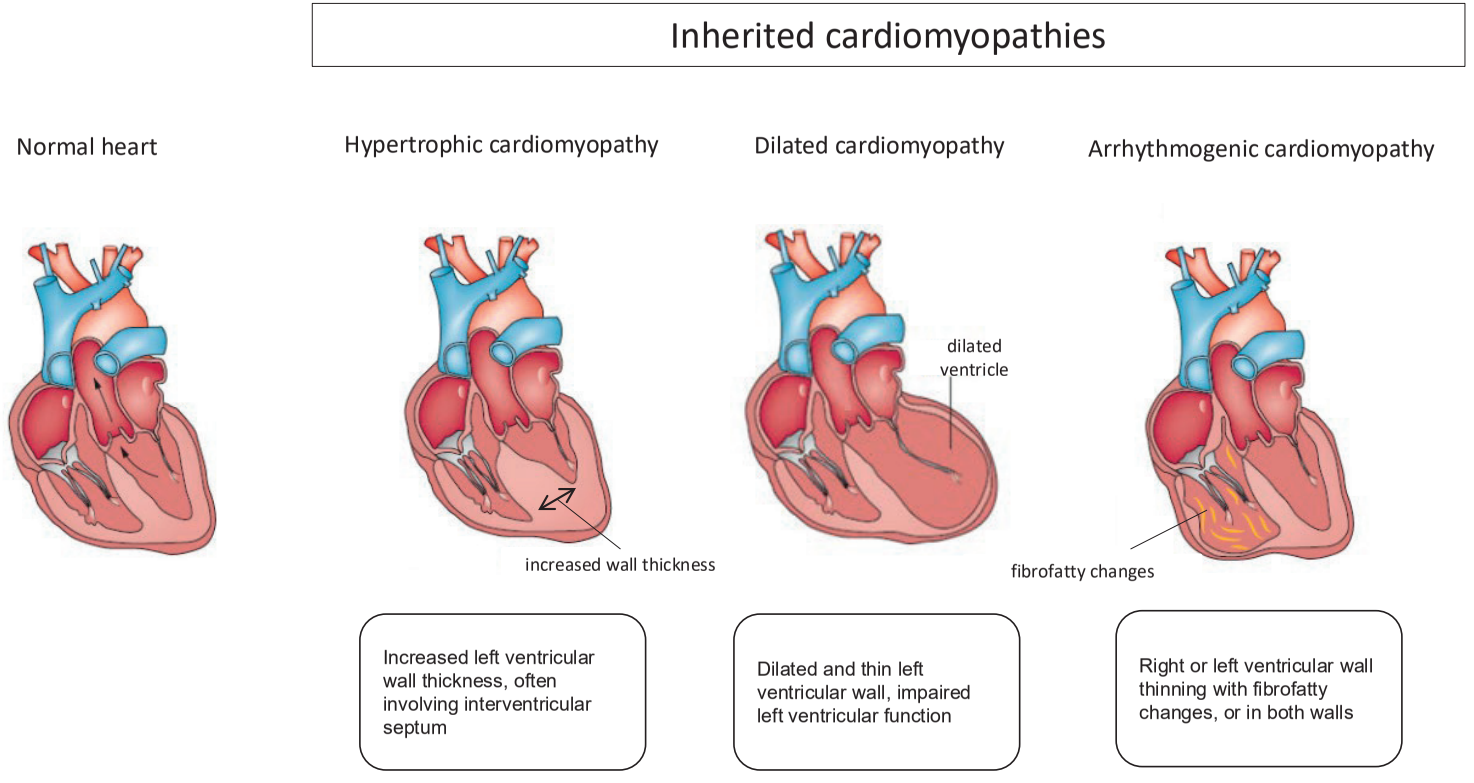

Figure 2. Overview of the most frequent occurring cardiomyopathies. Figure adapted from Wilde et al.[13]

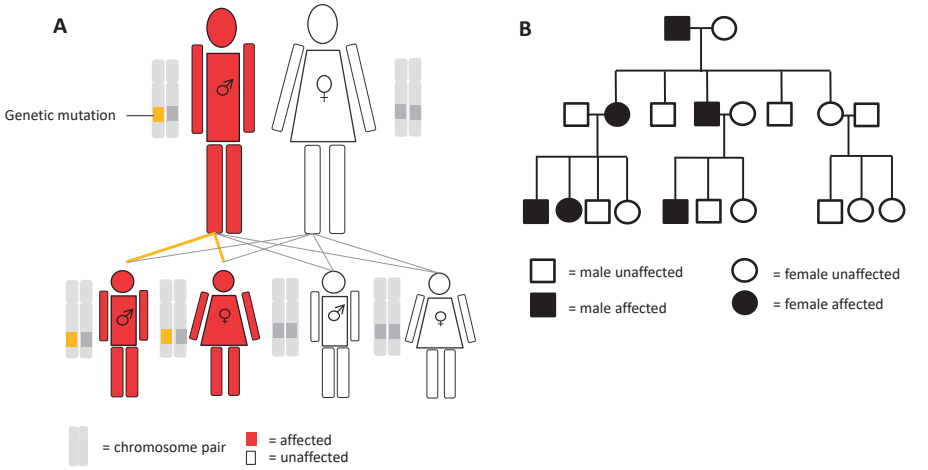

Figure 3. Autosomal dominant inheritance pattern. A) every person carries two copies of a gene and transmits one of those copies to the offspring. One of the two copies being mutated is enough for disease development. B) example of an autosomal dominant inheritance pattern in a three generation pedigree.

pleiotropy. For example the clinical phenotype of genetic variants in Plakophilin 2 *(PKP2)* can range from a pure structural phenotype (e.g. dilatation of the right ventricle) to a pure arrhythmogenic clinical phenotype with only electrical abnormalities being present.[14] The phenotypic variability described from a clinical perspective within families or patients with the same genetic variant is also called variable expression. Some carriers for example will have a very severe disease with the need for a heart transplant at a young age, while other carriers of the same variant will have minimal or no symptoms during their entire life.

Inherited cardiomyopathies are also characterized by incomplete and age-related penetrance. Incomplete penetrance refers to the phenomenon that only a certain proportion of individuals carrying a genetic pathogenic variant associated with a disease will actually develop the disease. Age-related means that this proportion of affected carriers increases over time/with age.

The model in which one genetic variant is responsible for the disease is not sufficient in explaining the incomplete penetrance and variable expression by itself. Additional factors are likely to play a role. These additional factors can vary from endogenous factors, like the inheritance of additional genetic variants ("genetic modifiers"), to exogenous factors, which include e.g. viral agents or consummation of alcohol. This more complex aetiology will be discussed in the next paragraph in further depth.

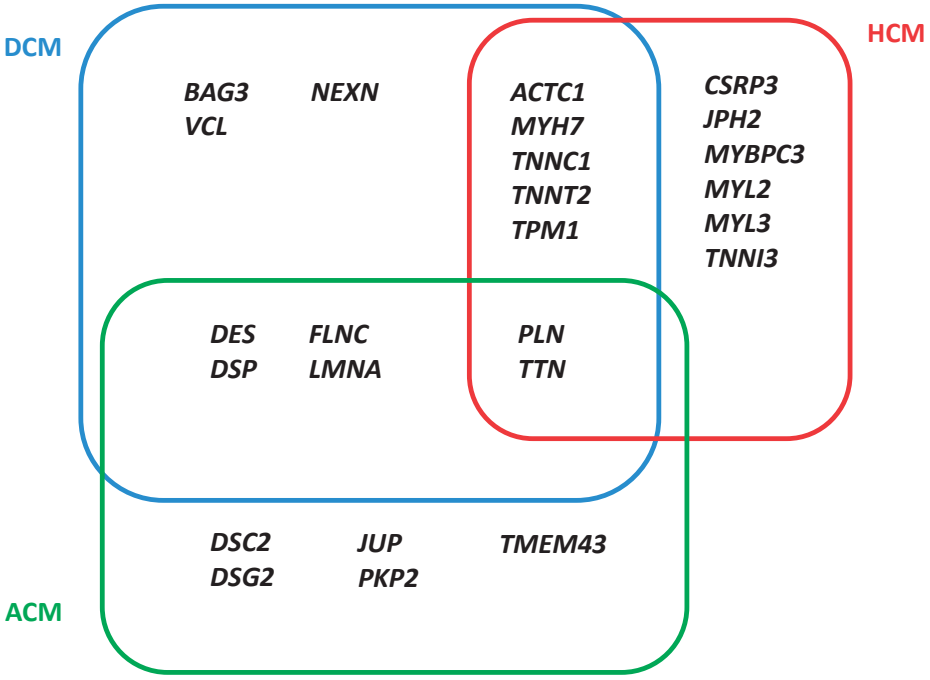

Figure 4. Genes identified to be associated with different subtypes of cardiomyopathy. The same gene can be associated with more than one subtype (pleiotropy). Adapted from van Spaendonck et al.[15]. The genes and the associated phenotypes in this figure are adapted from the HCM and ARVC Gene Curation Expert Panels,[16,17] a recent paper describing the genetic contribution in DCM[18], and a review regarding ACM.[19] ACM = arrhythmogenic cardiomyopathy, DCM = dilated cardiomyopathy, HCM = hypertrophic cardiomyopathy. Genes: *ACTC1* (Actin Alpha Cardiac Muscle 1), *BAG3* (BAG Cochaperone 3), *CSRP3* (Cysteine And Glycine Rich Protein 3), *DES* (desmin), *DMD* (dystrophin), *DSG2* (desmoglein-2), *DSC2* (desmocollin-2), *DSP* (desmoplakin), *EMD* (emerin), *FHL1* (Four And A Half LIM Domains 1), *FHOD3* (Formin Homology 2 Domain Containing 3), *FLNC* (Filamin C), *JUP* (junctional plakoglobin), *LMNA* (lamin A/C), *MYBPC3* (myosin-binding protein C), *MYH7* (β-myosin heavy chain), *MYL2* (regulatory myosin light chain), *MYL3* (essential myosin light chain), *NEXN* (Nexilin F-Actin Binding Protein), *PKP2* (plakophilin-2), *PLN* (phospholamban), *RBM20* (RNA Binding Motif Protein 20), *SCN5A* (cardiac sodium channel), *TMEM43* (Transmembrane Protein 43), *TNNC1* (Troponin C1), *TNNI3* (cardiac troponin I), *TNNT2* (cardiac tropinin T), *TPM1* (α-tropomyosin), *TTN* (titin), *VCL* (metavinculin).

To capture and define in a more standardized way the heterogeneous nature of this group of diseases a new nomenclature has recently been proposed called "The MOGE(S) Nomenclature", in which five features of the disease are described.[20] The morphofunctional characteristic (M), organ involvement (O), genetic or familial inheritance pattern (G), and an explicit etiological annotation (E) with details of genetic defect or underlying disease/cause and information about the functional status (S) (**Figure 5**). For example a patient carrying a pathogenic genetic variant in desmoplakin (*DSP*), who has dilated cardiomyopathy with a functional impairment (falling in category IV of the New York Heart Association classification) and has dermatologic abnormalities would be described as follows "$M_DO_{H+C}G_{AD}E_{g-}$

DSP[p.Gln250Profs*7]$_{SNU-IV}$". A major advantage of this relatively new classification system is that it is flexible and can be expanded, which makes it possible to incorporate future discoveries regarding the aetiology and manifestation of the various cardiomyopathic diseases.

This long history of classifications and definitions of cardiomyopathy reflects the continuing increase in knowledge regarding cardiomyopathies, which will be briefly discussed with regard to arrhythmogenic cardiomyopathy in the following paragraph, the subtype on which this thesis is focused.

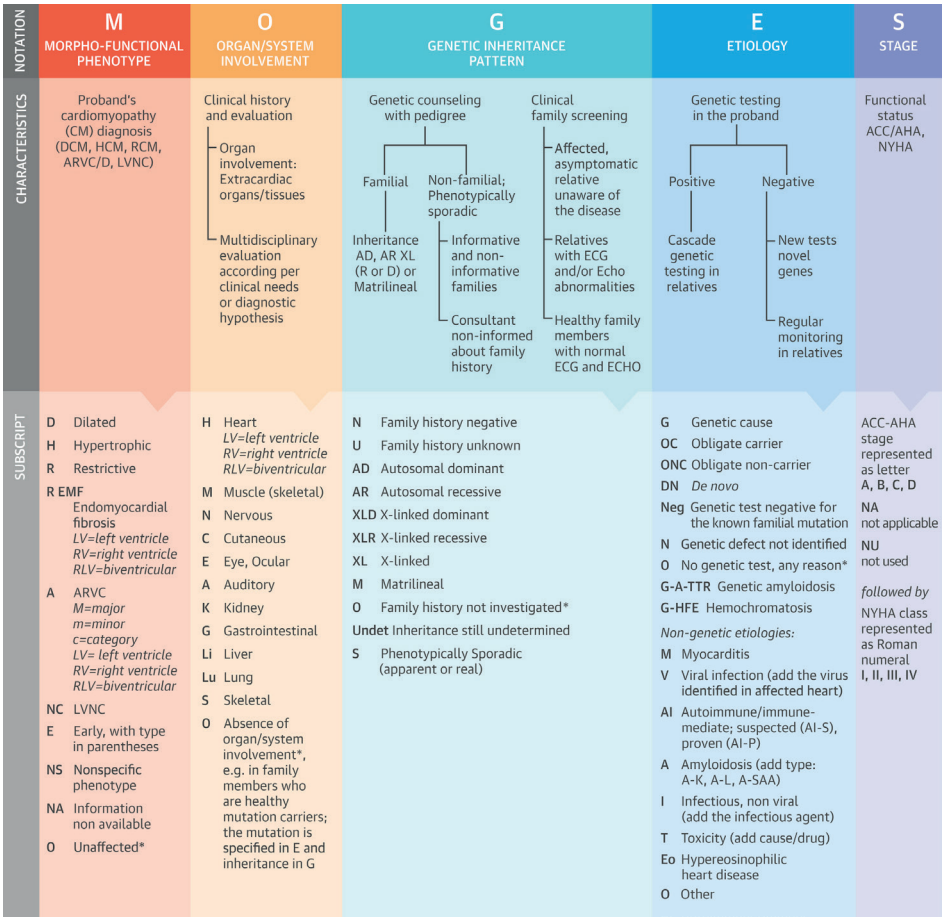

Figure 5. The MOGE(S) Nomenclature, adapted from Arbustini et al.[20]. ACC = American College of Cardiology, AHA = American Heart Association, ARVC/D = arrhythmogenic right ventricular cardiomyopathy/dysplasia, DCM = dilated cardiomyopathy, ECG = electrocardiogram, Echo = echocardiogram; HCM = hypertrophic cardiomyopathy. LVNC = left ventricular noncompaction, NYHA = New York Heart Association, RCM = restrictive cardiomyopathy.

Arrhythmogenic cardiomyopathy (ACM)

Arrhythmogenic cardiomyopathy is a disease that is characterized by progressive fibrofatty replacement of the heart muscle of the ventricles.[19]

The first description of familial arrhythmogenic right ventriular cardiomyopathy (ARVC), the best defined form of ACM, was published in 1736 in the book titled "*De Motu Cordis et Aneurysmatibus, Caput V, De Hereditaria ad Cordis Aneurysmata Constitutione: De Cordis Prolapse*"[21], which is translated to[22] "*On the Motion of the Heart and on Aneurysms, chapter 5, hereditary predisposition to cardiac aneurysma and bulgings"*. A four generation family is described to have symptoms of palpitations, heart failure, sudden death and dilatation and aneurysms of the right ventricle of the heart.

In 1982 a series of 24 adult cases with arrhythmogenic right ventriular cardiomyopathy and 10 adult cases from a literature review were published by Marcus et al.[23] The term arrhythmogenic right ventricular dysplasia (ARVD) was coined to describe this condition, the clinical features consisting of a ventricular tachycardia with left bundle branch block configuration and inverted T waves in the right precordial electrocardiogram (ECG) leads. Morphologic abnormalities consisted of an enlarged right ventricle and right ventricular aneurysms. The myocardium was replaced by fatty tissue and intramyocardial fibrosis. It was thought that these changes of the right ventricle originated from a developmental defect, hence the use of the terminology "dysplasia".

However, arrhythmogenic right ventricular dysplasia is not a congenital abnormality, but a cardiomyopathy which develops over time.[24–26] The term arrhythmogenic right ventricular cardiomyopathy (ARVC) was introduced and proposed in the 1995 cardiomyopathy nomenclature and classification of the World Health Organization.[27]

The diagnosis of arrhythmogenic right ventriucular cardiomyopathy (ARVC) is based on the revised 2010 Task Force Criteria.[28] There are six categories, including functional and structural findings, histology, repolarization and depolarization abnormalities, presence of arrhythmia and family history. If a patient fulfils two major criteria or four minor criteria from different categories the diagnosis of ARVC is made. The necessity of these six different categories delineate the wide clinical variable expression of ARVC. While the initial term i.e. ARVC describes it to be a disease of the right ventricle, involvement of the left ventricle has been observed for the first time in a pathological study in 14 out of 30 ARVC hearts.[24] Another study showed that in a cohort consisting of 200 probands and relatives, fulfilling the modified diagnostic criteria for ARVC at that time, more than 80% had signs suggestive of left ventricular involvement.[29] Expression of the disease was categorized according to the pattern of ventricular involvement, which led to three definitions under the umbrella of

arrhythmogenic cardiomyopathy (ACM): 1) the classical arrhythmogenic right ventricular cardiomyopathy (ARVC) with isolated right ventricular disease or in combination with mild left ventricular involvement, 2) left-dominant arrhythmogenic cardiomyopathy (also referred to as ALVC), with early and pronounced left ventricular involvement, with no or relatively mild disease of the right ventricle, 3) and a biventricular subtype, which is characterized by involvement of both ventricles in a parallel manner.[29]

The broader term arrhythmogenic cardiomyopathy (ACM) encompasses these three forms with predisposition to arrhythmia and fibrofatty myocardial replacement being the denominators in all three.[30]

Arrhythmogenic cardiomyopathy is often a genetic disease with genetic variants associated with the disease found in more than 50% of the patients.[31] Nowadays new DNA-sequencing technologies implemented in routine patient care allow for screening of a multitude of genes at a low cost. Subsequently an increasing number of genetic variants are being identified. One of the major challenges currently is to correctly interpret these variants, since many of the variants are not disease causing.[32]

Like cardiomyopathies in general, arrhythmogenic cardiomyopathy (ACM) often follows an autosomal dominant inheritance pattern. According to this inheritance pattern, a single pathogenic genetic variant causes the disease. However, studies have shown that there is significant incomplete penetrance in individuals carrying a genetic variant associated with ACM.[33,34] This implicates that other factors are likely to contribute to disease expression, such as other genetic variants. These are likely to play a role in disease expression. Carrying more than one genetic variant associated with ACM has been described to increase penetrance and appears to be an important risk factor for life threatening arrhythmia, like sudden cardiac death.[35] Such more complex inheritance is also observed for hypertrophic cardiomyopathy (HCM), where carrying more than one pathogenic genetic variant is associated with greater disease severity.[36] A more complex inheritance pattern is also reported in dilated cardiomyopathy (DCM), where families with evidence of bilineal inheritance reflecting a multigenic cause in DCM were reported.[37]

A clear autosomal dominant inheritance pattern of the disease indicates that there is a pathogenic genetic variant with a large effect, i.e. sufficient to cause the disease in every carrier. The relationship between the effect size and the occurrences of genetic variants is depicted in **Figure 6A**. Only very rare variants, shown at the left side of the figure, are likely to have large effect sizes. If the effect size of the genetic variant is large enough to reach the threshold of disease expression by itself, depicted by the most left bar in **Figure 6B,** it is a truly monogenetic Mendelian disease. However age-related incomplete penetrance, variable

expression and evidence of additional genetic modifiers playing a role indicates the presence of a more complex genotype underlying the disease (as indicated by the other two bars in **Figure 6B**) and that a truly 100% Mendelian familial cardiomyopathy is likely to never exist since there will always be some variability between carriers.

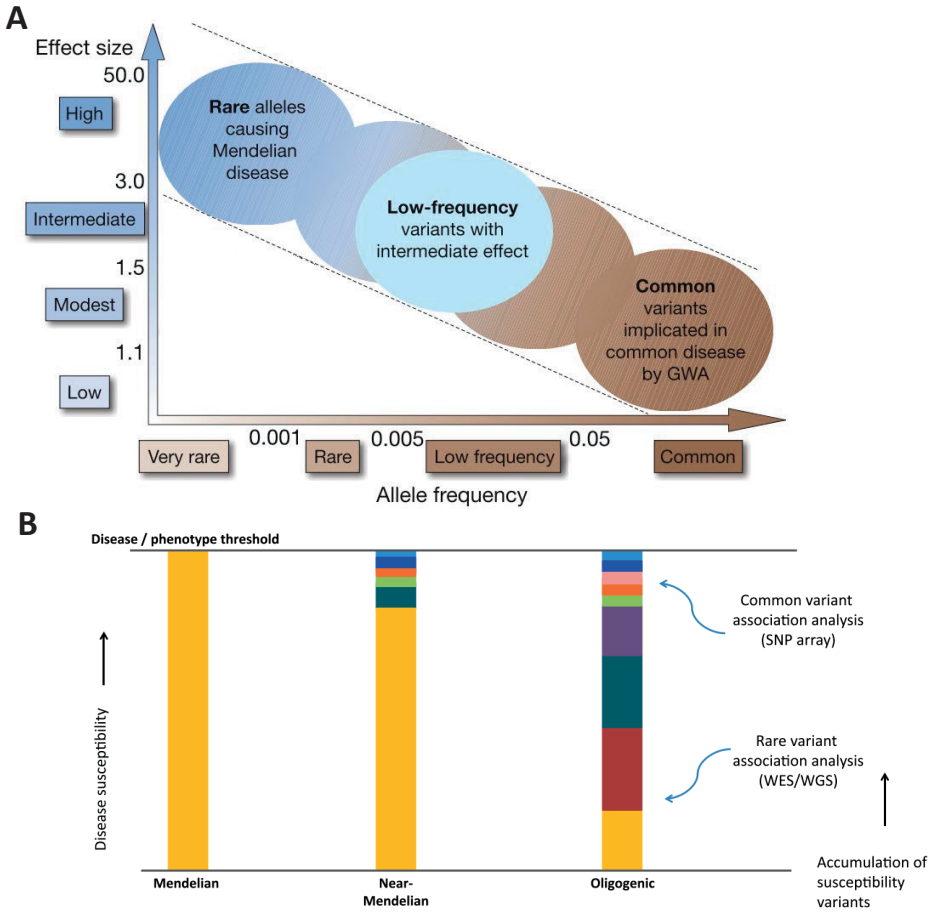

Figure 6. Variant effect size and disease development. (A) Relationship of effect size and allele frequency of genetic variants and (B) threshold model of the complexity of genetic architecture involved in inherited cardiac disease development. A specific coloured bar represents a genetic variant, where a larger bar reflects a larger effect size. GWA = genome wide association, SNP = single nucleotide polymorphism WES = whole exome sequencing, WGS = whole genome sequencing. Figures are adapted from McCarthy et al.[38] and Bezzina et al.[39]

However, also external factors and other internal factors appear to modify disease expression in arrhythmogenic cardiomyopathy (ACM). There is evidence that exercise is an important factor in disease penetrance. For example it has been demonstrated in endurance athletes who have a genetic variant predisposing to ACM have earlier onset of disease, a higher

likelihood of heart failure, and greater risk of arrhythmia.[40,41] Internal factors, like sex hormones, more specifically elevated testosterone levels in males and decreased estradiol levels in females, were independently associated with arrhythmia in arrhythmogenic right ventricular cardiomyopathy patients.[42] The same principle of disease modifiers has previously been shown to apply to other subtypes of cardiomyopathy, and thus appears to be a common feature of this disorder. Here are some examples. Hypertension has been identified to be a major risk factor for the development of hypertrophic cardiomyopathy (HCM) in individuals carrying a certain genetic variant in the *MYL2* gene.[43] In dilated cardiomyopathy (DCM) caused by truncating *TTN* variants excess alcohol intake was associated with more impaired left ventricular function with an absolute reduction of the ejection fraction of 8.7%.[44] Chemotherapy, especially anthracyclines, have cardiotoxic effects and can cause cancer therapy-induced cardiomyopathy (CCM). In a recent study researchers demonstrated that there is an enrichment of truncating variants in the titin gene (*TTN*tv) in cancer therapy-induced cardiomyopathy (CCM) patients and CCM patients with *TTN*tv experienced more heart failure and atrial fibrillation. Experimental models of cardiomyocytes with *TTN*tv were consistent with these data and showed sustained contractile dysfunction compared to wild type.[45] In some cases dilated cardiomyopathy (DCM) is diagnosed shortly after or during the pregnancy and is categorized as peripartum cardiomyopathy (PPCM). In a subset of PPCM patients cardiologic evaluation of first-degree relatives showed undiagnosed DCM and a family history of cardiomyopathy was reported in 16.5% in another cohort of peripartum cardiomyopathy (PPCM) patients.[46,47] In different cohorts with PPCM it was shown that a subset of PPCM patients had the same genetic pathophysiology as patients with DCM, with enrichment of *TTN*tv mutations being common in both groups. [48,49]

Together these findings point to a more complex aetiology of cardiomyopathies in general, including arrhythmogenic cardiomyopathy (ACM), in which more than one factor plays a role in the pathophysiology. By taking both the internal factors (genetic complexity, sex, comorbidities) and external factors (alcohol, chemotherapy, viruses) into account a more complex model than "one genetic variant one disease" emerges. This concept is illustrated in **Figure 7** in which the penetrance of genetic variants in the sarcomere genes for hypertrophic cardiomyopathy (HCM) is represented, depending on internal and external modifiers. The penetrance increases with age and additional factors play a role in the disease expression.[50] Such a model would also better capture the true nature of arrhythmogenic cardiomyopathy (ACM) and eventually lead to more individually tailored care for ACM patients.

Finally, there are families and patients with arrhythmogenic cardiomyopathy (ACM) in which still no genetic variant has been identified. Thirty-seven percent of 439 probands of a combined database of Dutch and US patients did not have an identifiable genetic variant in one of the known genes to be associated with ACM.[51] Identifying novel genes

and genetic variants in those gene-elusive families is of great importance since it has several consequences. For example if a genetic variant is identified it can be used to identify family members at risk. Those family members at risk can be screened by the cardiologist at regular intervals to enable early detection of disease. If disease develops early treatment can be started to possibly prevent worsening of the disease. Identifying novel genes and genetic variants will also provide new insight into the mechanisms underlying ACM. This may in turn aid development of new therapies which specifically target these mechanisms.

Although there are many more mysteries to be resolved with regard to arrhythmogenic cardiomyopathy (ACM) this thesis is focused on the interpretation of genetic variants, improving the understanding of the complexity of ACM and identifying new genetic regions involved in the underlying pathophysiology.

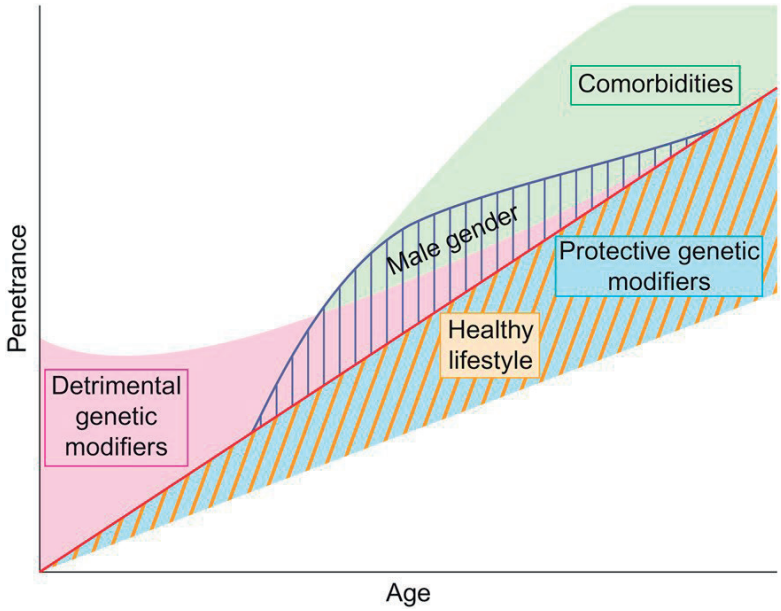

Figure 7. Penetrance of a pathogenic genetic variant in a sarcomere gene associated hypertrophic cardiomyopathy and the impact of various factors on the penetrance. Figure is adapted from Helms et al.[50]

OUTLINE OF THIS THESIS

As an extension to this introduction, **Chapter 1** provides an overview of the current knowledge on arrhythmogenic cardiomyopathy (ACM) with regard to the pathologic substrates, the genetic basis and the underlying pathophysiological mechanisms.

In Part 1 of this thesis the challenges, strategies and importance of the interpretation and characterization of genetic variants are explored. We also touch upon the underlying genotypic complexity of ACM. Two genetic variants, in lamin A/C (*LMNA*) and in titin (*TTN*), are extensively characterized in **Chapter 2** and **Chapter 3**, respectively. In **Chapter 2** novel insights are provided into possible mechanisms of dilated cardiomyopathy (DCM) caused by genetic variants in *LMNA*, whereas in **Chapter 3** we demonstrate that the phenotype of certain *TTN* variants is characterized by (paroxysmal) atrial fibrillation, even in the absence of gross structural and/or functional abnormalities of the heart and that in a substantial part of the DCM patients another external DCM related factor (e.g. alcohol or chemotherapy) was present in addition to the *TTN* variant. In **Chapter 4** the phenotypic spectrum associated with carrying genetic truncating variants in Desmoplakin (*DSPtv*) is described. Thanks to a large collaborative effort it was possible to combine the results of one of the largest cohorts of carriers of *DSPtv* variants, considered to be pathogenic or likely pathogenic, with an extensive literature review regarding *DSP*. Evidence is provided that the location at which such a genetic variant occurs plays an important role in disease severity and shows that there is an enrichment of genetic truncating variants in a specific *DSP* region when compared to controls. In **Chapter 5,** previous findings are confirmed showing that carrying more than one genetic variant in arrhythmogenic cardiomyopathy (ACM) related genes influences the disease severity, illustrating the multifactorial nature of ACM.

Part 2 of this thesis is focused on the identification of new genetic regions associated with arrhythmogenic cardiomyopathy (ACM). It was shown that variants in genes encoding the sarcomere (**Chapter 6**), the contractile unit of the heart muscle, and variants in the gene encoding Plectin (**Chapter 7**), a cytolinker protein, have no major role in the development of ACM. Although it cannot be excluded that some variants may have an effect.

Lastly, the key findings of the thesis are summarised and future perspectives regarding the challenges in the interpretation of genetic variants, the discovery of novel genetic regions implicated in the underlying disease mechanisms and the increase in understanding the various factors involved in disease expression with regard to arrhythmogenic cardiomyopathy are being presented at the end of this thesis.

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Chapter 1

Arrhythmogenic cardiomyopathy: pathology, genetics, and concepts in pathogenesis

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ABSTRACT

Arrhythmogenic cardiomyopathy (ACM) is a rare, heritable heart disease characterized by fibro-fatty replacement of the myocardium and a high degree of electric instability. It was first thought to be a congenital disorder, but is now regarded as a dystrophic heart muscle disease that develops over time. There is no curative treatment and current treatment strategies focus on attenuating the symptoms, slowing disease progression, and preventing life-threatening arrhythmias and sudden cardiac death. Identification of mutations in genes encoding desmosomal proteins and in other genes has led to insights into the disease pathogenesis and greatly facilitated identification of family members at risk. The disease phenotype is, however, highly variable and characterized by incomplete penetrance. Although the reasons are still poorly understood, sex, endurance exercise and a gene-dosage effect seem to play a role in these phenomena. The discovery of the genes and mutations implicated in ACM has allowed animal and cellular models to be generated, enabling researchers to start unravelling its underlying molecular mechanisms. Observations in humans and in animal models suggest that reduced cell-cell adhesion affects gap junction and ion channel remodelling at the intercalated disc, and along with impaired desmosomal function, these can lead to perturbations in signalling cascades like the Wnt/β-catenin and Hippo/YAP pathways. Perturbations of these pathways are also thought to lead to fibro-fatty replacement. A better understanding of the molecular processes may lead to new therapies that target specific pathways involved in ACM.

1. INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC), which is now considered a subform of arrhythmogenic cardiomyopathy (ACM) with right ventricular (RV) predominance, is a heritable condition characterized by fibro-fatty replacement of the myocardium that predisposes patients to ventricular arrhythmias (VA), which are frequently life-threatening, and to slowly progressive ventricular dysfunction.[1–4] Structural involvement of the RV predominates,[5] although left-dominant forms of ACM are also well-recognized.[6] Patients typically present in their second to fifth decade with symptoms associated with VA.[7] Sudden cardiac death may be the presenting symptom in up to 50% of index cases.[8] The diagnosis is based on International Task Force criteria[9] and mutations in genes encoding proteins of the cardiac desmosome are found in up to 60% cases.[1,10] Cardiac desmosomes are composed of a symmetrical group of proteins (cadherins, armadillo proteins, and plakins) that provide mechanical connections between myocytes. However, non-desmosomal genes have also been identified.[11] The current management strategies focus on lifestyle advice (restriction of physical exercise), attenuating symptoms, and slowing disease progression with anti-arrhythmic and heart failure medications, catheter ablation, and implantable cardioverter defibrillator (ICD) implantation. In cases of end-stage heart failure or refractory VA, a heart transplantation may be required.[12] Unravelling the genetic basis of ACM has led to the generation of animal and cellular models, enabling researchers to uncover the molecular mechanisms underlying ACM and even to discover new therapies.[13] This review will discuss the pathological findings, the genetic basis and the proposed mechanisms underlying ACM.

2. PATHOLOGICAL FINDINGS IN ACM

2.1 Morphological features

In ACM, part of the myocardium is replaced by fibrous and fatty tissue with either localized or diffuse myocardial atrophy due to cumulative myocyte loss.[14] The pathological hallmarks of the disease, the fibro-fatty replacement and myocyte atrophy, are usually distinctly present in the RV but may also occur in the left ventricle (LV), and can be segmental or patchy. Traditionally, the typical localization in the RV was described as the "triangle of dysplasia",[14,15] consisting of the RV inflow tract, RV outflow tract, and RV apex. However, recent cardiac magnetic resonance data[16] has revealed that limited ACM preferentially affects the basal inferior RV, with involvement of the RV apex only in advanced cases as part of global RV involvement. LV involvement has been observed in 76-84% of ACM cases[6,14], with a predilection for the thin posterolateral and posteroseptal areas.

Typically, the LV is affected to a lesser extent than the RV; however, there are disease variants characterized by predominant LV involvement, these are also referred to as arrhythmogenic left ventricular cardiomyopathy (ALVC).[17]

Involvement of the ventricular septum is rare, probably because it is not a subepicardial structure. The fibro-fatty scar tissue progresses from the subepicardial muscle layer towards the endocardium, ultimately resulting in transmural lesions with focal or diffuse wall thinning (**Figure 1**). This implies the ventricular wall is weakened, especially the relatively thin, free RV wall, which may lead to typical aneurysmal dilatation.

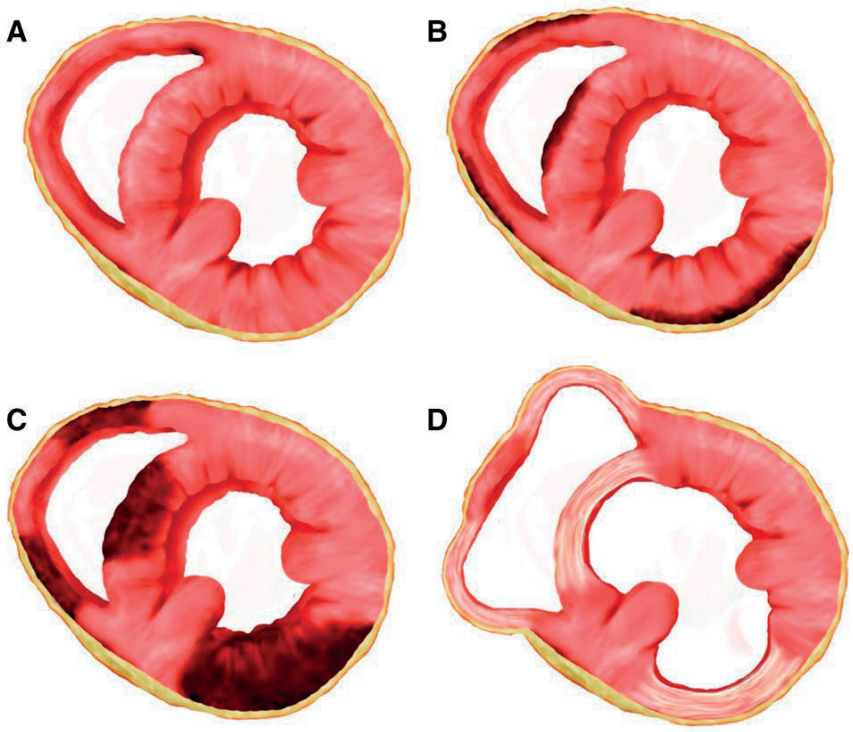

Figure 1. Development of arrhythmogenic cardiomyopathy (ACM) over time. Evidence from the *Dsg2*^{N271S} mouse model for ACM. A. At birth a structurally normal heart is presence. B. Early myocardial injury start on the epicardial side, extends transmurally (C) and is followed by wall thinning with fibrous repair and aneurysm development (D). Figure adapted from Basso et al.[104]

Microscopic examination typically shows islands of surviving myocytes, with fibro-fatty tissue in between.[14,17] These changes may account for intraventricular conduction delay and re-entry circuits triggering VA. Affected cardiomyocytes show non-specific degenerative features of myofibrillar loss and hyperchromatic changes in nuclear morphology.[14,17] Cardiomyocyte death (acquired injury), by either apoptosis[18] and/or necrosis,[19] accounts for

the progressive loss of the ventricular myocardium. These changes may be accompanied by inflammatory infiltrates, seen in up to 67% of hearts at autopsy.[14] Importantly, active inflammation might account for worsening of electrical instability and the onset of life-threatening arrhythmias. Whether the inflammatory cells are reactive to cell death or a primary event due to infection[20,21] or non-infective immune factors needs to be investigated.

2.2 Clinical utility of RV endomyocardial biopsy

RV endomyocardial biopsy (EMB) may be useful for the diagnosis of ACM, through an *in vivo* histological demonstration of fibro-fatty replacement. Moreover, EMB may provide additional information to rule out phenocopies, such as myocarditis or sarcoidosis, particularly in sporadic cases in which non-invasive evaluation remains inconclusive. The optimal EMB site is the RV free wall, which may, however, be severely thinned due to ACM.

In a normal heart, with increasing age and body weight, intramyocardial fat is, to a limited extent, present in the RV. Therefore, adipose tissue should be accompanied by replacement fibrosis and myocyte degeneration to be a sufficient morphologic diagnostic feature of ACM.[22]

In addition to conventional histology, immunohistochemical analysis can be a valuable tool, because plakoglobin (PG) signal levels at intercalated disks can be diffusely diminished in most ACM patients, also in samples from the LV or interventricular septum, irrespective of the underlying mutation.[23] However, the reliability and validity of this test for routine clinical practice still has to be confirmed.[24]

3. GENETIC BASIS OF ACM

Clustering of ACM within families was appreciated early.[25] Recognition that the cardiac phenotype of Naxos disease, a rare, familial, cardio-cutaneous condition, overlapped with familial ACM[26] was a key insight. Following the discovery that mutations in *JUP*, encoding PG, was the cause of Naxos disease[27], the ACM-associated mutations in the desmosomal genes were rapidly unveiled, including *DSP* encoding desmoplakin[28], *PKP2* encoding plakophilin-2[29], *DSG2* encoding desmoglein-2[30], and *DSC2* encoding desmocollin-2.[31]

Up to two-thirds of ACM patients harbour mutations in these desmosomal genes.[1,7] Heterozygous mutations resulting in premature termination of the protein product and/or abnormal splicing in *PKP2* are the most prevalent.[10,32] Inheritance of desmosomal mutations follows an autosomal dominant pattern with age-related, incomplete penetrance and variable expressivity. However, ACM patients with multiple mutations (compound heterozygosity and digenic) are not uncommon and their occurrence ranges widely (4-21% reported in

various cohorts).[7,32–34] This range is likely related to how stringently missense variants are adjudicated and how many genes are sequenced.[35] Cases with homozygous mutations are also seen.[36,37]In addition, there are pedigrees in which siblings of the index case are more likely to be affected than their parents or their parents' siblings. These phenomena raise the suspicion that other genetic and/or environmental factors may play a modifying role.[38]

Although most reported ACM-associated pathogenic variants are in desmosomal genes (as in 95.5% of the variants reported in the ARVC Genetic Variant Database[10]), extra-desmosomal mutations have been identified in a few patients. The first of these was the p.S358L founder mutation in *TMEM43*, encoding transmembrane protein 43, which was identified in patients in Newfoundland and Europe.[39,40] Pathogenic mutations have also been reported in genes associated with other cardiomyopathies and arrhythmia syndromes including desmin (*DES*)[41], titin (*TTN*)[42], lamin A/C (*LMNA*)[43], phospholamban (*PLN*)[44], $Na_v1.5$ (*SCN5A*)[45] and filamin C (*FLNC*).[46] Together, these discoveries reflect the clinical and genetic overlap of ACM with dilated cardiomyopathy at one phenotypic extreme[47] and with arrhythmia syndromes at the other. Supporting this concept, pathogenic ACM-associated *PKP2* missense mutations also have been identified in Brugada syndrome patients.[48]

Genes encoding proteins in the 'area composita' (composed of desmosomes, adherens junctions, ion channels and gap junctions) have also emerged as potentially important in the pathogenesis of ACM. Mutations in *CTNNA3*, encoding αT-catenin, have been identified in families with classic ACM.[49] Recently, two families, with right-predominant ACM, were found to have likely pathogenic mutations in *CDH2*, encoding cadherin-2, a calcium-dependent cell surface adhesion molecule.[50]

Mutations in transforming growth factor β3 (TGFβ3)[51] and the cardiac ryanodine receptor-2 (*RYR2*)[52] genes have been described in ACM, although this association needs to be confirmed.

Finally, there are some ACM cases with no identifiable mutation. In the largest study of ACM, among 439 index cases, 37% had no identifiable mutation in the desmosomal genes, *PLN*, or *TMEM43*.[7] Among these gene-elusive cases, only one-fifth had evidence of familial disease. A recent meta-analysis confirmed a lower prevalence of family history among ACM patients without desmosomal mutations.[53] This raises the question whether these gene-elusive cases have a primarily monogenic disease or whether they represent an oligogenic form of ACM with unknown, low-penetrant genetic variants and/or with external factors playing a role in their disease pathogenesis. Recent research showed that gene-elusive ACM cases without a positive family history were disproportionately observed in high-level endurance athletes,[54,55] which points to exercise as a key lifestyle risk factor in these cases.

3.1 Genotype-phenotype association in ACM

Several clinically useful genotype-phenotype associations have been identified. Broadly, neither the cardiac phenotype nor clinical course differ substantially between ACM patients with and without a mutation.[7] A recent meta-analysis identified inverted anterior precordial T-waves (V_{1-3}) but not structural abnormalities, epsilon waves, or arrhythmias with a left-bundle branch block morphology, as being more common among ACM patients with desmosomal mutations.[53] Patients with mutations do have earlier onset of ACM.[7,53,56]

In addition to an increased penetrance, carrying multiple mutations seems to be an important risk factor for malignant VA and sudden death.[57] Similarly, in 577 desmosomal, *PLN*, and *TMEM43* mutation carriers, the 4% of patients with multiple mutations had significantly earlier occurrence of malignant VA and more frequent LV dysfunction, class C heart failure, and transplantation.[32] Together these data suggest there is a gene-dosage effect in ACM.

Other associations between genotype and ACM phenotype include a higher prevalence of LV involvement and heart failure among ACM patients with *FLNC, DSP* and *PLN* mutations.[32,46,58] The TMEM43 p.S358L founder mutation is associated with high disease penetrance and arrhythmic risk among male carriers.[39]

Table 1 provides an overview of the genes implicated in ACM and the yield of genetic testing. Caution is warranted as variants in ACM-related genes are also often found in the general population.[59]

3.2 Penetrance of ACM mutations

Familial ACM is characterized by incomplete age-related penetrance and significantly variable expressivity.[60] With the expansion of genetic testing for ACM, increased numbers of at-risk mutation carriers are now being identified, so that understanding the risk conveyed by the presence of an ACM-associated variant is critical. The penetrance of ACM-associated mutations is likely to be overestimated, as families reported in genetic studies will have higher than typical penetrance and more affected individuals, making them attractive for genetic research. Such families likely share additional genetic or envirnomental factors that put them at increased risk. In a report of over 500 desmosomal mutation carriers,[32] roughly only one-third met diagnostic Task Force Criteria.

Data from unselected populations with incidentally detected desmosomal variants suggests that penetrance in the general population may be considerably lower. A recent publication[61] showed that among 18 individuals with incidentally identified pathogenic ACM mutations and 194 cases with rare variants of uncertain significance, neither cardiac diagnoses reported in the electronic medical record nor cardiac tests evaluated by ARVC experts showed higher rates of abnormalities than the control population.

Table 1. Overview of the genes implicated in ACM and the yield of genetic testing.

Gene	Protein	% index cases	Notes	References
Desmosomal				
PKP2	plakophilin-2	20-46%	Most prevalent in the majority of ACM populations	7,29,33,43,53
DSP	desmoplakin	3-15%	Autosomal dominant inheritance associated with ACM Autosomal recessive inheritance associated with Carvajal Syndrome (cardiocutaneous)	7,28,43,53
DSG2	desmoglein-2	3-20%		7,30,43,53
DSC2	desmocollin-2	1-8%		7,31,43,53
JUP	plakoglobin	0-1% (except in Naxos, Greece)	Autosomal dominant inheritance associated with ACM. Autosomal recessive inheritance Associated with Naxos disease (cardiocutaneous)	7,27,43,53,82
Area composita				
CTNNA3	αT-catenin	0–2%	2/76 Italian probands	49
CDH2	cadherin-2	2%?	Single report of 2/74 Italian families without desmosomal mutations	50
Other or overlapping syndromes				
PLN	phospholamban	0-1% (except in Dutch populations)	Dutch founder mutation	7,44,47
TMEM43	transmembrane protein 43	0-2% (except in Canadian populations)	Canadian (Newfoundland) founder mutation	7,39,40
SCN5A	$Na_V1.5$	2%		10,45
LMNA	lamin A/C	0-4%	Overlap with dilated cardiomyopathy	33,43
DES	desmin	0-2%	1 of two cases detected with pathogenic *PKP2* mutation	33,41
FLNC	filamin C	3%	7/219 Southern European ARVC patients left-dominant cardiomyopathy with myocardial fibrosis	46
TTN	titin	0-10%	Overlap with dilated cardiomyopathy	42

3.3 Interplay of genotype and exercise in ACM pathogenesis

While there is no clear explanation for phenotypic heterogenity in ACM, even among carriers of the same mutation, there is increasing evidence that exercise plays a major role in disease penetrance and arrhythmic risk. A history of participation in endurance exercise is associated with increased likelihood of disease penetrance in a dose-dependent fashion.[62] Desmosomal mutation carriers who were endurance athletes also have earlier onset of ACM, worse structural abnormalities, higher likelihood of heart failure, and greater arrhythmic risk.[62,63]

There is evidence that strongly suggests exercise is also associated with gene-elusive ACM. A study suggested that ultra-endurance athletes may develop a predominantly exercise-induced form of ACM.[64] Two research groups showed that ACM patients without a desmosomal mutation had done considerably more intense exercise prior to clinical presentation than desmosomal mutation carriers.[54,55]

An emerging paradigm suggests there is a threshold for phenotypic expression of ACM depending on the relative amount of exercise undertaken.[54,65] As shown in **Figure 2**, we hypothesize that individuals born with a very high genetic risk, such as carriers of multiple mutations, require little (or perhaps no) exercise for ACM disease expression. Ultra-endurance athletes may develop a predominantly exercise-induced form of ACM[64], although we suspect only a subset of this population is susceptible.

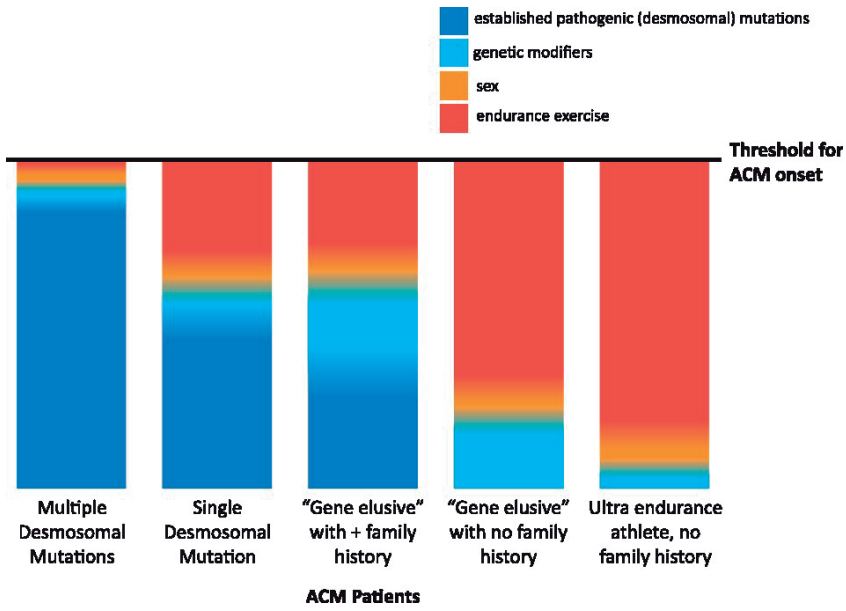

Figure 2. Threshold model for phenotypic expression of arrhythmogenic cardiomyopathy (ACM). Based on figure from Sawant et al.[54]

4. PATHOGENESIS

Multiple aetiopathogenic models have been proposed for ACM. The initial model explained the disease as a manifestation of an embryonic maldevelopment (dysplasia) of the RV. That ACM is not a congenital abnormality, like Uhl's disease, but a cardiomyopathy which develops over time, is supported by the pathological findings described above in section 2.1.[14,17] This assertion is further supported by an ACM mouse model, which demonstrated that loss of the myocardium starts after birth.[19]

Chronic inflammation could also contribute to the development of ACM. Experiments in mice (BALB/c strain) inoculated with Coxsackie virus B3 showed myocardial necrosis and inflammatory infiltrates with subsequent exclusive RV fibrosis.[66] While one report demonstrated that cardiotropic viruses are present in a subset of sporadic ACM cases,[20] another cohort that included almost half familial ACM cases, did not uncover any viral material in the hearts.[21] This suggests that a viral aetiology could be possible in sporadic cases, whereas a genetic substrate is more likely in familial ACM.[67]

The discovery that desmosomal gene mutations cause ACM offered important leads in understanding the mechanisms responsible for the disease. As mentioned in section 3, desmosomes together with the adherens junctions (AJ), gap junctions and ion channels form the area composita at the intercalated disc (ID) (**Figure 3A**). This structure is important for the electromechanical coupling of cardiomyocytes and plays a role in multiple intracellular signalling cascades.[68] Firstly, mutations affecting the desmosomal proteins could lead to a decreased mechanical coupling between the cells. Cardiomyocytes are especially subject to mechanical stress and decreased coupling can lead to detachment of the cardiomyocytes with subsequent cell death, inflammation and loss of myocardium. Ultrastructural abnormalities of the desmosomes and ID, reflecting an impaired cell-cell coupling, have been reported in hearts of ACM patients.[69] Secondly, considering the interaction between components of the desmosome, ion channels[70] and gap junctions,[71] desmosome dysfunction could also lead to the remodelling of these proteins at the ID causing abnormal electric coupling between cardiomyocytes. Remodelling of Cx43 and cardiac voltage-gated sodium channel ($Na_V1.5$) has been demonstrated with immunofluorescence in human ACM hearts.[72] These observations suggest that gap junction or ion channel remodelling may increase susceptibility to arrhythmias. Lastly, altered signalling pathways due to remodelling at the ID could also contribute to the pathogenesis of ACM.

Most of the experimental models have focused on genetic defects in the desmosomal proteins (**Figure 3**). The major components of the desmosomes are the cadherins (DSG2 and DSC2), the armadillo proteins (PG and PKP2) and the cytolinker protein DSP.[73] The cadherins have

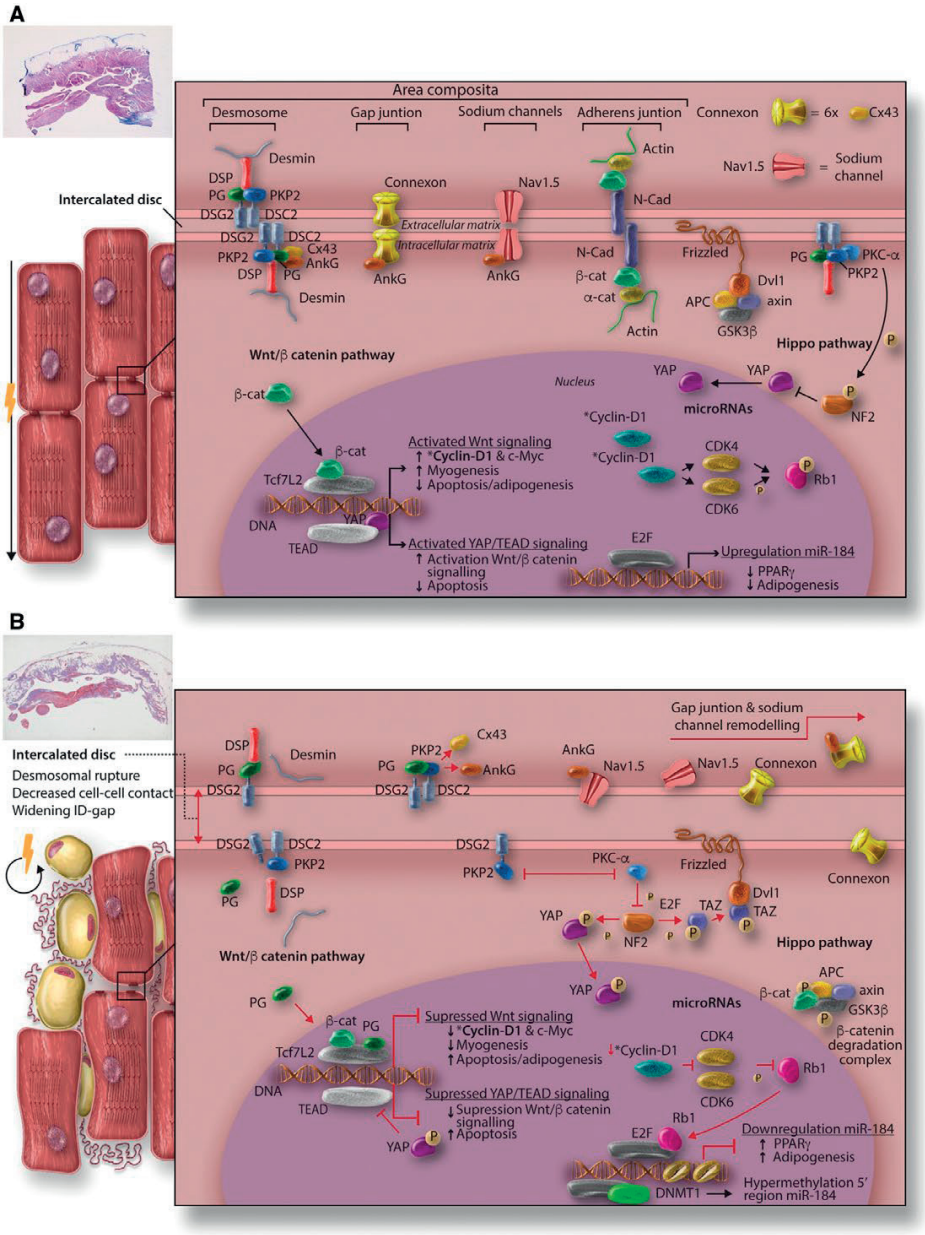

Figure 3. A. Overview of the "area composita" and B. proposed remodelling of desmosomal proteins, gap junctions and ion channels at the intercalated d and aberrant signalling pathways involved in arrhythmogenic cardiomyopathy substantiated by the various experimental models. Histological images of normal right ventricle (A) and right ventricle affected by ACM (B). Histological images are adapted from Basso et al.[14] Pathways are based on Corrado et al, Hu et al, Gurha et al and Vermij et al.[4,68,95,96]

an extracellular domain, which bind to the cadherins from adjacent cells and are important for the adhesive properties of the cell-cell contact. The intracellular domains of the cadherins bind to the armadillo protein, which are indirectly connected to the intermediate filament desmin by DSP. In addition to a structural role in the desmosome, the armadillo proteins participate in different signalling pathways.[73]

4.1 Cell-cell coupling

Cell-cell coupling has been studied extensively in multiple ACM models. Experimental models mimicking a deficiency of one of the desmosomal proteins in a cardiac-restricted or constitutive fashion showed that a deficiency of any of these desmosomal proteins can lead to ultrastructural abnormalities of the ID and desmosomes.[74–78] Similar findings were obtained *in vitro* in cellular studies that demonstrated decreased cell-cell adhesion upon downregulation of PKP2 or PG.[70,79] In addition, overexpression of mutations in different desmosomal proteins, simulating a dominant negative effect, also led to ultrastructural defects at the ID.[80–82] Impaired cell-cell coupling was also demonstrated to play a key role in the pathogenesis of ACM in mice overexpressing the *Dsg2* N271S mutation ($Dsg2^{N271S}$), which is homologous to the human mutation DSG2-N266S. In $Dsg2^{N271S}$ mice, widening of the ID preceded the occurrence of fibrosis and necrosis. In some observations this widening co-occurred with focal lysis of the cardiomyocytes at the points of attachment to the desmosomes.[81] *Dsp* cardiac-specific knock-out mice (*Dsp*-cKO), exhibit many features seen in the human ACM phenotype (including fat deposition and arrhythmic instability). Rupture of the desmosomes, widening of the ID, and loss of myocyte adhesion was observed as an early manifestation of the phenotype.[83] Collectively, these data underline the necessity of proper desmosomal function for the stable coupling of cardiomyocytes (**Figure 3B**).

4.2 Gap junction and ion channel remodelling

The cross-talk between dysfunction of desmosomal proteins and of components of cardiac electrical function has been studied in several models. At the ID, the desmosomes, adherens junctions, gap junctions and ion channels interact with each other and function as one unit.[68]

PKP2 has been shown to physically interact with Cx43, ankyrin-G (AnkG) and $Na_V1.5$ *in vitro*.[70,84] Ankyrin-G is a cytoskeletal adaptor protein and is an important component of the voltage-gated sodium channel complex.[70] Silencing of PKP2 in neonatal rat ventricular myocytes led to a reduced signal for Cx43, $Na_V1.5$ and AnkG at the ID.[70,84] Although no reduced signal of Cx43 and $Na_V1.5$ was seen at the ID in heterozygous *PKP2* knock-out mice ($Pkp2^{+/-}$), they did show altered sodium current kinetics and were prone to ventricular tachycardia when provoked by flecainide, without having histological cardiac alterations.[48] A marked reduction of immunoreactive signals of Cx43 and $Na_V1.5$ was also seen in human induced pluripotent stem cells (hiPSCs) derived from patients with *PKP2* mutations.[13] *In*

vivo mislocalization of Cx43, represented by punctate distribution instead of a continuous organization pattern of Cx43, was observed in mice overexpressing *Pkp2*^{R735X}, but only after they were subjected to exercise.[85] In addition to PKP2, DSG2 also co-immunoprecipitates with $Na_V1.5$.[81] Hearts from *Dsg2*^{N271S} mice that were studied *ex vivo* prior to the development of cardiomyopathic changes demonstrated reduced cardiac conduction velocities and increased arrhythmia inducibility, possibly mediated by a disturbed *Dsg2*-$Na_V1.5$ interaction.[81] DSC2 was also shown to physically interact with Cx43[71] and a specific *DSC2* mutation led to a decreased binding affinity for Cx43, indicating that *DSC2* mutations can alter Cx43 function. Experimental models, both *in vitro* as well as *in vivo*, modelling PG and DSP deficiency and mutations with dominant negative effect therein, demonstrated that Cx43 remodelling also occurred when these genes are affected.[13,79,80,83,86] In line with observations in *Dsg2*^{N271S} mice, heterozygous *Dsp* knock-out mice (*Dsp*$^{+/-}$) exhibited conduction delay and increased susceptibility to inducible ventricular tachycardia, without overt cardiac structural abnormalities. The observed mislocalization and reduced expression of Cx43 was noted as a possible underlying mechanism.[87]

An altered inward rectifier potassium current (I_{k1}), which is mediated by the potassium channel subunit Kir2.1, was seen in zebrafish overexpressing the mutant c.2057del2 *JUP* (PG-2057del2). Immunostaining of PDZ domain-containing synapse-associated protein-97 (SAP97) demonstrated a reduction of this protein. SAP97 mediates the trafficking of PG, Kir2.1 and $Na_V1.5$ to the intercalated disc, suggesting a possible role for these ion channels in the disease process.[13] That proteins at the area composita function as one unit is emphasized in a double knock-out mouse in which PG and β-catenin were deleted, were Cx43 remodelling preceded the highly arrhythmogenic phenotype of the mice.[88] Of note, Cx43 remodelling was not observed in a homozygous cardiac-restrictive PG deficient mouse model (Car *Pg*$^{-/-}$) with electric instability, while there was Cx43 remodelling in other Car *Pg*$^{-/-}$ mice, who did not have any electric abnormalities.[86,89] In conclusion, these studies in different cellular and mouse models support the view that the interaction of desmosomal proteins with gap junctions and ion channels at the area composita leads to conduction abnormalities and electrical instability upon disruption of desmosomal function (**Figure 3B**).

4.3 Intercalated disc remodelling and signalling pathways

4.3.1 Wnt/β-catenin pathway

Suppression of the canonical Wnt/β-catenin pathway can lead to an enhanced adipogenesis. β-catenin is an activator of Wnt signalling by activating T cell/lymphoid-enhancing binding (Tcf/Lef) transcription factor. Since PG, which is also known as γ-catenin, shares functional and structural properties with β-catenin, it is postulated that nuclear translocation of PG can interfere with this pathway by binding to a different site on Tcf7L2 than β-catenin does (**Figure 3B**). In cultured DSP-deficient atrial myocytes (HL-1 cells) there was an

increase of PG in the nuclear fraction, with a subsequent decrease in Tcf/Lef1-mediated gene transcription followed by a supressed canonical Wnt signalling (represented by an increase in adipogenetic transcriptional regulators). It is therefore believed that this mechanism may underlie fibro-fatty replacement in ACM. The heterozygous cardiac-specific DSP knockout (Car $Dsp^{+/-}$) mouse, with an ACM phenotype including electric instability and accumulation of fat droplets in the myocardium, showed an increase of PG in the nuclear fraction and suppressed Wnt signalling as seen in HL-cells.[90] This translocation of PG was also seen in mice overexpressing the mutant PG-2057del2, with a subsequently supressed Wnt signalling (represented by downregulation of the Wnt target genes (c-Myc and cyclin-D1)).[91] Further support of a role of the Wnt/β-catenin pathway in ACM was demonstrated in hiPSC-CMs from a patient with a *PKP2* mutation, which showed nuclear translocation of PG and a decreased β-catenin activity.[92] A decrease in expression of the Wnt target gene cyclin-D1 was also noted in PKP2 knockdown HL-1 cells.[93] Indirect evidence of supressed Wnt signalling was shown in homozygous mice lacking exons 4-5 of DSG2 ($Dsg2^{exon4\text{-}5/\ exon4\text{-}5}$).[94] Inhibition of the glycogen synthase kinase-3 beta (GSK3β), which targets β-catenin for degradation, reversed the adverse remodelling of the desmosomal proteins and gap junctions and prevented cardiac myocyte injury and cardiac dysfunction. As GSK3β targets degradation of β-catenin, inhibition of GSK3β should lead to activation of the canonical Wnt/β-catenin pathway. The observations that inhibition of GSK3β normalizes the desmosomal protein remodelling and improves the cardiac phenotype in the $Dsg2^{exon4\text{-}5/exon4\text{-}5}$ mice supports the concept that *DSG2* mutations could suppress the canonical Wnt/β-catenin pathway, most likely by disrupting the desmosome complex and leading to increased nuclear translocation of PG.[94]

Whether the aberrant Wnt signalling is a common pathway in ACM is a topic of debate, since PG remodelling was not seen in another set of hearts from ACM patients.[24] Kant et al suggested that the reduced PG immunofluorescence signal was due to epitope masking rather than remodelling. They also stated that target genes (CTGF and cyclin-D1) of the Wnt/β-catenin signalling, which should be downregulated during suppression, were upregulated in six human hearts with ACM.[24]

4.3.2 Hippo/YAP pathway

The Hippo/YAP pathway was also shown to be involved in experimental models with different mutations. PKP2 functions as a scaffold protein for the protein kinase C alpha (PKC-α). PKC-α inactivates neurofibromin (NF2), which is located upstream of the Hippo pathway. When NF2 is activated, it phosphorylates and then deactivates Yes-associated protein 1 (YAP), a transcription factor. Subsequently, phosphorylated YAP (pYAP) can contribute to suppression of Wnt/β-catenin signalling (**Figure 3B**).[93,95] When PKP2 is not present, PKC-α (which needs PKP2 as a scaffold) is significantly reduced in PKP2 knock-down HL-1 cells. Also, NF2 was activated and levels of increased pYAP were demonstrated. This

activation of NF2 was also observed in cardiac-restricted *Dsp*$^{+/-}$ mice and mice overexpressing PG-2057del2.[93]

4.3.3 microRNAs

Recently a new mechanism was proposed as being involved in ACM. In knock-down PKP2 HL-1 cells, transcriptome analysis showed that microRNA-184 (miR-184) was downregulated, although it is normally upregulated by E2F transcription factors. Cyclin-D1 was also downregulated, which normally deactivates retinoblastoma (RB1) protein.[96] In the case of downregulation of cyclin-D1, RB1 levels are increased and inhibit E2F transcription factors, which leads to a decrease in levels of miR-184. However, the downregulation was only partially explained by the diminished levels of cyclin-D1. Another factor contributing to the diminished levels appeared to be that the genomic region of miR-184 was hypermethylated by DNA (cytosine-5)-methyltransferase 1 (DNMT1). This could be due to the E2F/RB1 complex, as it has been shown that this complex can recruit DNA methyltransferases (**Figure 3B**).[97] Diminished levels of miR-184 cause an increase in expression of peroxisome proliferator-activated receptor gamma (PPARγ), which is an inducer of adipogenesis and should not normally be activated in cardiomyocytes.[96] This supports a prior discovery that hiPSC-CMs from patients with desmosome mutations require, besides normal activation of PPARα, abnormal activation of PPARγ to induce ACM features *in vitro*.[92] This downregulation of miR-184 was confirmed in mice overexpressing PG-2057del2.[96] Of note, miR184 overexpression or downregulation did not affect transcriptional activities of Hippo and canonical Wnt pathways. Whether this downregulation plays a role in the other desmosomal ACM models remains to be investigated.

4.4 Calcium handling deficits

Abnormal calcium handling may also contribute to ACM as experimental models showed perturbed calcium handling in hiPSCs-CMs with the homozygous *PKP2* c.2484C>T mutation.[92] A recent study in human hearts with ACM revealed that mRNA levels for phospholamban (PLN), a protein involved in the intracellular calcium homeostasis, was significantly upregulated.[98] Additionally, mutated PLN is well known to cause ACM in humans.[47] These findings indicate that abnormal calcium handling could play a role in the pathogenesis and it is important that this topic should be studied further.

4.5 Exercise

The effect of exercise has been studied retrospectively in humans with ACM and prospectively in several ACM mouse models; all studies consistently demonstrated that exercise induces or exacerbates the cardiac phenotype.[62,85,94,99,100] Mice overexpressing the mutant *Dsp* R2843H (*Dsp*^{R2483H}) showed a blunted activation of AKT1 in response to exercise. This blunted response could be due to sequestration of PG at the insoluble part of the cells.[100]

The consequence of this response, including adverse cardiac remodelling, remains unknown, although it is speculated that this is due to perturbed Wnt signalling.[100] *In vitro* studies have looked at the consequences of mechanical stress in cells expressing mutated forms of PG or PKP2: the cells failed to upregulate PG and N-cadherin when subjected to shear force.[79] In addition, cells overexpressing PG also showed increased apoptosis when subjected to uniaxial stress.[13] In conclusion, there is experimental evidence that exercise *in vivo* or induced mechanical force *in vitro* leads to an altered response in desmosomal mutations. How the altered responses eventually lead to ACM is not known but it is a topic of great interest.

4.6 Other proteins involved in ACM

Mutations in other proteins of the area composita (see section 3) have also been associated with ACM. Recently, rare missense variants in *SCN5A* were identified in 6 out of 287 (2%) ACM patients and hiPSCs were generated to assess the functional consequences of one of these variants (p.Arg1898His). The peak sodium current density was reduced, and a reduced density of $Na_v1.5$ and N-Cadherin at the contact site of the cells was observed.[45]

Another group reported two mutations in αT-catenin protein as associated with ACM. This protein is important for integrating the cadherin-catenin complex. *In vitro* studies of one mutation showed a decrease of binding affinity of αT-catenin to β-catenin and PG. Immunofluorescence showed abnormal localization of αT-catenin through the cytoplasm. No translocation was observed for PG or PKP2. The mechanisms underlying ACM caused by mutations in this gene need further study.[49]

Two new mouse models were generated recently in which Rho-kinase inhibition before birth or deficiency of inhibitor of apoptosis-stimulating protein of p53 (iASPP) led to an ACM phenotype. Similar features to the other ACM mouse models were observed, however, they occurred in the Rho-kinase model only when subjected to inhibition before birth.[101,102] Based on these findings, the role of Rho-kinase inhibition and deficiency of iASPP in the pathogenesis of ACM warrants further investigation. Of note, p53 was recently found to be significantly upregulated in ACM patients.[98]

5. TRANSLATIONAL ASPECTS

Besides major breakthroughs in understanding the pathophysiology, modelling the disease in experimental settings has led to the discovery of a possible pharmacological therapy.[13] Via high throughput screens of zebrafish embryos expressing the mutant PG-2057del2, the compound SB216763 improved the cardiac phenotype. This compound, an GSK3β inhibitor, prevents degradation of β-catenin and could therefore enhance the supressed canonical Wnt

signalling. It also prevented desmosomal protein and gap junction remodelling in neonatal rat ventricular cardiomyocytes expressing the mutant PG-2057del2 and reversed these processes in hiPSCs with *PKP2* mutations.[13] This compound was later tested in two mice models with different genetic mutations and showed that the adverse cardiac remodelling could be prevented.[94]

New technologies also offer unique possibilities to study and model ACM. Human cardiomyocytes, derived from hiPSC-CMs that are generated from patients, capture the exact genetic background and mutation status of the patient and should therefore model the disease more accurately and in a personalized fashion. However, *in vitro* hiPSC-CMs do not mature and lack the complex environment of the heart *in vivo*. Recently, by introducing hiPSCs-CM (with two different *PKP2* mutations) into neonatal rat hearts *in vivo*, it was shown that hiPSCs-CM can mature into adult cardiomyocytes. These cells captured the disease phenotype, as was shown by ultrastructural abnormalities of the intercalated disc, increased apoptosis, and accumulation of fat.[103] This new model means we can now investigate the disease processes underlying ACM with a human and patient-specific genetic mutation in the complex environment of the mammalian heart and it also provides a possible platform for *in vivo* drug testing.

6. CONCLUSION

Results from experimental and human studies have yielded valuable insights into the pathogenesis of ACM. Impaired mechanical coupling seems to be a uniform finding in the models with different desmosomal mutations. In addition, gap junction and ion channel remodelling seems to play a major role, even before gross structural abnormalities occur, manifesting as electric instability. However, there are models that do capture the electric instability, but they do not show gap junction remodelling, which suggests that these processes need further study. A suppressed Wnt/β-catenin signalling, by nuclear localization of plakoglobin, is supported by models with mutations in different desmosomal proteins, although other pathways also contribute to ACM. The proposed cascades leading to ACM and supported by the experimental models are shown in **Figure 4**.

Mutations in non-desmosomal genes are also implicated in ACM; modelling these in an experimental setting could provide more information on the underlying mechanisms. Furthermore, no mutation has been found in most of the sporadic cases of ACM so far. Possible environmental factors (e.g. cardiotropic viruses or endurance exercise) or innate factors (immune system) may play a role, but these require further investigation.

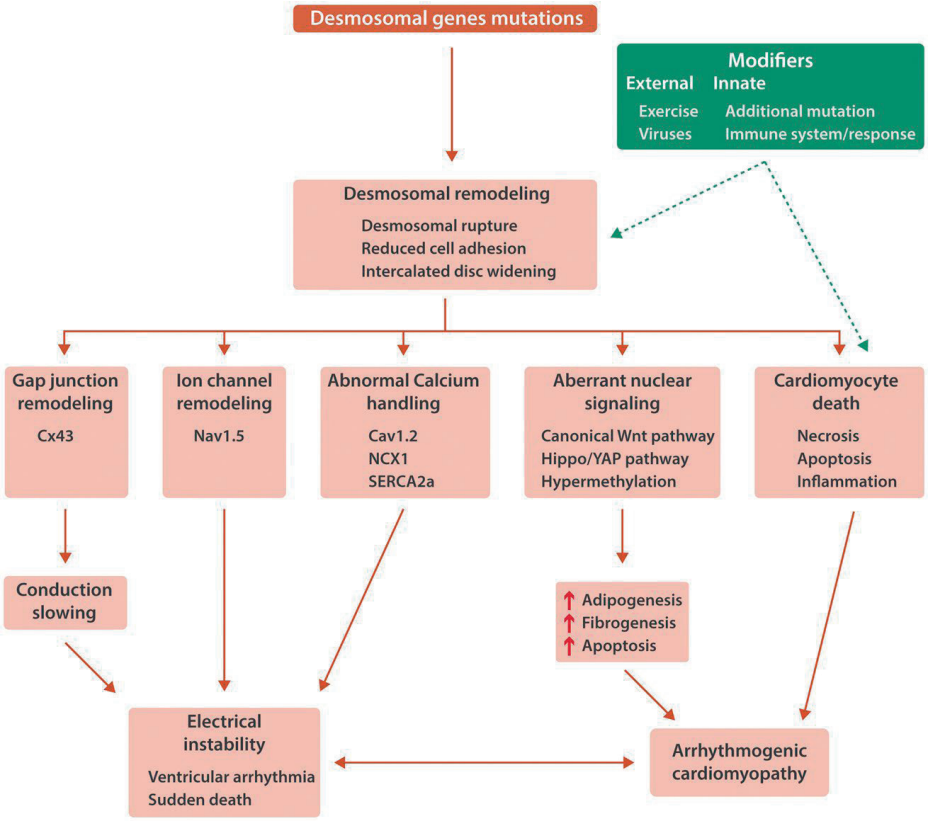

Figure 4. Proposed cellular and molecular cascades underlying arrhythmogenic cardiomyopathy, supported by evidence from experimental models. Based on figure from Basso et al.[104]

Future research to improve our understanding of how genetic and non-genetic factors interact to trigger disease onset will be key to managing ACM patients. It is critical that we expand our understanding of the molecular mechanisms through which exercise interacts with expression of abnormal protein or reduced protein expression to cause the pathologic features of ACM.

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Chapter 2

Lamin A/C-related cardiac disease: Late onset with a variable and mild phenotype in a large cohort of patients with the lamin A/C p.(Arg331Gln) founder mutation

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ABSTRACT

Background – Interpretation of missense variants can be especially difficult when the variant is also found in control populations. This is what we encountered for the *LMNA* c.992G>A (p.(Arg331Gln)) variant. Therefore, to evaluate the effect of this variant, we combined an evaluation of clinical data with functional experiments and morphological studies.

Methods and Results – Clinical data of 23 probands and 35 family members carrying this variant were retrospectively collected. A time-to-event analysis was performed to compare the course of the disease with carriers of other *LMNA* mutations. Myocardial biopsies were studied with electron microscopy (EM) and by measuring force development of the sarcomeres. Morphology of the nuclear envelope was assessed with immunofluorescence on cultured fibroblasts.

The phenotype in probands and family members was characterized by atrioventricular conduction disturbances (61% and 44%, respectively), supraventricular arrhythmias (69% and 52%, respectively) and dilated cardiomyopathy (74% and 14%, respectively). LMNA p.(Arg331Gln) carriers had a significantly better outcome regarding the composite endpoint (malignant ventricular arrhythmias, end stage heart failure or death) compared to carriers of other pathogenic *LMNA* mutations. A shared haplotype of 1 Mb around *LMNA* suggested a common founder. The combined LOD score was 3.46. Force development in membrane-permeabilized cardiomyocytes was reduced due to decreased myofibril density. Structural nuclear *LMNA*-associated envelope abnormalities, i.e. blebs, were confirmed by EM and immunofluorescence microscopy.

Conclusion – Clinical, morphological, functional, haplotype and segregation data all indicate that LMNA p.(Arg331Gln) is a pathogenic founder mutation with a phenotype reminiscent of other *LMNA* mutations, but with a more benign course.

INTRODUCTION

The *LMNA* gene encodes for the intermediate filament proteins lamin A and C. *LMNA* mutations are associated with a wide spectrum of phenotypes ranging from progeroid syndromes, muscular disease and lipodystrophy to isolated cardiac disease (dilated cardiomyopathy (DCM), conduction disorders) and phenotypes consisting of combinations of these different features.[1] Although their precise role is unknown, LMNA proteins are believed to play an important role in the structural integrity of the cell nucleus and in gene regulation.[2]

LMNA is one of the genes most frequently involved in genotyped DCM.[3] Sinus node dysfunction, atrioventricular conduction disorders, supraventricular and ventricular arrhythmias often precede or accompany DCM.[4] *LMNA*-related cardiac disease is associated with a high incidence of major cardiac events like sudden cardiac death (SCD), appropriate implantable cardioverter-defibrillator (ICD) therapy or end stage heart failure. DCM patients with an *LMNA* mutation are, in general, believed to have a poor prognosis compared with non-*LMNA*-mutation DCM patients.[5,6]

Currently, with all the new DNA sequencing technologies implemented in routine patient care, increasing numbers of DNA variants are being identified.[7] Classifying a variant as "pathogenic" has important implications for genetic counselling, the identification of family members at risk, clinical management and sometimes even clinical risk-stratification.[8] However, assigning "pathogenicity" to a variant is often challenging, particularly when the variant is found in ostensibly healthy controls.

In the GoNL database, which contains genome sequencing data of approximately 500 unrelated Dutch subjects, *LMNA* c.992G>A (p.(Arg331Gln)) was found four times, but in low quality calls. We therefore had difficulty assigning the correct label to this variant. Although *in silico* prediction models predicted that this substitution is deleterious or probably damaging, we sought to find additional evidence for the potential pathogenicity of this mutation by evaluating clinical data, studying morphology of the nuclear envelope and analyzing functional effects on the myocytes and fibroblasts of mutation carriers.

METHODS

Mutation analysis

Probands with the LMNA p.(Arg331Gln) variant were identified by next generation sequencing (NGS) using targeted panels (the list of genes screened is available on request) or by Sanger sequencing.[9,10] Variants found with NGS were confirmed by Sanger sequencing.

Variants in family members were analyzed by Sanger sequencing. Written informed consent was obtained from all participants according to the local medical ethics committees of our hospitals.

The online Genome of the Netherlands (GoNL) database and the Exome Aggregation Consortium dataset were searched for the LMNA p.(Arg331Gln) variant to check for its presence in the general population.[11,12]

Clinical evaluation

We collected retrospective clinical data on 23 probands (A to W), who carried the LMNA p.(Arg331Gln) variant and on 35 family members carrying this variant. When available, data on medical history, physical examination, 12lead electrocardiogram (ECG), 24-hour ambulatory ECG (Holter) and/or exercise-ECG (X-ECG), transthoracal echocardiography, magnetic resonance imaging (MRI) of the heart, myocardial perfusion scintigraphy and coronary angiography were collected. In cases of atrial fibrillation (AF) or pacemaker (PM) rhythm, earlier ECGs were analyzed for conduction disorders.

Pedigrees of the families were constructed to study segregation of the variant with the phenotype. Family members were considered to have cardiac involvement if there was evidence of sinus node dysfunction, supraventricular and ventricular arrhythmias, atrioventricular and ventricular conduction delay, PM and/or ICD implantation, structural cardiac abnormalities (determined by echocardiography or MRI) or symptomatic heart failure in the absence of other known causes. For a more detailed description of the phenotypes and definitions used see Supplemental Materials.

Time-to-event analysis

Data of 56 carriers of a pathogenic *LMNA* mutation – not p.(Arg331Gln) – associated with DCM (31 probands and 25 family members) were used to compare outcome. Pathogenicities of these mutations were previously assessed using a clinical classification scheme described earlier.[13] Data of these *LMNA* mutation carriers have been analyzed and used in previous studies.[6,13,14] An event was defined as a composite of the following endpoints: appropriate ICD therapy, out of hospital cardiac arrest (OHCA), heart transplant/left ventricular assist device implantation (HTx/LVAD) or death. Appropriate ICD therapy was defined as antitachycardia pacing (ATP) or an ICD discharge for termination of ventricular tachycardia or fibrillation. A log-rank test was performed to evaluate the potential difference in outcome between the LMNA p.(Arg331Gln) mutation carriers and other *LMNA* mutation carriers, labeled "LMNA group". We also compared the outcome of p.(Arg331Gln) carriers to those of individuals with only a missense *LMNA* mutation (16 probands and 11 family members) from the "LMNA group", labeled "LMNA missense only".

Haplotype analysis and genealogy

To evaluate whether the mutation originated from a common founder, 12 microsatellite markers around *LMNA* were analyzed. Verification of the phase and reconstruction of the haplotype was made possible by analyzing DNA samples of relatives. Calculation of the age of the haplotype was performed as described before with the assumption that a generation equals 20 years.[15] An estimation of the marker frequency in the general population of the first recombinant markers on both sides of the *LMNA* gene in the probands was made by analyzing these markers in 96 unrelated control individuals. Markers D1S305 and D1S2624 were used for this purpose.

To find common ancestors in these different families, we also performed genealogical searches using community registries and official records of births, marriages and deaths.

Linkage analysis

Linkage analysis was performed in the families D, E, G, I, L, M, P and Q. For this purpose we used the linkage program GRONLOD.[16] The model assumptions we used are described in the Supplemental Materials.

Nuclear morphology of LMNA p.(Arg331Gln) fibroblasts

For detailed information about the immunofluorescence staining, see Supplemental Materials. Fibroblasts obtained from a skin biopsy from a patient carrying the LMNA p.(Arg331Gln) variant were stained with the antibody JoL2 for detection of Lamin A/C and then counterstained using DAPI. Structural abnormalities of the nuclei were scored based on abnormalities of nuclear shape and according to the following categories: normal, presence of herniations (blebs), honeycomb structures and/or presence of donut-like nuclear invaginations. They were also compared to nuclear morphology data available from control dermal fibroblast cultures.

Electronic microscopic imaging of the nucleus

See Supplemental Materials for a detailed explanation of the electron microscopy (EM) imaging. Two myocardial biopsies of patients carrying the LMNA p.(Arg331Gln) mutation were fixed with Karnovsky's Fixative, embedded in Epon, and cut into 70 nm sections. They were then viewed with a FEI Tecnai T12 Electron Microscope.

Maximal force development of the sarcomeres

For a detailed description, see Supplemental Materials. Single cardiomyocytes from patients carrying LMNA p.(Arg331Gln) and from control hearts were membrane-permeabilized and glued between a force transducer and piezo motor. Force development was induced by transferring the cell to solutions of calcium with different concentrations (ranging from

physiologic concentrations to a saturating calcium concentration). Force development was recorded with the force transducer. In a later stage, maximal force generation was corrected for myofibril density, measured on EM images.

Statistical analysis

Descriptive statistics are reported as frequency or mean ± standard deviation. We used Kaplan-Meier survival to determine the cumulative event-free survival in LMNA p.(Arg331Gln) carriers. We used the Log-rank test to compare the outcomes for LMNA p.(Arg331Gln) carriers to those of other pathogenic *LMNA* mutation carriers. For the Kaplan-Meier survival analyses, we used MedCalc Statistical Software version 17.1 (MedCalc Software bvba, Ostend, Belgium). An independent two-sided t-test was used to compare the nuclear irregularities. The data was analyzed with the Statistical Package for Social Sciences (SPSS software version 23.0 (IBM Corp., Armonk, NY, USA)). Force development between groups was compared by Student's t-test after normal distribution was confirmed by Shapiro-Wilk normality test. Statistical analysis on force development was performed by GraphPad Prism 5 software. Data of the force measurements and myofibril density are shown as mean ± standard error of the mean. A p-value <0.05 was considered to represent a significant difference between groups.

RESULTS

Mutation analysis

The NGS cardiomyopathy panel was performed in 22 probands. Fourteen additional variants were found in 13 probands with the targeted cardiomyopathy panel, of which one was labelled as 'pathogenic' and the others as 'variant of unknown significance' (**Supplementary Table 1**). Screening of the major lipodystrophy genes (*CAV1*, *PLIN1, PPARG, AKT2*) with whole exome sequencing (WES) was negative for the proband A-III. Analyses of cardiomyopathy related genes screened with WES identified no additional mutation (a list of the screened genes is available on request).

The LMNA p.(Arg331Gln) variant was found twice in the Exome Aggregation Consortium dataset (allele frequency 0.0015%) and four times in the GoNL database (allele frequency 0.4%).[17,18]

Clinical evaluation

For a complete overview of clinical features in mutation carriers see **Table** and **Supplementary Table 2**. Twenty-three probands were identified, of whom 21 presented with cardiac symptoms, one with symptoms of a partial lipodystrophy and one was identified after family screening following sudden cardiac death. Thirty-five family members were identified as car-

rying the mutation. Sixteen family members were already known to have cardiac symptoms prior to genetic family screening (8 males, mean age of presentation 56 ± 7 years), 18 family members were evaluated for the first time in the course of family screening (9 males, mean age at first clinical examination 47 ± 12 years) and from one family member no cardiologic information was available. In both probands and family members, there was a high incidence of (paroxysmal) atrial fibrillation (52% and 42%, respectively) and atrioventricular conduction delay (61% and 44%, respectively). Ventricular arrhythmias were frequently reported in both groups, although the occurrence seemed to be higher in the proband group (83% vs. 40%). Twenty-two of 23 (96%) probands had structural abnormalities of the myocardium, of which 17 (74%) were classified as DCM. Structural abnormalities were present in only 11 (38%) family members, of which four were classified as DCM. The overall mean age at the diagnosis of DCM was 50 ± 15 years. End stage heart failure was seen in six carriers, of whom five received an HTx. Two patients died of heart failure, of whom one received an LVAD while awaiting a HTx. Two patients had an aborted cardiac arrest and two appropriate ICD therapy shocks were administered in total.

Explanted hearts of two patients after HTx showed extensive involvement of the right ventricle. The right ventricle even seemed to be predominantly involved in all three members of family Q. One family member fulfilled the revised Task Force criteria for borderline arrhythmogenic right ventricular cardiomyopathy (ARVC): he had one major criterion (regional akinesia and an end diastolic volume over 110 ml/m^2 measured by MRI) and one minor criterion (non-sustained ventricular tachycardia observed during X-ECG). In the other two family members, a widened right ventricle was observed (MRI; end diastolic volume of 103 and 109 ml/m^2) with a inhomogeneous contraction pattern. In one of them there was also focal bulging of the right ventricle. The left ventricle function was normal in all three subjects.

In family A the mutation seemed to segregate only with a partial lipodystrophy (**Supplementary Figure 1**). In both the proband (A-III) and her mother (A-II) there was loss of adipose tissue around the extremities and accumulation of adipose tissue on the abdomen, neck and face. Other manifestations were hypertension and hypertriglyceridemia. The proband (A-III) in addition had acanthosis nigricans. The mother (A-II) additionaly suffered from hepatic steatosis and diabetes mellitus type 2. The maternal grandmother had diabetes type 2 and was told to have the same physical appearance as A-II and to have unspecified cardiac problems. She died suddenly aged 72 years. During follow up, a dilated left ventricle (end diastolic dimension 62 mm) with systolic dysfunction (ejection fraction of 46%) was observed in the proband (A-III, **Supplementary Figure 1**).

Table. Summary of Characteristics of the Probands & Family Members Carrying the LMNA p.(Arg331Gln) Mutation.

Characteristics	Probands (N=23)	Family members (N=35)
Age presentation/evaluation, yrs (n = 23 & n = 29)*	47 ± (14)	51 ± (12)
Male	17 (74)	18 (51)
Symptoms		
Palpitations (n = 16 & n = 20) *	8 (50)	6 (30)
Syncope (n = 17 & n = 22) *	4 (24)	2 (9)
NYHA class ≥3 (n = 16 & n = 28) *	5 (31)	2 (7)
AV block (n = 18 & n = 25)*		
1st degree	8 (44)	10 (40)
2nd degree	3 (17)	1 (4)
Intraventricular conduction delay (n = 20 & n = 28)*		
LBBB	11 (55)	5 (18)
RBBB	3 (15)	1 (4)
Aspecific		4 (14)
Supraventricular arrhythmias (n = 23 & n = 31)*		
Paroxysmal atrial tachycardia	4 (17)	3 (10)
(Paroxysmal) atrial fibrillation	12 (52)	13 (42)
Ventricular arrhythmias (n = 23 & n = 30)*		
>500 PVCs		2 (7)
NSVT	13 (57)	9 (30)
VT/VF	6 (26)	1 (3)
PM and/or ICD implantation (n = 22 & n = 34)*	17 (77)	6 (18)
Cardiomyopathy (n = 23 & n = 29)*		
DCM	17 (74)	4 (14)
Mild DCM	5 (22)	6 (21)
Other structural abnormalities		1 (3)
HTx or end stage heart failure (n = 23 & n = 30)*	5 (22)	1 (3)
Comorbidity		
Hypertension (n = 23 & n = 29)*	4 (17)	8 (28)
Coronary artery disease (n = 23 & n = 28)*	1 (4)	3 (11)
Diabetes Mellitus type 2 (n = 23 & n = 27)*	2 (9)	1 (4)
Dyslipidemia (n = 23 & n = 27)*	1 (4)	3 (11)
Medication (n = 23 & n = 26)*		
Anti-arrhythmics	22 (96)	11 (42)
ACE inhibitor or ARB	22 (96)	6 (23)
Diuretics	14 (61)	7 (27)

Values are mean ± (standard deviation) or n (%) *number with available data (probands & family members). Data is summary of the data collected to last follow-up. ACE = Angiotensin-converting enzyme, ARB = Angiotensin receptor blocker; AV = Atrioventricular; DCM = Dilated Cardiomyopathy; HTx = Heart transplantation; ICD = Implantable cardioverter defibrillator; LBBB = Left bundle branch block; (NS)VT = (Non sustained) ventricular tachycardia; NYHA = New York Heart Association; PM = Pacemaker; PVCs = Premature ventricular beats; RBBB = Right bundle branch block; VT/VF = Ventricular tachycardia / Ventricular fibrillation.

Time-to-event analysis

Thirteen LMNA p.(Arg331Gln) mutation carriers reached the composite endpoint: appropriate ICD therapy, resuscitation, HTx/LVAD or death. Median survival, i.e. staying free of the composite endpoint, for the p.(Arg331Gln) group was 71 years (CI 95% = 58 to 84 years), which is in contrast to 57 years (CI 95% 53 to 61 years) for carriers of other *LMNA* mutations. Compared to other *LMNA* mutation carriers (both grouped and carriers of only missense mutations) the composite event occurred significantly later in the LMNA p.(Arg331Gln) mutation carriers (log-rank; p<0.001) (**Figure 1**). Information on type of *LMNA* mutation and number of carriers is given in **Supplementary Table 3**. No significant differences were found regarding sex or proband status, comorbidity or use of medication between the groups (**Supplementary Table 4**).

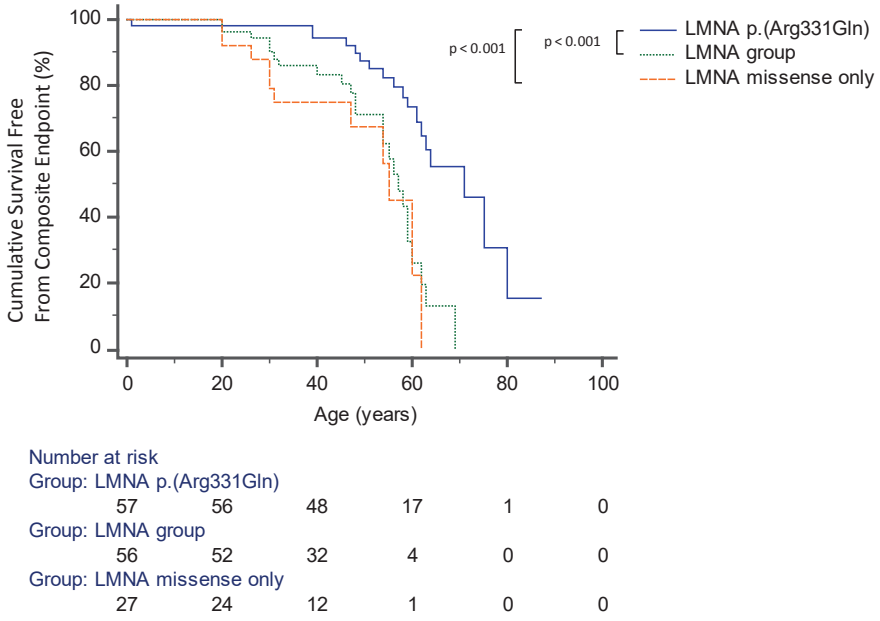

Figure 1. Kaplan-Meier survival analysis

Composite Endpoint = appropriate ICD treatment, resuscitation, HTx/LVAD, death. LVAD = left ventricular assist device. LMNA p.(Arg331Gln) carriers had a significantly better outcome compared to the "LMNA group", which was composed of carriers of different types of *LMNA* mutations. The outcome was also compared to a subgroup of the "LMNA group", which consisted of only *LMNA* missense mutations carriers, called the "LMNA missense only"group.

Haplotype and genealogy

Haplotype analysis was performed in 15 probands and nine family members. A shared haplotype of at least three markers was found covering a 1.00 Mb region surrounding *LMNA* in all the 15 probands (**Supplementary Table 5**). We calculated that the age of the haplotype containing the mutation is between 340 and 760 years old.

Through genealogical research, we found common ancestors in six families. We could genealogically link family A to family E six generations ago, family J to family M six generations ago (**Supplementary Figure 1** and **Supplementary Table 5**), and family U to family S four generations ago (pedigrees not shown).

Linkage analysis

A combined LOD score of 3.46 was found with linkage analysis in the families D, E, G, I, L, M, P and Q (Supplemental Materials). This adds to the likelihood that LMNA p.(Arg331Gln) is linked to the disease. Functional analysis should further substantiate the hypothesis that the observed mutation is the causal one. In family F the segregation was not conclusive. The cardiac phenotype of the LMNA p.(Arg331Gln) negative mother, who had a reduced left ventricular ejection fraction (46%) and frequent ventricular ectopic beats (±18000/24 hours), could also be explained by the fact that she is a carrier of the pathogenic *SCN5A* p.(Gly1319Val) mutation (F-I-1, **Supplementary Figure 1**),[19] as there are more cases described in which *SCN5A* mutations are associated with DCM.[20,21] The family history reported that the father of the proband, an obligate carrier of the LMNA p.(Arg331Gln) mutation, died at the age of 56 years and that the paternal grandfather had a pacemaker.

Nuclear morphology of LMNA p.(Arg331Gln) fibroblasts

Nuclear morphology was analyzed using immunohistochemical staining for lamin A/C in fibroblasts of an LMNA p.(Arg331Gln) carrier (proband I-II-1). Next generation cardiomyopathy panel analysis revealed no additional mutations in this patient. The morphology of 496 nuclei were analyzed. An irregular structure was observed in 22.0 ± 6.4% of the p.(Arg331Gln) nuclei, with a honeycomb-like nuclear structure the most frequently observed irregularity (13.6 ± 8.3%; **Figure 2**). Nuclear blebbing and donut-shaped nuclei were observed in 5.8 ± 3.7% and 2.0 ± 1.4% of the p.(Arg331Gln) fibroblasts, respectively. The findings are consistent with abnormalities of the nuclear membrane in other pathogenic *LMNA* mutations.[22] In contrast, the eight control fibroblast cultures displayed fewer nuclear irregularities, 5.9 ± 1.4% ($p < 0.01$), of which 0.9 ± 1.1% were honeycomb structures ($p < 0.01$), 2.1 ± 1.8% ($p = 0.04$) nuclear blebbing and 1.4 ± 0.7% (no significant difference) donut-shaped nuclei (data not shown).

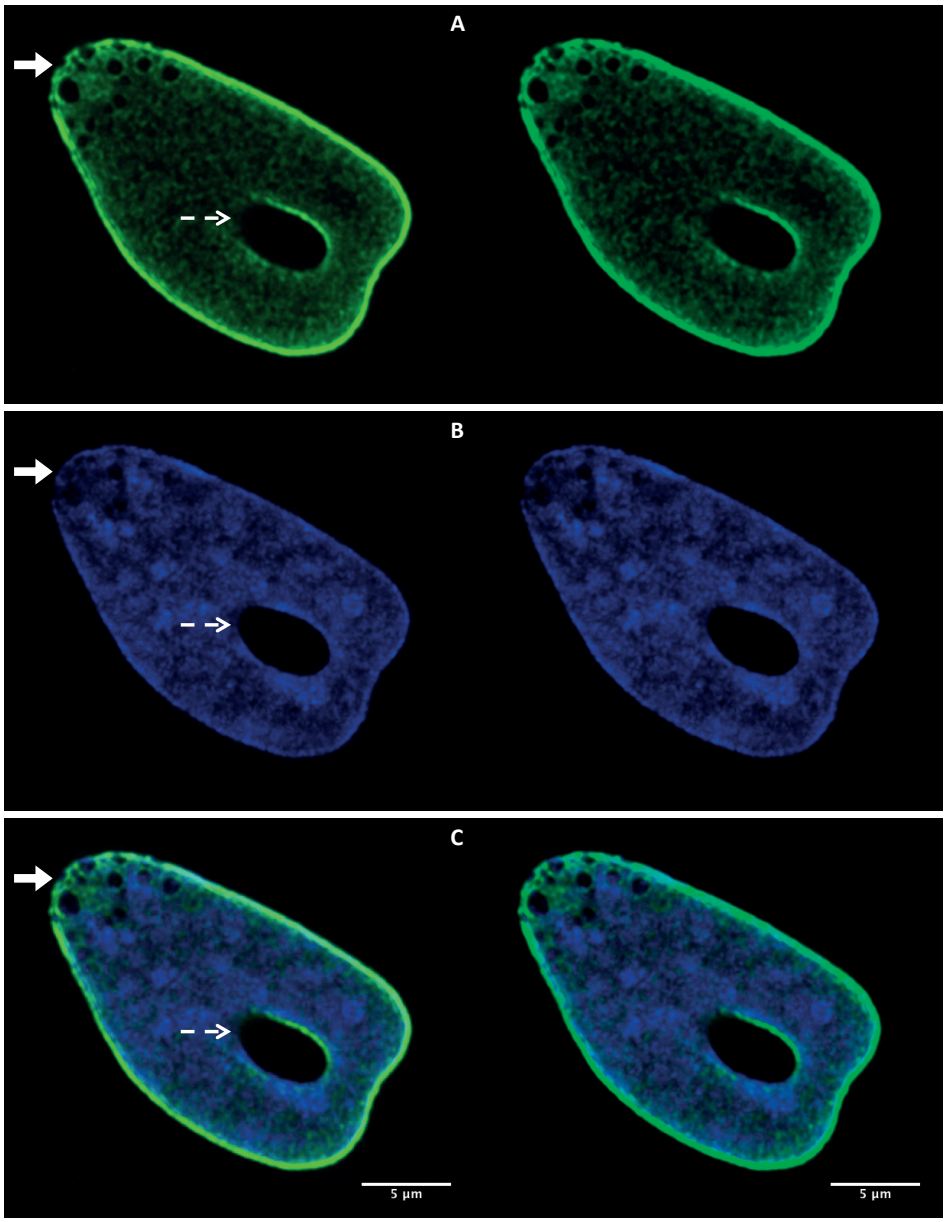

Figure 2. Nuclear envelope immunostainings of skin fibroblasts of an LMNA p.(Arg331Gln) carrier
A) Lamin A/C staining with antibody JoL2. B) DAPI staining. C) Lamin A/C staining and DAPI staining merged. Broken white arrow indicates donut-like nuclear invaginations. Continuous white arrow indicates honeycomb-like nuclear structure.

Electron microscopic imaging of the nucleus

EM of myocardial biopsies of two patients demonstrated the irregular and convoluted shapes of the enlarged nuclei of the cardiomyocytes (**Figure 3**). In patient P-III-2 (family-member), carrier of additional variant of unknown significance in the gene encoding desmoplakin (*DSP*) (DSP p.(Lys2706Met)), blebs of the nuclear membrane into the cytoplasm were observed (**Figure 3B**). In proband B, who carried two additional variants of unknown significance (LMNA p.(Arg156Leu) and TTN p.(Phe9717Serfs*23)), a discontinuous layer of heterochromatin of the inner nuclear membrane was observed in several areas of the nuclei. In this patient some small indications of blebs were observed, but larger ones were not evident. Since Lamin A and C play a role in the structural stability of the nuclear membrane, the ultrastructural defects of the nuclear membrane described above are often seen in conjunction with *LMNA* mutations.[23,24]

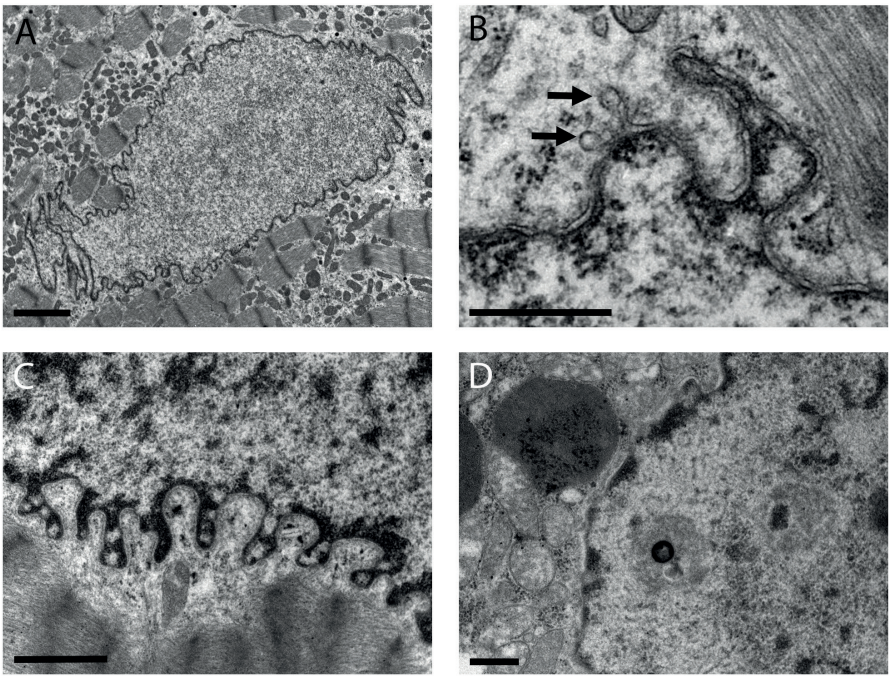

Figure 3. Electron microscopy of myocardium of Patients P-III-2 and B-III-1
A and B. Patient from family P. A) Nucleus of cardiomyocyte with convoluted shape. Bar = 2 μm. B) detail of nuclear membrane with small blebs of the nuclear membrane into the cytoplasm (arrows). Bar = 500 nm. C and D. Proband from family B. C) irregular shape of the nuclear membrane. Bar = 1 μm. D) detail of nuclear membrane showing a discontinuous layer of chromatin of the nuclear membrane, possible enlarged nuclear pores. Bar = 500 nm.

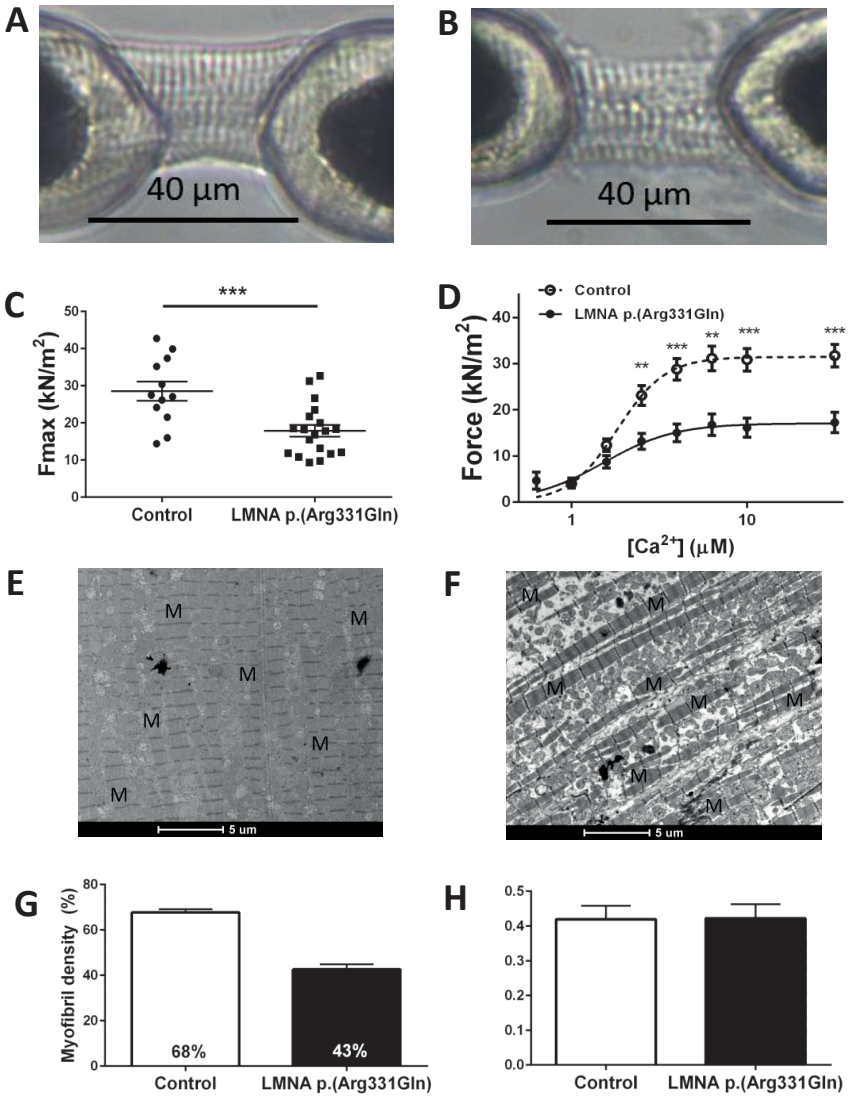

Figure 4. Maximal force development of the sarcomeres

A and B. Mechanical isolated cardiomyocyte of a control heart (A) and a cardiomyocytes of a patient with the LMNA p.(Arg331Gln) mutation (B) glued between a force transducer and piezo motor. C) Patients with the LMNA p.(Arg331Gln) mutation (N = 3, n = 19) showed a significantly decreased maximal force development compared with controls (N = 2, n = 12) (p = 0.002). D) Force development was significantly lower in p.(Arg331Gln) samples (N = 3, n = 11) compared to controls (N = 2, n = 9) over a range of submaximal (physiological) calcium concentrations. E, F and G) Myofibril density was lower (F) in the LMNA p.(Arg331Gln) patients (N = 3 (G)) compared to (E) control hearts (N=2 (G)). H) Maximal force development corrected for myofibril density was similar in the LMNA p.(Arg331Gln) patients (N = 3, n = 19) compared to the control hearts (N = 2, n = 12). For each sample, at least four electron microscopy images at a magnification of 2250x were used to calculate average the myofibril density. N= number of patients, n=number of cardiomyocytes.

Maximal force development of the sarcomeres

In **Figure 4A** a cardiomyocyte of a control heart and in **Figure 4B** a cardiomyocyte of a patient with the LMNA p.(Arg331Gln) variant is visualized at sarcomere length 2.2 μm. Cardiac tissue of three carriers (B-II-1, N and P-III-2) was used for this analysis. B-II-1 carried two additional variants and P-III-2 carried one additional variant of unknown significance (**Supplementary table 2**). After transfer of the cell to a solution containing an activating concentration of calcium, the cell developed force, which was recorded by the force transducer. Patients with the *LMNA* p.(Arg331Gln) mutation showed a significantly decreased maximal force development (17.9 kN/m^2) compared with controls (28.5 kN/m^2) ($p = 0.002$, **Figure 4C**). This indicates an effect of the variant through impairment of cardiomyocyte contractility. As shown in **Figure 4D**, force development was significantly lower in p.(Arg331Gln) variant samples compared to controls over a range of submaximal (physiological) calcium concentrations.

In some cases of hypertrophic cardiomyopathy a reduction of myofibril density underlies the lower force generating capacity.[25] We hypothesized that this could also be the case for the LMNA p.(Arg331Gln) variant. Myofibril density was calculated as a percentage of total cardiomyocyte area by EM, and myofibril density was found to be lower in the hearts of LMNA p.(Arg331Gln) patients (**Figure 4F**) when compared to control hearts (**Figure 4E**). Myofibril density was 43% in the LMNA p.(Arg331Gln) patient hearts compared to 68% in control hearts (**Figure 4G**). Maximal force development corrected for myofibril density was similar in the LMNA p.(Arg331Gln) patients compared with control hearts (**Figure 4H**). This indicates that the decreased force generation observed in the p.(Arg331Gln) mutation in LMNA is probably due to the reduced myofibril density.

DISCUSSION

Interpretation of missense mutations is especially challenging when a variant is also present in a control population, the situation we encountered here for the LMNA p.(Arg331Gln)) variant. Although we are not the first to describe the LMNA p.(Arg331Gln) variant, we were able to collect the largest cohort of carriers to date. In a previous report this variant was found in a patient who was compound heterozygous (carrier of the LMNA p.(Glu347Lys)). In addition, another variant at the same position, LMNA p.(Arg331Pro), was described to be associated with DCM, conduction delay and limb-girdle muscular dystrophy.[26] In another report the parents of the proband were not screened for the mutation and were seemingly unaffected (only the father had atrial fibrillation).[27] Extensive evaluation of clinical, segregation and functional data helped us to classify this mutation as truly pathogenic and we decided to communicate this as such to the carriers of this mutation. Moreover, its associated

phenotype is consistent with that described in carriers of other *LMNA* mutations but milder in terms of significant clinical events (malignant arrhythmias, end stage heart failure, or death).

The phenotype in our cohort is characterized by a high incidence of (paroxysmal) atrial fibrillation ((p)AF) and atrioventricular conduction delay in both probands and family members ((p)AF, 52% and 42%: AV-block, 61% and 44%). These findings are consistent with the clinical findings of two studies of 269 and 299 *LMNA* mutation carriers.[4,6] These *LMNA* mutation carriers demonstrated a high prevalence of atrial tachyarrhythmia (36%) and conduction disease (47%).[6] More specifically, Fatkin et al described four missense mutations in the rod domain, where the p.Arg331Gln variant is also situated, with a phenotype (AV-conduction delay, atrial fibrillation, sinus bradycardia and DCM) similar to that seen in our cohort.[28] However, malignant ventricular arrhythmias (appropriate ICD therapy, SCD, OHCA, ventricle fibrillation) did not seem to occur as often (12%) in our cohort compared to *LMNA* patient series described in literature, where malignant ventricular arrhythmias were observed in 24% to 28% of the cases.[6,29] The milder phenotype in our carriers is corroborated by the relatively infrequent occurrence of appropriate shock and ATP therapy (only two shocks and three ATP therapies in 86 patient years). In both cases of ICD shock, there were additional factors that could have played a role (poor LV systolic function and evidence of an old myocardial infarction). This is in contrast to the observation in patients with other *LMNA* mutations where 28% to 42% of the carriers seemed to benefit (appropriate therapy) from ICD implantation[6,8] The diagnosis of DCM in our cohort was made relatively late in life (50 $\pm$ 15 years), compared to that of the group DCM patients carrying other pathogenic *LMNA* mutations, for whom an age of onset of 40 $\pm$ 10 years is described.[29] Structural abnormalities were only apparent in eleven (38%) family members. However, 65% of the family members had electric disturbances of the heart (evidence of sinus node dysfunction, cardiac (AV) conduction delay, atrial or ventricular arrhythmias, with no structural abnormalities of the heart (yet)). In *LMNA* mutations, it is a well-known phenomenon that electric abnormalities, like conduction delay and arrhythmias, often precede the structural abnormalities.[4,28] Regular follow-up is warranted because these initial electric abnormalities could be the first signs of structural abnormalities, which could be followed by an impaired function and LV dilatation.

One notable aspect of our study is the pleiotropic effect of the LMNA p.(Arg331Gln) mutation, which is demonstrated by the differences in phenotypes between the families. This is most striking in family A where the mutation seemed to segregate with a partial lipodystrophy (PLD) phenotype. When PLD is associated with *LMNA* mutations, the disease is also referred to as FLDP2 (familial partial lipodystrophy type 2), which is an autosomal dominant disease that mostly results from missense mutations in the C-terminal region of the *LMNA* gene and is characterized by progressive abnormal subcutaneous adipose

distribution.[30] However mutations in the N-terminal head and alpha-helical rod domain in which the FLDP2 is accompanied by cardiomyopathy and conduction disorders have also been described.[31] The apparent absence of this lipodystrophic phenotype in the other families suggests there is another possible genetic cause. However, screening of other major lipodystrophy genes was normal. Other results of the pleiotropic effects of this mutation are the findings of the apparent solely RV involvement in family Q. Recently, another LMNA mutation (p.(Leu140_Ala146dup)) was described as associated with both ARVC and DCM.[32] In two other studies, genetic screening in patients with ARVC revealed five missense mutations and one nonsense mutation in *LMNA* in the absence of mutations in the desmosomal genes.[33,34] Although DCM was the predominant form of cardiomyopathy in our cohort, RV involvement was seen in 57% of the patients with DCM and the available pathology reports in two probands describe extensive right ventricle involvement. This suggests that LMNA related disease may mimic ARVC. The heterogenous phenotype might be influenced by additional genetic factors (**Supplementary Table 2**), yet this series of patients is too small to systematically evaluate this.

Marker analysis showed a common haplotype of 1 Mb, suggesting a founder mutation. Slippage during replication of DNA in one of the ancestors could explain the difference in length of marker D1S1153 found in the two groups.

Although most of the families were small, a dominant autosomal inheritance pattern could be observed. The calculated combined LOD score was well over 3, an additional observation suggesting pathogenicity of this variant. A limitation of the segregation analysis is the fact that we counted a subject as affected when he or she displayed one of the phenotypes commonly observed with *LMNA* mutations. As described earlier, the phenotype can be highly variable and some of the phenotypes, e.g. AF and conduction disease, are also found in relatively high frequencies in the general population in absence of *LMNA* mutations. To take this into account we calculated the LOD scores with a phenocopy frequency (e.g. AF 10%, conduction disease 10%) higher than expected for the general population (see Supplemental Materials). Still, that resulted in a LOD score of more than 3. Non-segregation was observed possibly once and has been described before in a large *LMNA* family.[35] In family F the cardiac phenotype of the mother (F-I-1) could be explained by a pathogenic *SCN5A* mutation, as it is recognized that *SCNA5A* mutations can also cause DCM.[20] The same *SCN5A* mutation was also found in our laboratory in two unrelated patients with cardiomyopathy, while screening of 53 or 55 other cardiomyopathy-related genes revealed no additional mutations in those subjects (unpublished data).

Lamins A and C are important components of the nuclear envelope and, when mutated, abnormalities of the nuclear envelope can be observed.[22] Irregular nuclear structures were

significantly more frequently observed in fibroblasts of a LMNA p.(Arg331Gln) carrier. The nuclear abnormalities observed in the p.(Arg331Gln) fibroblasts are in line with the nuclear irregularities observed in fibroblasts of other established pathogenic mutations in *LMNA*, thereby supporting its pathogenicity.[22,36,37,38]

Like the abnormal nuclear structures in the fibroblasts observed using immunofluorescence, ultrastructural investigation on diseased cells in cardiac tissue with EM also support pathogenicity. The convoluted shapes of the nuclei, blebs, discontinuous layer of heterochromatin and possible enlarged nuclear pores are features commonly seen in other *LMNA* mutations.[23,24,39] It should be kept in mind that such structural defects can also be found in DCM patients without *LMNA* mutations.[40]

Apart from their function in nuclear stability, it has been suggested that lamin proteins are important for the structural integrity of the whole cell through interactions between nuclear lamina, the cytoskeleton and the extracellular matrix.[41,42] The lamin A/C coil 2B domain in which the p.(Arg331Gln) mutation is located is important for homodimerization of lamin proteins. Gangemi et al. indicated that the p.(Arg331Gln) mutation might affect lamina stability, because it has been predicted to impair dimerization of the lamin proteins due to loss of salt-bridge interactions.[43] This might explain the detrimental effect on the heart since correct assembly of dimers is essential for protein function. In addition, it is known that myofilaments in cardiomyocytes create nuclear deformation in the plane parallel to the myofilaments during contraction.[42] Therefore, the continuous mechanical stress during contractions in cardiomyocytes can have pathological effects on nuclear structure over time in patients with the p.(Arg331Gln) mutation. Our study supports this possibility by showing impairment of nuclear architecture and decreased myofibril density in patients with the p.(Arg331Gln) mutation causing a reduction in cardiomyocyte force development.

LIMITATIONS

The observational design of this study is prone to introduction of survival bias. Initially, genetic testing focused on the most severely affected cases and currently patients with a less severe phenotype are more easily referred for genetic testing. However, this study includes all patients that were identified since the start of *LMNA* screening in 2001 and therefore is likely to reflect both sides of the spectrum. The combined LOD score of more than 3 does not mean significant linkage due to a possible selection bias in the families selected. It should be considered a strong indication that the mutation segregates with the observed phenotypes.

CONCLUSION

Genetic and segregation data support the pathogenic effects of LMNA p.(Arg331Gln). Electron microscopy and immunofluorescence showed an effect on nuclear architecture. In addition, the LMNA p.(Arg331Gln) mutation causes decreased myofibril density resulting in reduced force development at saturating and physiological calcium concentrations. The clinical phenotype related to the LMNA p.(Arg331Gln) founder mutation is generally characterized by a phenotype (consisting of cardiac conduction delay, (atrial) arrhythmias, and dilated cardiomyopathy with a later onset and more favorable prognosis compared to other pathogenic *LMNA* mutations. Further research is needed to elucidate the role of other contributing factors leading to the clinical variability.

CLINICAL PERSPECTIVE

Dilated cardiomyopathy is characterized by an impaired systolic dysfunction and dilatation of the left ventricle. Although genetically heterozygous, *LMNA* is one of the most frequent genes found to be mutated in dilated cardiomyopathy. *LMNA*-related cardiac disease is associated with a high incidence of malignant ventricular arrhythmias and patients with an *LMNA* mutation are believed to have a poor prognosis. With the increasing use of next-generation sequencing based on dedicated gene panels, variants leading to an abnormal protein will be identified more often. Correct interpretation of these genetic variants is, however, challenging, especially when the variant is also found in control populations, as is the case for the LMNA p.(Arg331Gln) variant. After evaluating the clinical data, performing segregation analysis, and studying the nuclear morphology in cardiomyocytes and fibroblasts, we have classified this variant was as pathogenic. This allows for detection of asymptomatic mutation carriers and monitor them for early signs of disease and early intervention. Additionally, we show that LMNA p.(Arg331Gln) carriers seem to have a more favorable clinical course compared with patients with other *LMNA* mutations. This provides clinicians with the opportunity to address uncertainties regarding the disease course. Our data also provide novel insight into the possible pathogenesis of dilated cardiomyopathy caused by *LMNA* mutations, by showing that the decreased force development in the cardiomyocytes is likely to be because of secondary disease remodeling.

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SUPPLEMENTAL DATA

METHODS

Clinical evaluation

Sinus node dysfunction was defined as sinus bradycardia of less than 50 beats per minute on a resting ECG, chronotropic incompetence (inadequate increase of heart rate (≤ 80%) of expected value, during X-ECG) and pauses of more than three seconds observed with Holter monitoring during sinus rhythm or two seconds when atrial fibrillation was the underlying rhythm. Dilated cardiomyopathy (DCM) was defined by 1) diminished left ventricular contractile function (left ventricular ejection fraction <45% and or left ventricular shortening <25%) and 2) ventricular dilatation (left ventricular end-diastolic dimensions >95th percentile indexed for body surface area, age and sex in case of MRI).[1–3]. If only one of the two criteria was fulfilled (either left ventricular dilatation or dysfunction), the patient was labeled as having 'mild DCM'. If bodyweight or end diastolic diameter was unavailable, we still recorded a diagnosis of DCM if this was stated in the clinical information. A diagnosis of borderline arrhythmogenic right ventricular cardiomyopathy (ARVC) was made when one major criterion and one minor criterion or three minor criteria from different groups were fulfilled according the modified Task Force criteria.[4]

Linkage analysis 'GRONDLOD'

The aim of linkage analysis is to test if a genetic variant cosegregates in a genome wide significant way with observed phenotypes in affected families. *LMNA* mutations are associated with a clinically variable phenotype. The phenotypes observed are, however, not exclusively found in the presence of *LMNA* mutations and some of these phenotypes have a relatively frequent occurrence in the general population (increasing with age), atrial fibrillation and conduction disease for example. To take account for possible phenocopies and age-related penetrance, we used the linkage program GRONDLOD because this program has the advantage of easily defining genetic model assumptions with complex phenotype-genotype relations.[5] Families D, E, G, I, L, M, P and Q were used to calculate LOD scores at zero recombination frequency (**Supplementary figure 1**). Linkage analysis cannot prove that genetic variants are causally implicated in a disease, only that the co-segregation of a variant with a phenotype is more likely if the observed variant is associated with an assumed causative locus than when it is unlinked.

The following model assumptions were used for the computation:

We defined phenotypes as follows (abbreviations); unknown, normal, supraventricular tachycardia (SVT), atrial fibrillation (AF), conduction disease (CD), dilated cardiomyopathy (DCM), dilated cardiomyopathy with conduction disease (CD_DCM), cardiomyopathy with atrial fibrillation and conduction disease (AF_CD_CM) and ventricular arrhythmias

(VA). For family Q a phenotype cardiomyopathy unspecified' (CM_unsp) was used because although there were apparent structural abnormalities in all three family members, only one fulfilled the diagnosis for borderline ARVC. Because no definite diagnosis of a cardiomyopathy could be made, we allowed for a higher frequency of phenocopies (10%).

The disease gene is considered to be a rare variant and the allele frequency was set to 0.001 and the normal variant to 0.999.

The probabilities for a certain phenotype given an genotype were as follows:

Explanation: the first number after the curved bracket is the disease locus, the second and third numbers describe the autosomal marker phenotype and the disease phenotype follows between the double quotes. The last number reflects the assumed probability of the genotype conditional on the disease status genotype.

phen_gen(1,1,1,"SVT",0.3) // allows for 30% phenocopies

phen_gen(1,1,2,"SVT",0.5) // 50% penetrance for carriers

phen_gen(1,2,2,"SVT",1)

phen_gen(1,1,1,"AF",0.1) // allows for 10% phenocopies

phen_gen(1,1,2,"AF",0.5) // 50% penetrance for carriers

phen_gen(1,2,2,"AF",1)

phen_gen(1,1,1,"CD",0.1) // allows for 10% phenocopies

phen_gen(1,1,2,"CD",0.5) // 50% penetrance for carriers

phen_gen(1,2,2,"CD",1)

phen_gen(1,1,1,"AF_CD",0.05) // allows for 5% phenocopies

phen_gen(1,1,2,"AF_CD",0.4) // 40% penetrance for carriers

phen_gen(1,2,2,"AF_CD",1)

phen_gen(1,1,1,"DCM",0.005) // allows for 0.5% phenocopies

phen_gen(1,1,2,"DCM",0.5) // 50% penetrance for carriers

phen_gen(1,2,2,"DCM",1)

phen_gen(1,1,1,"CD_DCM",0.001) // allows for 0.1% phenocopies

phen_gen(1,1,2,"CD_DCM",0.4) // 40% penetrance for carriers

phen_gen(1,2,2,"CD_DCM",1)

phen_gen(1,1,1,"AF_CD_CM",0.001) // allows for 0.1% phenocopies

phen_gen(1,1,2,"AF_CD_CM",0.4) // 40% penetrance for carriers

phen_gen(1,2,2,"AF_CD_CM",1)

phen_gen(1,1,1,"VA",0.2) // allows for 20% phenocopies

phen_gen(1,1,2,"VA",0.5) // 50% penetrance for carriers

phen_gen(1,2,2,"VA",1)

phen_gen(1,1,1,"CM_unsp",0.1) // allows for 10% phenocopies

phen_gen(1,1,2,"CM_unsp",0.5) // 50% penetrance for carriers

phen_gen(1,1,2,"CM_unsp",1)

Nuclear morphology of LMNA p.(Arg331Gln) fibroblasts

Fibroblasts from a skin biopsy taken from one patient (proband I-II-1) with the LMNA p.(Arg331Gln) were cultured for immunostaining. Early passage cells (p3-5) were seeded at a low density and were allowed to attach for two days before fixation. Detection of lamin A/C was performed using antibody JoL2 (kindly provided by Dr. C. Hutchison, Durham University, UK) as described previously.[6] The nuclei were counterstained using DAPI. Imaging was performed by means of an inverted confocal microscope (Leica SPE) mounted on a DMI4000 inverted microscope. Per fibroblast culture, the morphology of at least 5x100 nuclei was scored by two independent researchers. Nuclear morphology scores were based

on abnormalities of nuclear shape and irregular immunostaining for lamin A/C. Nuclei were scored according to the following categories: normal; presence of herniations (blebs); and/or honeycomb structures (visualized by immunolabelling with JoL2); presence of donut-like nuclear invaginations.

Electronic microscopic imaging of the nucleus

Myocardial biopsies of the apex of the heart of two patients (family-member P-III-2 and proband B) obtained during implantation of a LVAD were studied with electron microscopy (EM). Myocardium was fixed in Karnovsky's Fixative and embedded in Epon, and 70 nm sections were cut. The sections were mounted onto formvar-coated copper grids (Stork Veco, Eerbeek, the Netherlands) and stained with a 5% solution of uranyl acetate, followed by Reynold's lead citrate. Sections were viewed with a FEI Tecnai T12 Transmission Electron Microscope (FEI, Hillsboro, Oregon, USA).

Maximal force development of the sarcomeres

Samples:

Force measurements in membrane-permeabilized single cardiomyocytes and EM images were acquired from three patients with the LMNA p.(Arg331Gln) mutation. Myocardial biopsies of the apex of the heart were obtained prior to LVAD implantation in two patients. In one patient, explanted left ventricular myocardial tissue was collected during heart transplantation. Left ventricular cardiac tissue of two patients who died of a non-cardiac cause served as controls.

Skinned cardiomyocyte force measurements:

Force development of sarcomeres was measured in single membrane-permeabilized cardiomyocytes mechanically isolated from heart tissue as previously described[7] and corrected for cross sectional area. Mechanically isolated cardiomyocytes were glued between a force transducer and piezo motor and stretched to a sarcomere length of 2.2 μm. Force development was recorded with the force transducer attached to the cell. When force development reached a plateau, the cell was shortened by 30% of its length in order to detach cross-bridges and determine the total force generated. Total force development was calculated by the difference between force at plateau and force at slack length. The cell was then transferred back to a relax solution where it was again shortened by 30% to calculate passive force development. Maximal force development was calculated by subtraction of passive force from total force at a saturating calcium concentration of 31.6 μM. In addition, force development was also measured at a range of submaximal (physiological) calcium concentrations.

Myofibril density:

EM pictures were analyzed at a magnification of 2250x. Myofibril density was calculated as a percentage of cardiomyocyte area by ImageJ software. For each sample 4 or 5 images were used to calculate average myofibril density. Maximal force was corrected for myofibril density by dividing the maximal force generated by individual cardiomyocytes by the average myofibril density of the corresponding sample.

Supplementary Table 1. Other Genetic Variants identified.

Gene	Position	Protein	Effect	Classification	Description
ANKRD1	c.651+1G>A		Splice site mutation	VUS	No cardiac abnormalities in the family this variant originated from (family M, Supplementary Figure 1).
DSC2	c.942+3A>G		Splice site mutation	VUS / Likely benign	RT-PCR revealed no aberrant splicing of mRNA in proband carrying this additional mutation.
DSP	c.8500C>T	p.(Arg2834Cys)	Missense	VUS	Did not segregate with phenotype "Mild DCM, conduction disease and atrial fibrillation" in the family where this mutation was found (family G, supplementary Figure 1).
DSP	c.8117A>T	p.(Lys2706Met)	Missense	VUS	Found 5x in ExAC. South Asian background; 3/16512 alleles. European (Non-Finnish) background; 2/66626 alleles.
LMNA	c.467G>T	p.(Arg156Leu)	Missense	VUS	
	c.1879C>T	p.(Arg627Cys)	Missense	VUS	No nuclear envelope abnormalities were found in fibroblasts of the carrier (brother of proband) with only this variant.

Supplementary Table 1. Other Genetic Variants identified. (*continued*)

Gene	Position	Protein	Effect	Classification	Description
LDB3	c.1885G>A	p.(Ala629Thr)	Missense	VUS	
MYH6	c.3809G>A	p.(Arg1270His)	Missense	VUS	Did not segregate with phenotype "sinus node dysfunction"
RYR2	c.8147A>T	p.(Lys2716Ile)	Missense	VUS	Did not segregate with phenotype "atrial fibrillation" in the family where this mutation was found (family G, supplementary Figure 1).
SCN5A	c.3956G>T	p.(Gly1319Val)	Missense	Pathogenic	Associated with Brugada syndrome.[8] Could be responsible for cardiac phenotype in the mother of proband (family F, supplementary Figure 1).
TTN	c.16452delA	p.(Lys5484Leufs*20)	Frameshift	VUS / Likely benign	Resided in isoform novex-3 transcript, minor small cardiac isoform. Low expression in heart therefore possibly "likely benign".
	c.29148delC	p.(Phe9717Serfs*23)	Frameshift	VUS	Resided in exon 103 in the I-band. Not incorporated in major cardiac isoform N2B.
	c.61688T>A	p.(Ile20563Asn)	Missense	VUS	
	c.102275G>A	p.(Arg34092His)	Missense	VUS	Found 6x in European (non-Finnish) population (6/66688 alleles). Not incorporated in major cardiac isoform N2B.

ANKRD1 = Ankyrin Domain 1; *DSC2* = Desmocollin 2; *DSP* = Desmoplakin; *LDB3* = LIM Domain-Binding 3; ExAC = The Exome Aggregation Consortium; *LMNA* = Lamin; *MYH6* = Myosin heavy chain 6; *RYR2* = Ryanodine receptor 2; *SCN5A* = Cardiac sodium channel α subunit ; *TTN* = Titin;VUS = Variant of unknown significance.

Supplementary Table 2. Individual LMNA p.(Arg331Gln) carrier characteristics ordered by pedigree.

Subject	Sex	Indication genetic screening	Presentation (Age, Yrs.)	Follow-up (Yrs.)	CCD/ECG Abnormalities	SVA	VA	LVF* (EF)	LVEDD* Other
A-II	F		Atypical symptoms (65)	2	SND	No	No	Normal	41 mm
A-III	**F**	**PLD**	**Symptoms of PLD (39)**	**2**	**No**	**No**	**No**	**46%**	**62 mm RWMA**
B-III-1	**M**	**DCM**	**Dyspnea on exertion (35)**	**13**	**LBBB AV-block (2nd) Low voltage**	**No**	**NSVT**	**20%**	**60 mm**
B-III-2	F		Screening (39)	0	No	No	No	55%	50 mm
B-III-3	F		Screening (47)	0	LAHB	No	No	Normal	50 mm
C	**M**	**DCM**	**Palpitations, AF (61)**	**16**	**LBBB SND**	**AF**	**NSVT**	**30%**	**63 mm RWMA**
D-II-1	F		MI (62)	11	LBBB AV-block (1st)	AF	NSVT	Normal	.
D-III-1	**M**	**DCM**	**AF (46)**	**13**	**LBBB AV-block (1st) Low voltage**	**AF**	**NSVT**	**40%**	**Dilated† RWMA LGE-**
D-III-2	M		AF (52)	8	IV cond. delay (124 ms) AV-block (1st)	AF	>500 PVCs/ 24h	27%	58 mm RWMA
E-II-1	**M**	**DCM**	**Syncope, AF (54)**	**2**	**LAHB, RBBB SND**	**AF**	**NSVT**	**37%**	**62 mm LGE+**
E-II-2	M		Screening (55)	0	SND PVCs on ECG	No	No	Normal	54 mm
E-II-3	M		pAF (48)	4	AV-block (1st) Low voltage	pAF	No	61%	51 mm
F-II-1	**M**	**Suspected ARVC**	**Fatigue (40)**	**2**	**RBBB SND**	**No**	**No**	**49%**	**59 mm LGE-**
G-II-1	**M**	**DCM**	**NSVT, AF (53)**	**8**	**LBBB AV-block (1st)**	**AF**	**NSVT**	**28%**	**66 mm**

RVF* (EF)	RVEDD* Other	Hypertension	Dyslipidemia	DM type 2	CAD	Additional variant(s)	BMI (kg/m^2)	Outcome	Comment
Normal	.	+	+	+	-	-	24,7	PLD Beta-blocker Diuretic	
.	**.**	**+**	**+**	**-**	**-**	**-**	**34,3**	**PLD** **Mild DCM**	**Beta-blocker** **ATr antagonist** **Diuretic**
Impaired	**.**	**-**	**-**	**-**	**-**	**+**	**22,8**	**DCM** **CRT-D** **LVAD, HTx**	***TTN* c.29148delc** ***LMNA* c.467G>T** **PA: Heart weight 400 gram, extensive diffuse fibrosis**
Normal	Normal	-	-	-	-	+	21,5	Asymptomatic	*TTN* c.29148delc
Normal	Normal	-	-	-	-	-	26,3	CCD	
Impaired	**Dilated** **RWMA**	**-**	**-**	**-**	**-**	**-**	**22,6**	**DCM** **CRT-P**	
Normal	.	+	+	-	+	+	.	CVA, death (80)	*TTN* c.61688T>A Beta-blocker
26%	**.**	**-**	**-**	**-**	**+**	**+**	**22,9**	**DCM** **CRT-D**	***TTN* c.61688T>A**
.	.	+	-	-	-	.	30,6	DCM Recovery LVF LV hypertrophy	Beta-blocker ATr antagonist Diuretic
54%	**Dilated**	**-**	**-**	**-**	**-**	**+**	**20,4**	**DCM** **PM, ICD**	***MYH6* c.3809G>T**
Normal	Normal	**-**	**-**	**-**	**-**	-	23,1	CCD	
Normal	**.**	**-**	**-**	**-**	**-**	+	27,8	CCD SVA	*MYH6* c.3809G>T Flecainide
40%	**TFC 2010+** **Pron. trab.**	**-**	**-**	**-**	**-**	**+**	**21,0**	**Mild DCM / Possible ARVC** **PM, ICD**	***SCN5A* c.3956G>T** **ACE inhibitor**
Poor	**.**	**-**	**-**	**+**	**-**	**+**	**26,2**	**DCM** **CRT-D** **HTx**	***RYR2* c.8147A>T** ***DSP* c.8500C>T** **PA: Heart weight 470 gram, interstitial fibrosis**

Supplementary Table 2. Individual LMNA p.(Arg331Gln) carrier characteristics ordered by pedigree. (*continued*)

Subject	Sex	Indication genetic screening	Presentation (Age, Yrs.)	Follow-up (Yrs.)	CCD/ECG Abnormalities	SVA	VA	LVF* (EF)	LVEDD* Other
G-II-2	M		LBBB, pAF (56)	7	LBBB AV-block (2nd) Low voltage	pAF	NSVT	30%	54 mm LGE+
G-II-3	F		Screening (56)	3	LBBB AV-block (1st)	No	No	49%	47 mm LGE-
G-II-4	F		IV cond. delay (60)	4	LBBB AV-block (1st)	pAF	No	50%	53 mm
G-III-1	M		Screening (36)	0	No	No	NSVT	Normal	45 mm
H	**M**	**DCM**	**pAF (64)**	**7**	**.**	**AF**	**No**	**26%**	**58 mm**
I-II-1	**F**	**DCM**	**Atypical chestpain (54)**	**0**	**LBBB AV-block (1st)**	**No**	**NSVT**	**40%**	**58 mm**
I-II-1	F		CAD (60)	9	IV cond. delay (QRS 120 ms) AV-block (1st)	pAF	No	16%	57 mm RWMA LGE+
I-III-1	M		Screening (32)	2	IV cond. delay (QRS 145 ms)	No	No	Normal	56 mm
I-III-2	M		Screening (44)	0	No	No	No	Normal	Normal
J-I-1	M	Obligate carrier	.	.	.	.	.	.	.
J-II-1	**M**	**DCM**	**Dyspnea on exertion, palpitations (60)**	**4**	**LBBB AV-block (1st)**	**pAF**	**NSVT**	**36%**	**64 mm LGE+**
J-II-2	M		Screening (61)	2	RBBB, LAHB AV-block (1st)	No	No	Normal	56 mm
J-III-1	F		Screening (28)	0	No	No	No	Normal	48 mm
K	**M**	**DCM**	**pAF (56)**	**11**	**LBBB AV-block (1st) Low voltage**	**AF**	**NSVT**	**31%**	**60 mm Pron. trab. LGE+**

RVF* (EF)	RVEDD* Other	Hypertension	Dyslipidemia	DM type 2	CAD	Additional variant(s)	BMI (kg/m^2)	Outcome	Comment
Normal	.	+	–	–	–	+	31,9	Mild DCM CRT-D	*RYR2* c.8147A>T *DSP* c.8500C>T
49%	43 mm	–	–	–	–	+	28,3	CCD	*RYR2* c.8147A>T
Normal		+	–	–	–	–	26,3	Mild DCM CCD SVA	Beta-blocker ACE inhibitor Diuretic
.	.	–	–	–	–	–	23,6	VA	
Normal	**.**	**+**	**–**	**–**	**–**	**+**	**24,8**	**DCM CRT-D Recovery LVF**	***TTN* c.102275G>A Beta-blocker ACE inhibitor Diuretic**
.	**.**	**–**	**–**	**–**	**–**	**–**	**19,4**	**DCM CRT-D**	
Normal	Normal	+	–	–	+	–	22,4	DCM CRT-D	Alcohol abuse
Normal	41 mm	–	–	–	–	–	20,7	Mild DCM CCD	Heavy physical exercise
Normal	.	–	+	–	–	–	.	Asymptomatic	
.	.	.	.	.	.	.	.	Liver cirrhosis, dead (48)	
Poor	**Dilated**	**+**	**–**	**+**	**–**	**–**	**25,8**	**DCM CRT-D ATP therapy**	
Normal	43 mm	–	–	–	–	–	27,8	Mild DCM	
Normal	Normal	–	–	–	–	–	29,4	Asymptomatic	
.	**LGE+**	**–**	**–**	**–**	**–**	**+**	**30,0**	**DCM CRT-D**	***TTN* c.16452delA**

Supplementary Table 2. Individual LMNA p.(Arg331Gln) carrier characteristics ordered by pedigree. (*continued*)

Subject	Sex	Indication genetic screening	Presentation (Age, Yrs.)	Follow-up (Yrs.)	CCD/ECG Abnormalities	SVA	VA	LVF* (EF)	LVEDD* Other
L-I-1	**M**	**DCM**	**LBBB (56)**	**19**	**LBBB**	**AF**	**NSVT**	**35%**	**.**
L-I-2	M	Obligate carrier	Family History: -Rhythm disorder (35)	-	Family History: -Bradycardia -PM	.	.	.	.
L-I-3	F	Obligate carrier	LBBB	-	LBBB	AF	NSVT	.	.
L-II-1	M		Screening (49)	3	AV-block (1st)	No	No	58%	50 mm Pron. trab.
L-II-2	F		Screening (60)	0	No	Runs of PACs	No	64%	50 mm Pron. trab.
M-I-1	F		Syncope (62)	6	Low voltage PVCs	pAF	>1000 PVCs/ 24H	Normal	50 mm LGE-
M-I-2	F		AF (74)	0	SND	AF	NSVT	Normal	52 mm
M-I-3	F	.	Screening (60)	0	No	Runs of PACs	No	Normal	44 mm
M-I-4	M	Obligate carrier	-	.	-	pAF	.	.	.
M-II-1	M		Screening (36)	2	No	No	NSVT	53%	.
M-II-2	F		Screening (43)	2	No	Runs of PACs	No	59%	50 mm
M-II-3	F		Screening (42)	0	Low voltage	No	No	Normal	44 mm
M-II-4	M		Screening (39)	0	No	No	No	Normal	43 mm

RVF* (EF)	RVEDD* Other	Hypertension Dyslipidemia DM type 2 CAD Additional variant(s)	BMI (kg/m^2)	Outcome	Comment
.	.	- - - - -	23,1	**DCM CRT-D ATP therapy ossible hypertensionnge- emailed.**	
.	.	. . . . .	.	Death (62)	
.	.	. . . . .	.	CCD PM	
Normal	Normal	- - - - -	23,5	CCD	
Normal	38 mm	- - - - -	24,4	SVA	
Normal	.	- - - - -	23,5	SVA VA, ICD	Beta-blocker
Normal	Normal	- - - - -	21,5	Mild DCM VA, ICD SVA	Radiotherapy
Normal	Normal	- - - - -	26,4	SVA	
.	.	- - - - -	.	Sepsis, death (63)	Metastasized Esophagus ca. Autopsy: Hypertrophic heart Heart weight 460 gram
.		- - - - -	22,3	VA	
.		- - - - -	26,1	SVA	
Normal		- - - - -	29,0	Low voltage	
.		- - - - -	24,0	Asymptomatic	

Supplementary Table 2. Individual LMNA p.(Arg331Gln) carrier characteristics ordered by pedigree. (*continued*)

Subject	Sex	Indication genetic screening	Presentation (Age, Yrs.)	Follow-up (Yrs.)	CCD/ECG Abnormalities	SVA	VA	LVF* (EF)	LVEDD* Other
M-III-1	**F**	**DCM**	**OHCA (1)**	**2**	Low voltage	**No**	**VF**	**34%**	**35 mm LGE+**
N	**F**	**DCM**	**Dyspnea (44)**	**4**	**RBBB**	Run of PACs	**VT**	**27%**	**66 mm**
O-I	M	Obligate carrier	AF (61)	-	.	AF	NSVT	.	.
O-II	**M**	**CM**	**Palpitations, NSVT (44)**	**2**	**AV-block (1st) Low voltage**	**Runs of PACs**	**NSVT**	**47%**	**63 mm, Apical thinning, LGE+**
P-II-1	F	Obligate carrier		.	.	.	.	.	.
P-II-2	F		Palpitations, AF (59)	14	IV cond. delay (QRS 126 ms)	AF	VF	38%	64 mm
P-III-1	**M**	**CM**	**Angina Pectoris (47)**	**5**	**LBBB AV-block (1st) Sinus node dysfunction**	**Runs of PACs**	**NSVT**	**48%**	**Dilated† NCCM LGE-**
P-III-2	M		Palpitation, AF (41)	5	SND Low voltage	AF	NSVT	20%	59 mm
Q-II-1	**M**	**Possible ARVC**	**Screening (41)**	**11**	**Incomplete RBBB**	**Runs of PACs**	**NSVT**	**59%**	**Dilated† LGE+**
Q-II-2	M		Palpitations (45)	14	Incomplete RBBB, AV-block (1st)	No	No	58%	Normal

RVF* (EF)	RVEDD* Other	Hypertension Dyslipidemia DM type 2 CAD	Additional variant(s)	BMI (kg/m^2)	Outcome	Comment
45%		– – – –	**+**	**14,9**	**DCM ICD Partial recovery LVF**	*ANKRD1* c.651+1G>A Beta blocker ACE inhibitor Diuretic
Normal		**– – – – –**		**29,8**	**DCM CRT-D Appr. ICD shock LVAD, HTx**	**PA: DCM phenotype, irregular shape of the nuclei, interstitial fibrosis, small area of fibrofatty replacement in epicardial area of the left ventricle**
.	.	. . . .	.	.	Death (71)	
Normal	**.**	**+ – – – –**		**32,8**	**Mild DCM SVA VA**	**Beta blocker ATr antagonist**
.	.	. . . .	.	.	Family History: Sudden death (56)	Family history: -PM indication -Heart failure
Impaired	.	+ – – + –		30,6	OHCA, ICD DCM	
46%	**TFC+ NCCM**	**– – – –**	**+**	**29,2**	**Mild DCM / NCCM**	***DSP* c.8117A>T Beta blocker ATr antagonist**
Normal	.	– – – –	+	24,9	LVAD Death due to heart failure (46)	***DSP* c.8117A>T** PA: Unregularly nuclei, fibrosis, DCM phenotype
47%	**Focal bulging, Basally dilated**	**– – – – –**		**27,8**	**Mild DCM**	**Beta-blocker**
47%	Inhom. contraction pattern	– – – – –		25,9	CCD	Beta-blocker

Supplementary Table 2. Individual LMNA p.(Arg331Gln) carrier characteristics ordered by pedigree. (*continued*)

Subject	Sex	Indication genetic screening	Presentation (Age, Yrs.)	Follow-up (Yrs.)	CCD/ECG Abnormalities	SVA	VA	LVF* (EF)	LVEDD* Other
Q-II-3	M		Screening (44)	11	AV-block (1st)	No	NSVT	55%	Dilated† LGE+
R	**M**	**DCM**	**Dyspnea (52)**	**6**	**LBBB AV-block (1st)**	**No**	**.**	**35%**	**56 mm RWMA LGE+**
S	**M**	**DCM**	**Syncope (42)**	**0**	**No**	**No**	**VT**	**37%**	**62 mm**
T	**M**	**DCM**	**DCM (50)**	**4**	**LBBB**	**pAF**	**VF**	**30%**	**71 mm**
U	**F**	**DCM**	**DCM (48)**	**7**	**AV-block (2nd)**	**pAF**	**No**	**20%**	**52 mm**
V	**F**	**VT and sudden cardiac death in family**	**Syncope (31)**	**5**	**Low voltage**	**pAF**	**VT**	**48%**	**56 mm**
W	**M**	**VT and possible DCM**	**AV-bock (2nd) (48)**	**13**	**AV-block (2nd)**	**AF**	**VT**	**35%**	**57 mm**

Probands displayed in **bold;** *Measurements are based on lowest measured ejection fraction and largest end-diastolic diameter; †Left ventricular end-diastolic volume > 95th percentile indexed for body surface area, age and sex measured with MRI. ACE = Angiotensin-converting enzyme; AF = Atrial fibrilliation; ARVC = Arrhythmogenic right ventricular cardiomyopathy; Appr. = appropriate; ATrR = Angiotensin receptor ATP = Antitachycardia pacing; AV = Atrioventricular BMI = Body mass index; CAD = Coronary artery disease; CCD = Cardiac conduction delay; CRT-D / P = Cardiac resynchronization therapy defibrillator / pacemaker; CVA = Cerebrovascular accident; DCM = Dilated cardiomyopathy; DM = Diabetes Mellitus; kg/m^2 = kilograms/meter^2; ECG = Electrocardiogram; EF = Ejection fraction; F = Female; HTx = Heart transplant; ICD = Implantable cardioverter-defibrillator; CM = Cardiomyopathy; Inhom. = Inhomogeous; IV cond. = Intraventricular conduction; LAHB = Left anterior hemiblock; LBBB = Left bundle branch block; LVEDD= Left ventricular end diastolic dimension; LVAD = Left ventricular assist device; LVF = Left ventricular function; LGE = Late gadolinium enhancement; M = Male; MI = Myocardial infarction; mm = millimeter; NCCM = Non-compaction cardiomyopathy; PA = Pathology; pAF = Paroxysmal atrial fibrillation; PAC = Premature atrial contractions; PLD = Partial lipodystrophy; PM = Pacemaker; Pron. trab. Pronounced trabecularisation; PVCs = Premature ventricular complexes; NSVT = Non sustained ventricular arrhythmia; Presympt. = Presymptomatic; RBBB = Right ventricular bundle branch block RVEDD = Right ventricular end diastolic dimension; RWMA = Regional wall motion abnormalities; SND = Sinus node dysfunction; SVA = Supraventricular arrhythmia; Sympt. = Symptomatic; TFC2010+ = Fulfilling major criterion structural alteration right ventricle according Task Force Criteria 2010; VA = Ventricular arrhythmia; VF = Ventricular fibrillation; Yrs. = Years; 24H = 24 Hours.

RVF* (EF)	RVEDD* Other	Hypertension	Dyslipidemia	DM type 2	CAD	Additional variant(s)	BMI (kg/m^2)	Outcome	Comment
49%	TFC+	+	-	-	-	-	27,7	Mild DCM / Borderline ARVC	Beta-blocker
.	.	**-**	**-**	**-**	**-**	**+**	**27,8**	**DCM Death due to heart failure (58)**	*LMNA* c.1879C>T
Normal		**-**	**-**	**-**	**-**	**+**	**.**	**DCM**	*LDB3* c.1885G>A
Impaired	**.**	**-**	**-**	**-**	**-**	**-**	**19,4**	**DCM ICD HTx**	
Impaired	**.**	**-**	**-**	**-**	**-**	**-**	**26,3**	**DCM ICD LVAD, HTx**	
64%	.	**-**	**-**	**-**	**-**	**+**	**33,5**	**VA, ICD**	***DSC2* c.942+3A>G** **Calcium antagonist ACE inhibitor Diuretic**
Normal	**-**	**-**	**-**	**-**	**-**	**-**	**24,9**	**DCM PM, ICD ATP therapy**	**Beta-blocker ACE inhibitor**

Supplementary Table 3. Overview of *LMNA* mutations carriers (30 probands and 26 family members) included in survival analysis.

Mutation	Genomic position (GRCh37)	Number of probands	Number of Family members	Indication genetic screening probands
c.73C>T, p.(Arg25Cys)	156084782C>T	1	1	DCM
c.250G>A, p.(Glu84Lys)	156084959G>A	1	5	DCM
c.466C>A, p.(Arg156Ser)	156100517C>A	1		DCM
c.481G>A, p.(Glu161Lys)	156100532G>A	2		DCM
c.514-1G>A	156104193G>A	1	3	DCM
c.547C>T, p.(Leu183Pro)	156104227C>T	1		DCM
c.568C>T, p.(Arg190Trp)	156104248C>T	1		DCM
c.569G>A, p.(Arg190Gln) and c.746G>A, p.(Arg249Gln)	156104249G>A	1		DCM
c.624-626delGAA, p.(Lys208del)	156104304delGAA	1	3	DCM + LGMD
c.746G>A, p.(Arg249Gln)	156104702G>A	2		DCM
c.777T>A, p.(Tyr259*)	156104733T>A	1	2	DCM
c.855delG, p.(Ala287Leufs*193)	156105026delG	1		DCM
c.936+1delG	156105104delG	1		DCM
c.936+2T>G	156105105T>G	1		DCM
c.1004G>A, p.(Arg335Gln)	156105759G>A	1		DCM
c.1045C>T, p.(Arg349Trp)	156105800C>T	1	1	DCM
c.1130G>A, p.(Arg377His)	156105885G>A	1	1	DCM + EDMD
c.1130G>T, p.(Arg377Leu)	156105885G>T	2	3	DCM
c.1157+1G>A	156105913G>A	1		DCM
c.1157+46delC	156105958delC	1		DCM
c.1370delA, p.(Lys457Serfs*23)	156106217delA	1		DCM
c.1380+1G>A	156106228G>A	2	1	DCM
c.1513_1514insGA, p.(Thr505Argfs*44)	156106228G>A	1	1	DCM
c.1517A>C (p.His506Pro)	156106928_156106929insGA	1		DCM
c.1608+4A>G	156106932A>C	1	4	DCM
c.1-318-?_c.356+?del (del exon 1)	156107027A>G	1	1	DCM

DCM = Dilated cardiomyopathy; EDMD = Emery-Dreifuss Muscular Dystrophy; LGMD = Limb-girdle muscular dystrophy.

Supplementary table 4. Overview characteristics of LMNA p.(Arg331Gln) carriers and pathogenic *LMNA* mutations carriers other than the LMNA p.(Arg331Gln) included in survival analysis.

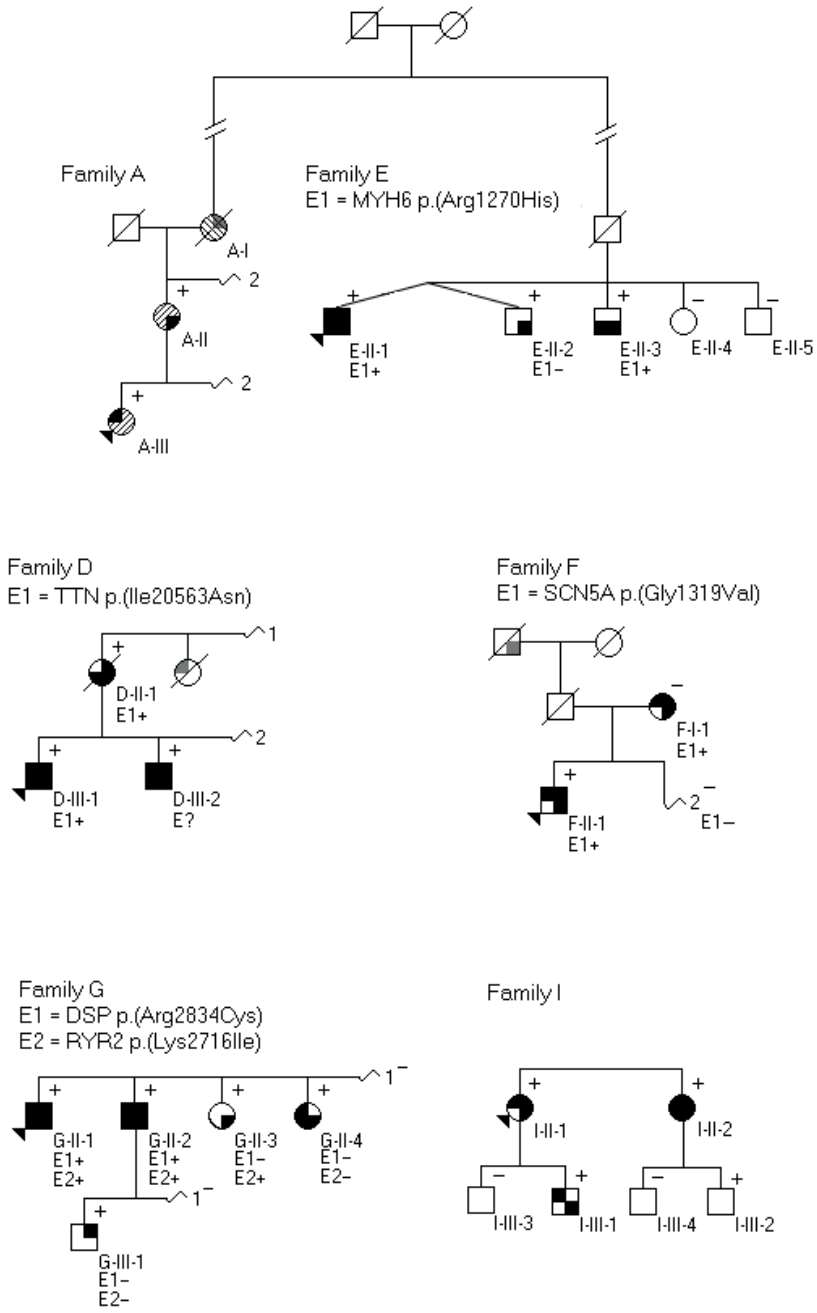
	LMNA p.(Arg331Gln)	LMNA group	P-value
Male	61% (35/57)	50% (28/56)	0.258
Proband status	40% (23/57)	55% (31/56)	0.133
Hypertension	23% (12/52)	20% (10/50)	0.811
Dyslipidemia	8% (4/50)	16% (8/50)	0.234
Diabetes Mellitus type 2	6% (3/50)	8% (3/52)	1
Coronary artery disease	8% (4/51)	0% (0/35)	0.142
Anti-arrhythmics	67% (33/49)	57% (30/53)	0.419
ACE inhibitor or ARB	57% (28/49)	59% (31/53)	0.844
Diuretics	43% (21/49)	51% (27/53)	0.431

Supplementary Table 5. Shared haplotype surrounding the *LMNA* gene in p.(Arg331Gn) probands.

Marker	Position	A⋆		E⋆		B		D		F		I		L		P	
D1S305	154,3	167	159	167	167	167	165	167	169	167	161	167	167	167	157	163	163
D1S2714	155,1	238	234	238	238	238	238	238	234	238	234	238	238	238	238	238	242
D1S1153	155,3	306	306	306	278	306	306	306	306	306	298	306	302	306	274	306	278
D1S303	155,6	183	189	183	179	183	183	183	187	183	187	183	183	183	179	183	187
D1S1595	155,7	287	275	287	291	287	279	287	275	287	275	287	287	287	279	287	287
LMNA p.(Arg331Gln)	156,1	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
D1S2624	156,6	207	203	207	203	207	203	207	203	207	201	207	203	207	207	207	201
D1S2625	157,5	422	422	422	422	412	420	412	414	422	422	422	424	422	422	426	420
D1S2626	158,0	212	210	212	218	212	212	212	214	212	214	212	214	212	212	212	212
D1S2627	158,5	310	304	302	312	302	300	302	302	302	300	302	300	306	298	314	308
D1S2635	159,2	146	138	142	142	150	144	142	142	150	146	152	146	152	146	146	154

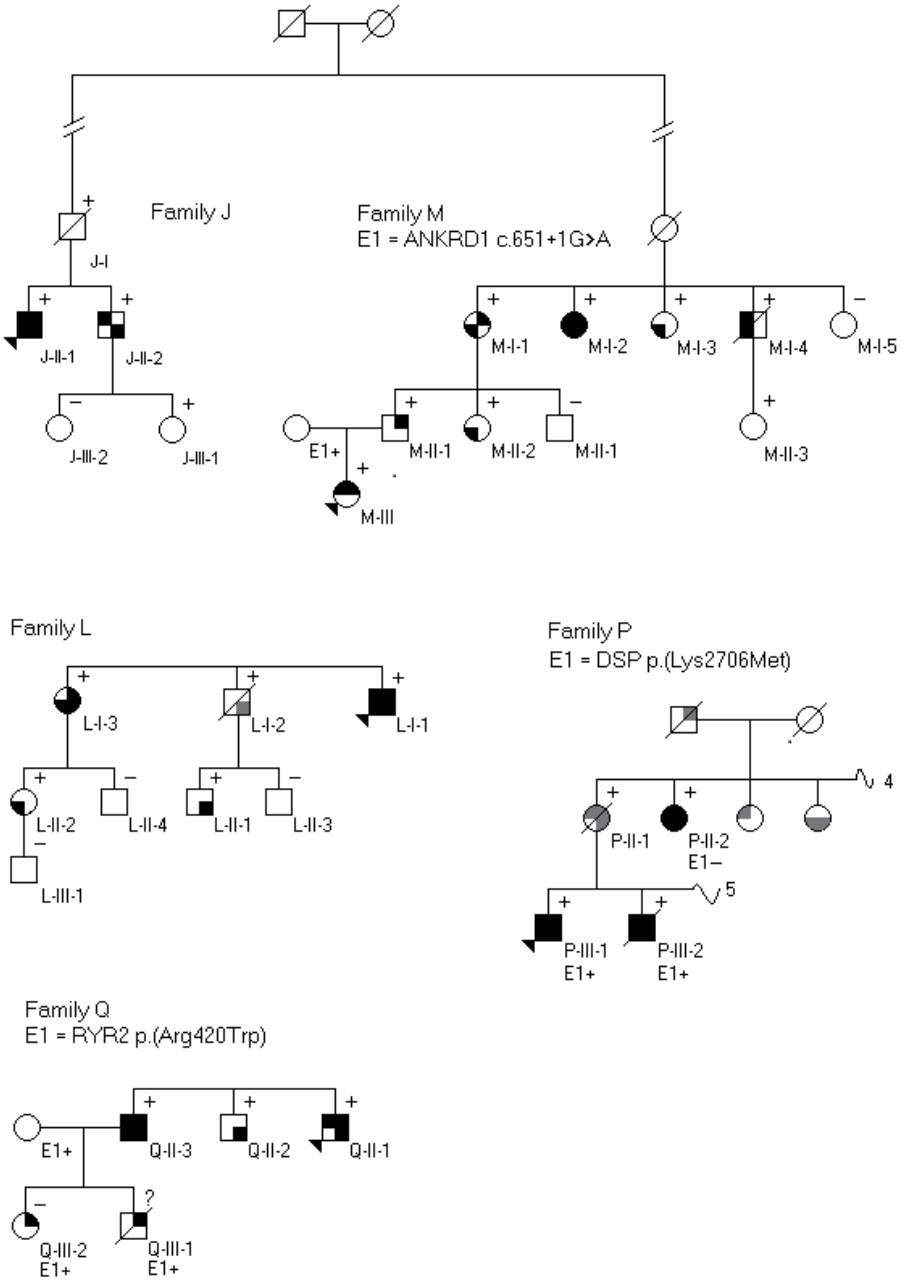
Markers	Position	C		G		J†		M†		K		N		O			
D1S305	154,3	169	159	167	167	167	157	167	165	167	161	167	159	167	153		
D1S2714	155,1	238	234	238	240	238	238	238	234	238	242	238	238	238	240		
D1S1153	155,3	310	310	310	310	310	302	310	306	310	286	310	298	310	286		
D1S303	155,6	183	183	183	183	183	181	183	179	183	179	183	181	183	183		
D1S1595	155,7	287	283	287	283	287	283	287	291	287	283	287	283	287	283		
LMNA p.(Arg331Gln)	156,1	+	-	+	-	+	-	+	-	+	-	+	-	+	-		
D1S2624	156,63	207	203	207	201	207	207	207	203	207	203	207	205	207	201		
D1S2625	157,54	422	422	422	422	422	414	422	418	422	424	422	422	422	414		
D1S2626	158,0	212	214	212	210	212	214	212	220	218	218	212	214	212	212		
D1S2627	158,57	310	298	302	306	300	300	300	298	302	300	302	296	302	300		
D1S2635	159,2	158	142	150	152	152	150	146	140	150	144	150	154	152	138		

Marker D1S1153 (light grey) differs in approximately one half of the probands. It is likely that this has occurred due to slippage during replication some time ago. All probands share a common haplotype of approximately 1 Mb around the LMNA gene. ⋆Families A & E have been genealogical linked and share common ancestors 6 generations back. †Families J & M have been genealogical linked and share common ancestors 6 generations back. Dark grey = phase of microsatellite markers.



Supplementary Figure 1. Overview Pedigrees

The mutation segregates with the phenotypes typical for *LMNA* mutations. Only in family F is the segregation not clear. In this family, the phenotype of the mother of the index is probably caused by the pathogenic SCN5A p.(Gly1319Val) mutation.



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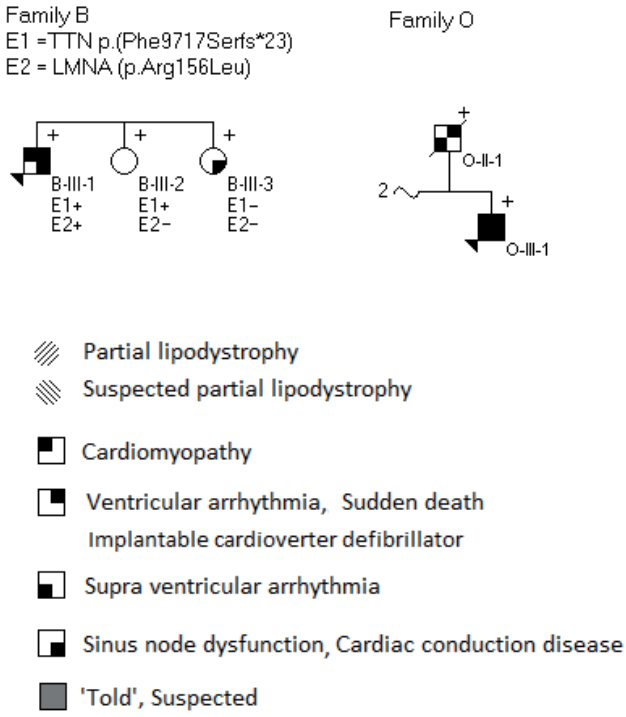

Supplementary Figure 1. Overview Pedigrees

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Chapter 3

The first titin (c.59926+1G>A) founder mutation associated with dilated cardiomyopathy

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Full manuscript is enclosed on which the research letter published in Eur J Heart Fail. 2018 Apr;20(4):803-806 is based.

ABSTRACT

Aim - Truncating titin *(TTN)* variants play an important role in the development of dilated cardiomyopathy (DCM), although the occurrence of truncating variants in the general population makes their interpretation challenging. Here we describe the *TTN* c.59926+1G>A splice-site variant located in the A-band that we have identified in multiple probands with DCM.

Methods and Results - Clinical and genetic data from 11 probands with the *TTN* c.59926+1G>A variant and 37 family members (20 carriers) were collected retrospectively. We studied the founder effect of the *TTN* variant by haplotype analysis and genealogical research. Birthplaces of ancestors were plotted to deduce the geographical origin of the variant. In 30 carriers of the *TTN* variant, DCM was the predominant clinical finding (67%: 20/30; mean age of onset 49±14 years). Paroxysmal atrial tachyarrhythmia also occurred frequently (53%; 16/30), with (paroxysmal)atrial fibrillation or paroxysmal atrial flutter most frequently observed (50%; 15/30). Male carriers were more often diagnosed with DCM than female carriers (93%; 14/15 versus 40%; 6/15). We identified a shared haplotype, suggesting this is a founder variant; this is also supported by genealogical links found between the families.

Conclusion - The *TTN* c.59926+1G>A variant is pathogenic and is the first reported *TTN* founder mutation associated with DCM. Carriers often suffer from DCM and atrial tachyarrhythmias.

INTRODUCTION

Titin (TTN), the largest known human protein, plays an important functional and structural role in the sarcomere.[1] It is encoded by the *TTN* gene, and variants in this gene have been shown to play a major role in dilated cardiomyopathy (DCM), with up to 25% of patients carry a pathogenic or likely pathogenic variant.[2,3] In DCM truncated *TTN* variants (*TTN*tv) are associated with early arrhythmic risk, composed of atrial fibrillation (AF), nonsustained and/or sustained ventricular tachycardia (VT).[4] Although *TTN*tv are significantly more often present in DCM patients than in ostensibly healthy controls (25% vs 3%),[2] the occurrence of *TTN*tv in controls makes correct interpretation difficult, as it is possible that the truncating variant could be a coincidental finding.[5,6] Although truncating variants in the A-band region of *TTN* or downstream of the internal Cronos promoter are generally believed to be pathogenic, segregation studies can provide additional, more conclusive evidence for their pathogenicity.[3,7]

Here we describe the *TTN* c.59926+1G>A splice-site variant identified in multiple probands and their relatives. To gain better insight in the genotype-phenotype relation and correctly assign pathogenicity of this variant, we retrospectively collected extensive genetic and clinical data of a large series of carriers of this variant and demonstrated a founder effect.

METHODS

Genetic analysis

We performed genetic analysis using panel-based next generation sequencing (NGS), as described previously.[8] Confirmation of the identified variant was done by Sanger sequencing. The specific variant was analysed in family members by Sanger sequencing. The variant was reported using Human Genome Variation Society nomenclature guidelines (http://varnomen.hgvs.org/) and NM_001267550.2 as the reference sequence.

Written informed consent was obtained from all participants following local medical ethics committee guidelines. Our study and all experiments conformed with the principles of the Declaration of Helsinki.

The Exome Aggregation Consortium dataset (ExAC) and the Genome of the Netherlands database (GoNL) were used as control populations.

Haplotypes

Eleven microsatellite markers surrounding *TTN* were analysed to evaluate whether the variant originated from a common ancestor. By analysing DNA samples of relatives we could reconstruct the haplotype and verify the phase. The haplotypes of the carriers were then compared to those of non-carriers. The age of the mutation was calculated as described previously.[9]

Clinical evaluation

Clinical data were collected retrospectively from 11 probands and 37 family members (20 mutation-positive) who were genetically screened in the Departments of Clinical Genetics, UMCG, Groningen, LUMC, Leiden, or AMC, Amsterdam. Medical history, physical examination, 12-lead electrocardiograms (ECG), 24-hour ambulatory ECG (Holter) and/or exercise-ECG (X-ECG), echocardiograms, magnetic resonance imaging (MRI) studies of the heart, myocardial perfusion scintigrams and/or coronary angiograms were collected if available. Left ventricular tissue sections (4 μm) obtained from the explanted heart of one affected carrier (E-IV-1) were stained with Masson's trichrome and hematoxylin & eosin for histological examination.

Subjects were diagnosed with DCM when they had a reduced left ventricular ejection fraction (<45%) and a widened ventricle (>117% of predicted). In the absence of information on end diastolic diameter, length or weight, DCM was noted when it was declared DCM by the cardiologist.

To evaluate if this predisposition to (paroxysmal) atrial fibrilliation ((p)AF) is inherent to this specific mutation or to *TTN*tv in general, we looked at age at diagnosis of DCM and (p)AF of 53 carriers of 16 other *TTN*tv residing in constitutively expressed exons.

Postal code analysis, genealogy, and Linkage analysis

Genealogical research to find common ancestors was performed using community registries and official records of births, marriages and deaths. We were able to trace back up to nine generations of family members.

To study the geographical distribution of the *TTN* variant carriers, the home location of carriers was plotted on a map of the Netherlands, using the four numbers of their postal codes. To deduce the region of origin of the variant, the birthplaces of the grandparents of the carriers from the oldest generation were also plotted for the ten families.

The linkage program GRONDLOD[10] was used to calculate the combined LOD score, taking into account the age-related penetrance, which is an important characteristic of DCM.

Linkage analysis was performed in five of the ten families (B, C, E, F and H). For a detailed description of the model assumptions please see the **Supplementary Material**.

Statistical analysis

Frequencies are expressed as numbers and percentages and continuous variables as mean ± standard deviation. The Mann-Whitney U test was used for between-group comparisons and Fisher's exact test for comparing frequencies. Data were analysed using SPSS version 23 (IBM Corp., Armonk, NY, USA). A P-value <0.05 was considered statistically significant.

RESULTS

Genetic analysis

The *TTN* c.59926+1G>A (chr2:179456704C>T, build GRCh37) splice-site variant was found in eleven probands. In ten of them the variant was identified by targeted NGS and in one proband (C-II-1) by Sanger sequencing, because of a genealogical link with family B (**Supplementary Figure 1**) in which the *TTN* variant had already been identified with NGS. The *TTN* c.59926+1G>A variant was identified with Sanger sequencing in 18/37 family members and a further two family members were indicated as obligate carriers. In total, 31 carriers (11 probands and 20 family members) and 17 non-carriers were studied.

Titin c.59926 + 1G >A was found only once in 966 alleles in GoNL (0.1%)[11] and was not present in ExAC.[12]

Multiple splice-site predicting software tools (SpliceSiteFinder-like, MaxEntScan, NNSPLICE, Genesplicer, Human Splicing Finder) predict that the consensus splice site at the 3' of exon-intron boundary of exon 302 (in transcript variant NM_001267550.2) is no longer recognized. This is predicted to lead to intron retention, resulting in a frameshift variant TTN p.(His19976Argfs*), or to exon skipping, with the loss of exon 302 (encoding 100 amino acids).

In proband F-IV-1, a second pathogenic variant was found in *SCN5A*, c.4213G>C; p.(Val1405Leu). This specific variant was described in a different study in a proband with Brugada syndrome.[13]

Haplotypes

Haplotype analysis of nine probands and seven family members revealed a shared region of approximately 4 Mb (**Supplementary Table 1**), indicating a common ancestor. We calculated the mutation to be 300-580 years old.

Postal code analysis, genealogy and linkage analysis

Most carriers, and the grandparents of the oldest generation carriers from the different families, originated from the eastern part of the Netherlands (**Figure 1A and B**). Genealogy going back five to nine generations in seven families revealed three pairs of common ancestors, all born within a 10 kilometre radius (**Figure 1B**). Segregation analysis yielded a combined LOD score of 3.6: strong evidence that this mutation is pathogenic.

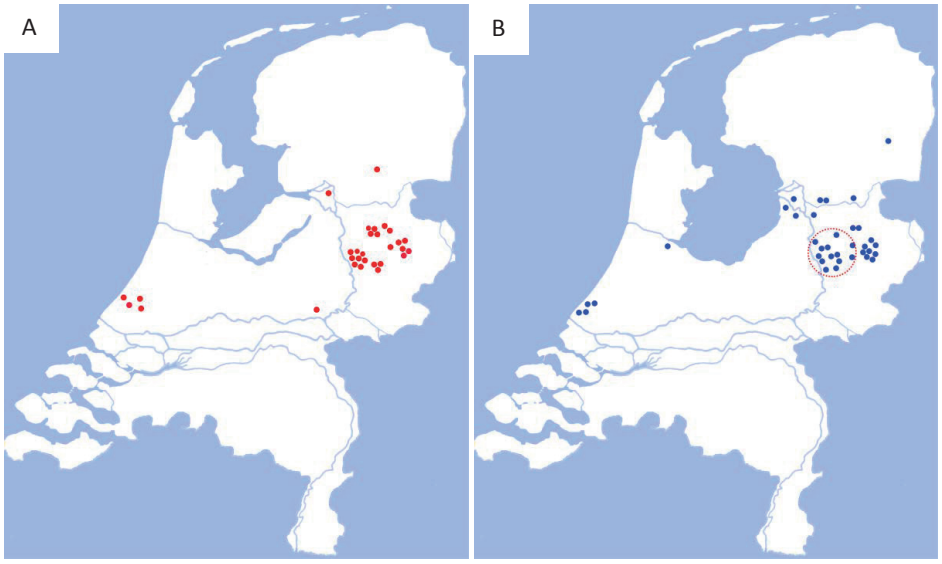

Figure 1. A. Carriers places of residence. B. Birthplaces of grandparents of carriers in the oldest generation of the different families. The six common ancestors linking seven of the ten families all originated from a small area in the southern part of the province of Overijssel indicated by the red circle (10 km radius).

Clinical evaluation

One obligate carrier was not included in the analyses as additional clinical data was unavailable because the first symptom was sudden cardiac death. The predominant finding in mutation carriers was DCM (67%: 20/30; mean age of onset 49±14 years). Atrial tachyarrhythmias were observed in 65% of DCM patients (13/20), with (p)AF or paroxysmal atrial flutter most frequently observed (60%; 12/20) (**Table 1**). One DCM patient had collapsed due to an AV-nodal re-entry tachycardia (AVNRT), which converted to sinus rhythm after administration of adenosine. Of note, left atrial enlargement was observed in echocardiography six months before the AVNRT. In three patients (p)AF preceded the DCM phenotype (by 11, 12, and 14 years). Males were overrepresented in the DCM group compared to carriers with no DCM ($p=0.003$).

Table 1. Clinical characteristics of 30 *TTN* c.59926+1G>A mutation carriers and 17 mutation negative family members.

	TTN c.59926+1G>A +		*TTN* c.59926+1G>A –
	DCM (N=20)	No DCM (N=10)	No DCM (N=17)
Age at diagnosis/evaluation	49 ±14	47 ±19	49 ±10
Proband	11 (55%)	0 (0%)	0 (0%)
Male	14 (70%)	1 (10%)	13 (62%)
Left ventricular ejection fraction[a], %	26 ±11	53 ±3[*]	57 ±4[*]
Left ventricular end diastolic dimension[a], mm	63 ±7	51 ±6[†]	50 ±4[†]
Atrial tachyarrhythmias	13 (65%)	3 (30%)	0 (0%)[‡]
(paroxysmal) atrial fibrillation/flutter	12 (60%)	3 (30%)	NA
Ventricular arrhythmias	9 (45%)	1 (10%)	0 (0%)
Non-sustained ventricular tachycardia	5 (25%)	1 (10%)	NA
Ventricular tachycardia/fibrillation	4 (20%)	0 (0%)	NA
Out of hospital cardiac arrest	2 (10%)	0 (0%)	0 (0%)
ICD-implantation	9 (45%)	0 (0%)	0 (0%)
Appropriate ICD-therapy	4 (20%)	NA	NA
Risk factors	11 (55%)	6 (60%)	3 (18%)
Hypertension	7 (35%)	2 (20%)	2 (12%)
Diabetes Mellitus	2 (10%)	0 (0%)	0 (0%)
Dyslipidaemia	3 (15%)	0 (0%)	2 (12%)
Coronary artery disease	2 (10%)	1 (10%)	0 (0%)
Obesitas	1 (5%)	2 (10%)	1 (6%)
Other[b]	3 (15%)	1 (10%)	0 (0%)

DCM, dilated cardiomyopathy; ICD, implantable cardioverter-defibrillator; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; NA, not applicable; *TTN,* titin.

[a] Measurements are based on the lowest measured ejection fraction and the largest end-diastolic diameter.

[b] Composition of chemotherapy, history of excess alcohol consumption, and severe mitral valve insufficiency.

[*] LVEF is available from five carriers and six non-carriers. In four carriers, left ventricular function is described as normal, of which one is described as >55%. In another carrier left ventricular function is described as moderate (45 – 55%). In the other 11 non-carriers left ventricular function is described as normal.

[†]LVEDD is missing from one carrier and available from 13 non-carriers; in the other four non-carriers, LVEDD is described as normal.

[‡]Holter monitoring only performed in three non-carriers.

To evaluate if the frequent occurrence of (p)AF occurred with left atrial widening, we collected information on atrial size (see **Supplementary Table 2** for risk factors, age at onset of (p)AF, DCM and age at left atrial measurements). Interestingly, in the DCM group, eight subjects had (p)AF without left atrial dilatation, including four without risk factors that could attribute to the development of (p)AF. In the carrier group without DCM, three subjects (30%) had (p)AF: two had no atrial dilatation and one had atrial dilatation measured while atrial fibrillation was her underlying rhythm (**Figure 2**).

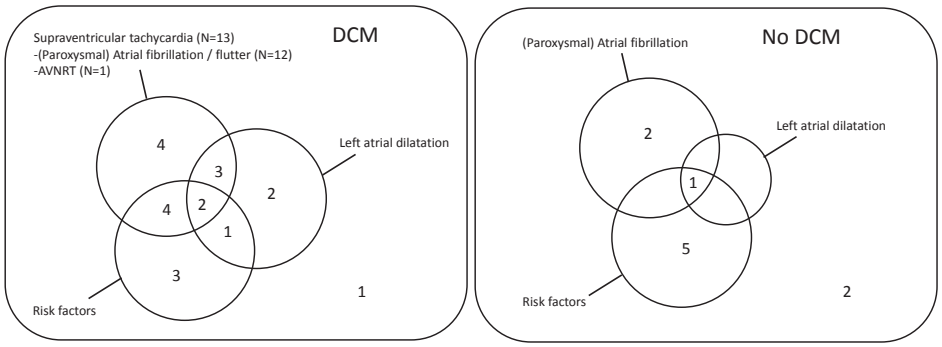

Figure 2. Venn-diagrams of carriers with and without dilated cardiomyopathy (DCM) showing the presence of risk factors, atrial arrhythmias and atrial dilatation. AVNRT = AV-nodal re-entry tachycardia.

Two carriers suffered from an aborted cardiac arrest and one obligate carrier died suddenly (ages 58, 75, and 33, respectively). Autopsy in the deceased patient revealed signs of cardiomyopathy. An implantable cardioverter-defibrillator (ICD) was implanted in nine carriers: two in the context of secondary intervention and seven for primary prevention. Four ICD carriers (44%; 4/9) received appropriate treatment (3x ICD-shock and 1x antitachycardia pacing) within 1-4 years after implantation. Two patients underwent a heart transplantation. Histological examination of one of the explanted heart revealed hypertrophic cardiomyocytes with moderate interstitial fibrosis (**Supplementary Figure 2**). These findings are compatible with histological findings in DCM, in general. Hypertension, diabetes mellitus, and dyslipidaemia seemed more frequently present in the DCM group, but significant differences could not be demonstrated given our small sample.

An additional stressor was reported in six carriers. The obligate carrier who died suddenly was reported to have used excessive amounts of alcohol, which was also suggested as a possible cause of DCM in another carrier. In two other carriers a history of an infectious period preceded the development of severe left ventricular impairment. One required intra-aortic balloon support, with myocarditis suspected but not evaluated, and his cardiac function largely recovered. In the other myocardial biopsy was negative for cardiotropic viruses, but his left ventricular function remained poor (EF 18%). In another carrier, severe DCM (EF 15%) became apparent after anthracycline therapy. Finally, one proband with DCM carried an additional pathogenic *SCN5A* mutation. He had no spontaneous type 1 Brugada ECG but did have *SCN5A*-related features: a right bundle branch block and delayed atrioventricular conduction (PR-interval 330 msec). Family history reported a high incidence of sudden death, but we could not investigate segregation because there were no additional genetic and clinical data available for family members.

DCM caused by *TTN*tv has been reported to respond relatively well to treatment,[14] which we also observed in some carriers. Cardiac function in one patient recovered after a short period of cardiogenic shock due to a suspected myocarditis. Two DCM patients responded well to treatment after a follow-up of six and four years (increases of ejection fraction of 20% to 45% and 43% to 50% and decreases in left ventricular end-diastolic dimensions of 65 mm to 53 mm and 60 to 47 mm, respectively).

The occurrence of (p)AF without or preceding DCM was interesting. The evaluation of the 53 carriers (16 probands and 37 family members) of 16 other *TTN*tv residing in consitutively expressed exons showed 19 asymptomatic carriers, 6 with only (p)AF (11%), 3 for whom (p)AF preceded DCM (6%) (by 1, 4 and 22 years), 11 in whom (p)AF was diagnosed the same year as DCM (21%), 6 who developed (p)AF after diagnosis of DCM (11%) and 8 with only DCM (15%). Together with the results from our cohort this indicates that (p)AF is an important part of the clinical disease spectrum caused by *TTN*tv, even if gross structural abnormalities are not yet present.

DISCUSSION

We evaluated the clinical and genetic background of the largest cohort reported to date of carriers with an identical *TTN* (founder) variant believed to be pathogenic because of segregation with the disease (LOD score = 3.6). To our knowledge, this is the first time a founder effect in *TTN* has been associated with cardiomyopathy. The phenotype is characterized by DCM and atrial tachyarrhythmias, specifically (p)AF. As (p)AF could be the first symptom, clinicians could consider performing ambulatory ECG (Holter) monitoring in asymptomatic carriers during cardiac follow-up. A role for *TTN* in the aetiology of AF is further supported by a recent genome wide association study.[15]

Phenotype

DCM was the main clinical finding (67%); it was occasionally severe, with two carriers eventually needing a HTx due to end-stage heart failure. This is in line with observations reported in the literature since most *TTN*tv are associated with DCM and a truncating *TTN* variant is found in up to 25% of familial DCM cases.[2,3]

However, there was a great variability in age of disease onset. The youngest DCM patient was a male presenting at age 25 years, while the oldest patient, also male, was diagnosed with DCM at age 76 years. This variability in age of onset was also observed in two large families (N = 19 and N = 15) with a single *TTN* truncating variant in the A-band region.[16,17]

Atrial tachyarrhythmias occurred frequently (53%, 16/30), with (p)AF or paroxysmal atrial flutter being the most frequently observed (50%; 15/30)). In some carriers (p)AF was the only clinical finding, while in others it preceded DCM. This finding was confirmed in 53 carriers of 16 other *TTN*tv residing in constitutively expressed exons. The high incidence of (p)AF has not previously been described in two large families,[16,17] however, in another more heterogeneous cohort studied by Akinrinade et al., atrial fibrillation was observed in 40% of carriers with a *TTN*tv who had DCM.[18] The observation that (p)AF occurred in *TTN*tv without DCM has not been described before and raises the possibility that *TTN*tv could play a role in the development of atrial fibrillation, in general.

In addition to atrial arrhythmias, *TTN*tv may also increase susceptibility for ventricular arrhythmias because two carriers had an aborted cardiac arrest, one obligate carrier died suddenly, and nearly half of the ICD carriers had received appropriate ICD therapy (4/9). Roberts et al. also showed that *TTN*tv-positive DCM patients experienced sustained VT more often than *TTN*tv-negative DCM patients.[3]

Sex differences

We also observed that DCM occurred more frequently in males (93% of males had DCM versus 40% of females, **Supplementary Table 3**), which seems counter-intuitive given autosomal dominant inheritance. However, in a large cohort of carriers of a different *TTN*tv (N = 94), males also seemed more severely affected with respect to rates of heart transplantations, implantation of left ventricular assist devices, and death from cardiac causes.[2] Since our cohort is relatively homogeneous (identical mutation), the significant sex difference underscores the important role of sex in the development of DCM in *TTN*tv. In different forms of cardiomyopathy, sex is known to influence the clinical manifestation.[19] In *TNNT2* and *LMNA*-related DCM, it is well-known that males are more often affected.[20,21] The numbers of cases were not large enough to evaluate a potential effect of risk factors or comorbidities that could explain the differences between males and females.

Recovery and additional factors

The partial or full recovery of left ventricular function in three carriers (E-V-3, I-II-2 and I-II-3) was remarkable. Jansweijer et al. showed that DCM caused by *TTN*tv responds relatively well to treatment in terms of improvement of left ventricular function.[14] A recovery of heart function was also described in two females with *TTN*tv and peripartum cardiomyopathy (PPCM).[22] Our finding thus supports the previous observations that cardiomyopathies caused by *TTN*tv are relatively amenable to treatment, with the possibility to recover.[14]

While we still do not understand which mechanisms underlie the incomplete penetrance, there are some indications from our cohort that additional stressors (cardiovascular risk fac-

tors, alcohol, myocarditis, chemotherapy, or an additional mutation, male sex) may trigger the development of DCM in a heart that is already susceptible by a *TTN*tv. In the cases of PPCM with *TTN*tv, it is possible that the pregnancy acted as an additional stressor leading to cardiomyopathy.[22] This multiple-hit model could also play a role in one of our female patients (H-II-3) with severe DCM. Chemotherapy may have been the additional stressor since the diagnosis and symptoms of DCM only became apparent after anthracycline therapy. Anthracyclines have cardiotoxic effects that may induce a cardiomyopathy in genetically predisposed persons, as seen in *MYH7* variant carriers or in the context of familial DCM.[23,24] In another female (from the same extended family H, H-II-1), her severe DCM was accompanied by a severe mitral valve insufficiency, which could have acted as an additional factor contributing to the development and severity of DCM. The additional stressor might also be excessive alcohol consumption (C-II-1 and J-II-1) or myocarditis (E-V-3). In a recent study of two rat models with *TTN*tv, it was shown that the heart is already in a compensated state due to the mutation and cannot cope with increasing stress from other factors.[6] These observations need systematic investigation in larger cohorts of different *TTN*tv or in a later stadium when more carriers of this mutation are identified.

Founder effect

Identification of this variant in multiple, seemingly unrelated probands in mainly the eastern part of the Netherlands suggested they had a common ancestor. The shared haplotype of at least 4 Mb pairs supports this. The relative large shared haplotype size suggests a more recent origin than other founder variants, like the PLN p.(Arg14del), LMNA p.(Arg331Gln) and MYL2 p.(Glu22Lys) variants, in which shared haplotypes of around 1 to 2 Mb pairs were found and estimated to be between 340 − 940 years old.[25,26,27]

Limitations

The relative small numbers and the retrospective nature of our study means we cannot draw definitive conclusions. Prospective studies in larger cohorts are needed to confirm our observations.

CONCLUSIONS

The *TTN* c.59926+1G>A variant is believed to affect heart function and is the first *TTN* founder variant associated with DCM to be described. Carriers show a variable phenotype of DCM and atrial tachyarrhythmias, which may even be the initial or sole manifestation. Male carriers are affected more often than female carriers.

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SUPPLEMENTAL DATA

SUPPLEMENTARY METHODS

Linkage analysis using 'GRONDLOD'

We performed linkage analysis to analyse if the genetic variant co-segregated in a genome-wide way with the observed phenotypes in the affected families. To take into account the possibilities of age-related penetrance, we used the linkage program GRONDLOD, because it has the advantage of defining genetic model assumptions in genetic diseases with reduced penetrance. Families B, C, E, F and I were used to calculate LOD scores at zero recombination frequency (Figure 1).

The following assumptions were used for the model computation. We defined phenotypes as follows: unknown, normal, dilated cardiomyopathy (DCM). The disease gene was considered to be a rare variant and the allele frequency was set to 0.001, which was also underscored by the allele frequency found in GoNL data and the normal variant to 0.999.[1,2]

The probabilities for 12 phenotypes, given a specific genotype, are given below.

Explanation: The first number after the bracket indicates the disease locus, the second and third numbers indicate the autosomal marker phenotype. The disease phenotype is indicated with double quotation marks. The last number reflects the assumed probability of the genotype conditional on the disease status genotype.

phen_gen (1, 1, 1, "DCM ", 0.005) // allows for 0.5% phenocopies

phen_gen (1, 1, 2, "DCM", 0.8) // 80% penetrance for carriers

phen_gen (1, 2, 2, "DCM", 1)

phen_gen (1, 1, 1, "normal_age_34yrs", 0.9)

phen_gen (1, 1, 2, "normal_age_34yrs", 0.9) // 90% chance of normal phenotype at age 34 years, while carrying the mutation

phen_gen (1, 2, 2, "normal_age_34yrs", 0.001)

phen_gen (1, 1, 1, "normal_46yrs", 0.9)

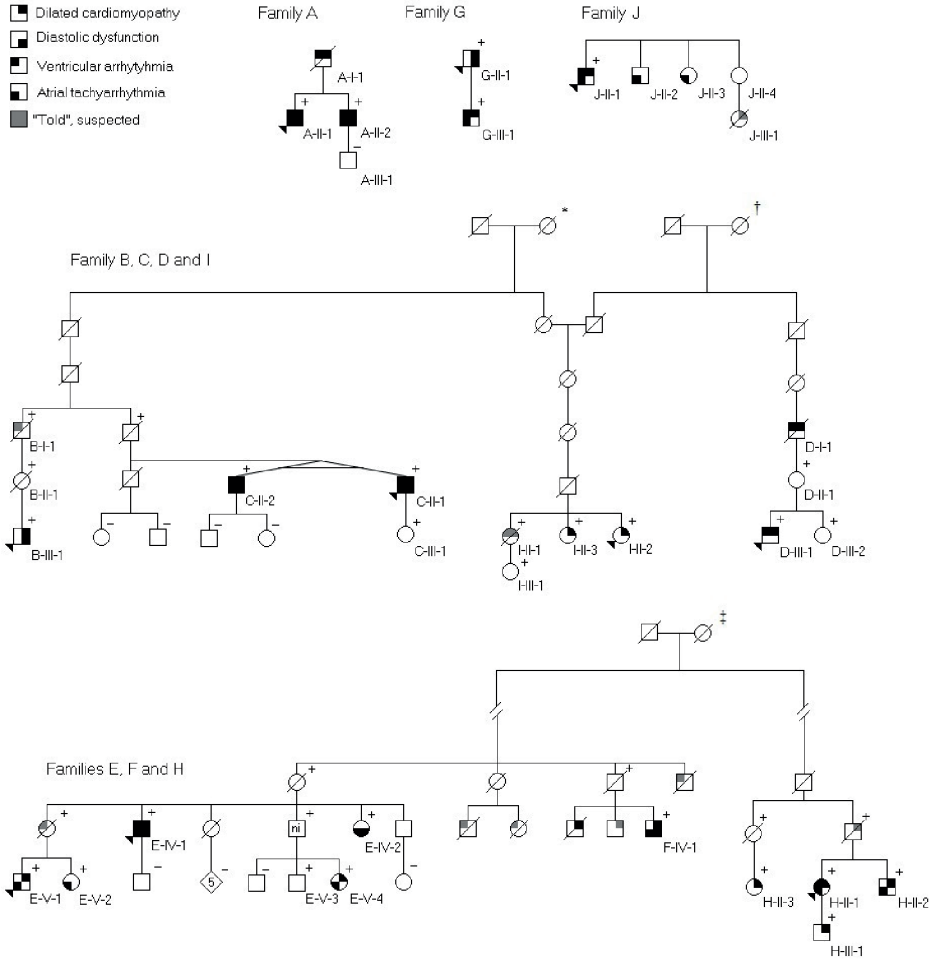
phen_gen (1, 1, 2, "normal_46yrs", 0.3) // 30% chance of normal phenotype, at age 46 years, while carrying the mutation

phen_gen (1, 2, 2, "normal_46yrs", 0.001)

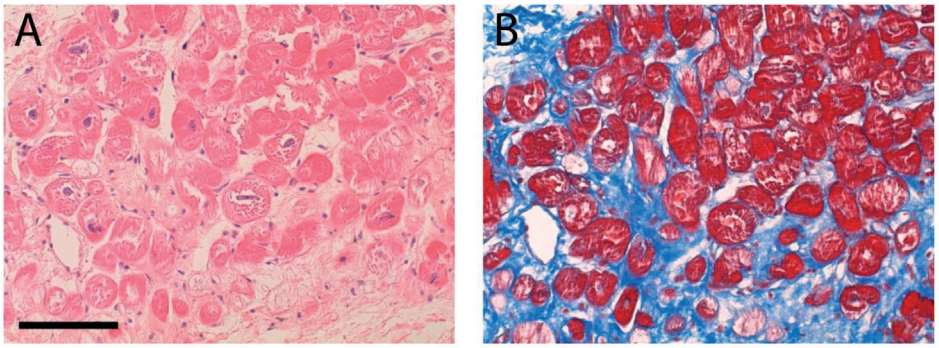
phen_gen (1, 1, 1, "unknown", 1)

phen_gen (1, 1, 2, "unknown", 1)

phen_gen (1, 2, 2, "unknown", 1)



Supplementary Figure 1. Overview of 10 family pedigrees showing *TTN* distribution. Figures in grey are suspected of having the represented phenotype from information reported during family history evaluation. *Common ancestors linking the families B, C and J go back 5 generations. †Common ancestor linking families D and J goes back 5 generations. ‡Common ancestors go back 9 generations to link families E, G and I.



Supplementary Figure 2. Histology of an explanted heart from a *TTN* c.59926+1G>A mutation carrier. A, Hematoxylin & eosin stain. Bar = 100 μm. B, Masson's trichrome stain showing the cardiomyocytes in red and fibrosis in blue.

Supplementary Table 1. Haplotype analysis in nine probands with the *TTN* c.59926+1G>A mutation.

		Probands																	
Marker	Position	A-II-1		B-III-1★		C-II-1★		I-II-1★,#		D-II-1#		E-V-1‡		F-IV-1‡		H-II-1‡		G-II-1	
D2S326	173,104,	164	162	166	158	168	158	166	168	168	166	166	162	160	160	160	160	166	168
D2S2307	175,462,	145	145	159	145	159	145	151	145	167	145	163	145	151	145	145	145	167	145
D2S2257	176,176	165	163	163	163	163	163	175	163	163	163	165	163	163	163	173	163	163	163
D2S2314	176,868,	90	104	102	92	104	92	90	92	92	92	192	104	92	104	104	104	92	92
D2S148	178,236	188	184	184	184	184	184	184	184	184	184	186	184	182	184	186	184	184	184
TTN c.59926+1G>A	179,396	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
D2S385	179,631	162	162	162	162	162	162	162	162	162	162	160	162	166	162	162	162	162	162
D2S2261	181,503,	142	122	116	122	116	122	114	122	122	122	140	122	140	122	140	122	122	122
D2S2310	182,172	258	248	246	248	254	248	246	248	258	248	258	248	248	248	246	248	248	248
D2S364	183,04	222	234	222	228	232	228	244	228	224	228	222	232	232	232	228	228	228	228
D2S2281	184,186	218	218	222	226	222	218	222	218	222	224	224	224	220	224	220	224	222	218
D2S2366	184,498	178	178	178	178	178	178	180	178	176	176	176	176	178	178	176	178	178	178

A shared haplotype of approximately 4Mb around the TTN mutation was found. Dark grey indicates the phase of microsatellite markers derived from members of family C. Light grey is the phase of microsatellite markers derived from members of family E and I.

★Families B, C and I could be genealogically linked, going back 3 and 5 generations, respectively. #Families D and H could be genealogically linked, both going back 5 generations. ‡Families E, F and H could be genealogically linked going back 3 and 9 generations, respectively.

Supplementary Table 2. Clinical information on the *TTN* c.59926+1G>A carriers.

Subject	Sex	Risk factors	SVT (Age, yrs)	DCM (Age, yrs)	Age echo, yrs	Left atrial description^	LAVI, ml/m2	Other left atrial measurements	Diastolic function^	Measurements
A-II-1	Male	No	pAF (39)	DCM (53)	39	Mildly dilated		PLAX: 42 mm		IVS thickness 8 mm LVPW thickness 8 mm
					43	Normal		PLAX: 31 mm 4chamber Major: 63 mm	Abnormal	E/A 0.73 IVS thickness 9 mm LVPW thickness 7 mm
					47	Normal		PLAX: 36 mm		IVS thickness 8 mm LVPW thickness 8 mm
A-II-2	Male	No	pAF (54)	DCM (54)	50	Normal		PLAX: 39 mm 4chamber Major: 54 mm		E/A 0.97 DT 268 ms IVRT 170 ms IVS thickness 7 mm LVPW thickness 8 mm
					61	Normal	36.0	PLAX: 38 mm 4chamber Major: 57 mm	Abnormal Grade 1	E/A 0.69 e' 5 cm/s IVS thickness 10 mm LVPW thickness 8 mm LV mass 98 g/m2
G-II-1	Male	Ht	No (62)	DCM (55)	57	Normal	25.0		Abnormal Grade 2	E/A 0.83 DT 144 ms e' 6 cm/s E/e' 11.00 IVS thickness 10 mm LVPW thickness 10 mm

Supplementary Table 2. Clinical information on the *TTN* c.59926+1G>A carriers. (*continued*)

Subject	Sex	Risk factors	SVT (Age, yrs)	DCM (Age, yrs)	Age echo, yrs	Left atrial description^	LAVI, ml/m2	Other left atrial measurements	Diastolic function^	Measurements
					59	Dilated		PLAX: 43 mm	Abnormal Grade 1	IVS thickness 11 mm LVPW thickness 10 mm LV mass 119 g/m2
G-III-1	Male	No	pAF (28)	DCM (26)	28	Normal	25.0		Normal	E/A 1.90 DT 235 ms e'19 cm/s IVS thickness 7 mm LVPW thickness 8 mm LV mass 74 g/m2
J-II-1	Male	Ht / CAD	pAF (75)	DCM (75)	75	Dilated	34.4		Abnormal	E/A 0.77 e' 4.7 cm/s E/e' 8.60 IVS thickness not hypertrophic LVPW thickness not hypertrophic
B-II-1★	Female	CAD	No (75)	No (75)						
B-III-1★	Male	Ht / Dyslip	No (60)	DCM (49)	54	Normal		PLAX: 35 mm	Normal	E/A 0.57 E/e' 8.90 IVS thickness 9 mm LVPW thickness 9 mm
					60	Dilated	50.0		Not assessable	IVS thickness 8 mm LVPW thickness 10 mm

Supplementary Table 2. Clinical information on the *TTN* c.59926+1G>A carriers. (*continued*)

Subject	Sex	Risk factors	SVT (Age, yrs)	DCM (Age, yrs)	Age echo, yrs	Left atrial description^	LAVI, ml/m2	Other left atrial measurements	Diastolic function^	Measurements
B-IV-1*	Female	Ht	No (38)	No (38)	38	Normal		PLAX: 30 mm	Normal	E/A 1.04 e' 11cm/s E/e' 7.50 IVS thickness 7 mm LVPW thickness 6 mm LV massa 42 g/m^2
B-IV-2*	Female	No	No (34)	No (34)	34	Normal		PLAX: 38 mm	Normal	E/A 1.05 e' 11cm/s E/e' 7.60 IVS thickness 7 mm LVPW thickness 7 mm LV massa 53.8 g/m^2
C-II-1*	Male	Alcohol	AF (53)	DCM (52)	60	Normal		4chamber Major: 54 mm	Abnormal relaxation	E/A 0.69 DT 220 ms IVS thickness 7 mm LVPW thickness 7 mm
C-II-2*	Male	Dyslip	pAF (59)	DCM (59)	59	Mildly dilated		PLAX: 40 mm 4chamber Major: 66 mm 4chamber Minor: 44 mm		E/A 0.78 DT 190 ms E/e' 7.00 IVS thickness 9 mm LVPW thickness 10 mm

Supplementary Table 2. Clinical information on the *TTN* c.59926+1G>A carriers. (*continued*)

Subject	Sex	Risk factors	SVT (Age, yrs)	DCM (Age, yrs)	Age echo, yrs	Left atrial description^	LAVI, ml/m2	Other left atrial measurements	Diastolic function^	Measurements
					64	Normal		PLAX: 38 mm	Abnormal relaxation	E/A 0.57 e' 8 cm/s E/e' 6.77 IVS thickness 7 mm LVPW thickness 9 mm
C-III-1★	Female	No	pAF (34)	No (36)	36	Normal	19.0		Normal	E/A 1.40 DT 236 ms e' 13 cm/s E/e'6.64 IVS thickness 8 mm LVPW thickness 8 mm
I-II-2★,#	Female	Obese	No (54)	DCM (41)	54	Normal	32.0		Abnormal relaxation	E/A 1.40 DT 90 ms IVS thickness 10 mm LVPW thickness 8 mm LV mass 79 g/m2
I-II-3★,#	Female	Ht	No (58)	DCM (40)	58	Normal	21.0		Not assessable	
I-III-1★,#	Female	Obese	No (33)	Mild DCM (33)	35	Normal	28.0			E/A 1.00 IVS thickness 10 mm LVPW thickness 10 mm

Supplementary Table 2. Clinical information on the *TTN* c.59926+1G>A carriers. (*continued*)

Subject	Sex	Risk factors	SVT (Age, yrs)	DCM (Age, yrs)	Age echo, yrs	Left atrial description^	LAVI, ml/m2	Other left atrial measurements	Diastolic function^	Measurements
D-II-1[#]	Female	Obese	No (54)	No (54)	54	Normal	29.0		Normal	E/A 1.46 DT 227 ms e' 9 cm/s E/e' 10.00 IVS thickness 10 mm LVPW thickness 10 mm LV mass 84 g/m2
D-III-1[#]	Male	No	No (28)	DCM (25)	27	Dilated		PLAX: 45.1 mm 4chamber Major: 72 mm		E/A 1.47 DT 141 ms IVS thickness 8 mm LVPW thickness 8 mm
D-III-2[#]	Female	No	No (27)	No (27)	27	Normal	29.0		Normal	E/A 1.07 DT 222 ms e' 22 cm/s E/e' 4.00 IVS thickness 6 mm LVPW thickness 7 mm LV mass 55 g/m2
E-IV-1[†]	Male	No	pAF (36)	DCM (48)	52	Dilated		PLAX: 47 mm		IVS thickness 9 mm
E-IV-2[†]	Female	MI	AF (67)	No (78)	67 (with AF)	Dilated		PLAX: 44 mm 4chamber Major: 64 mm		

Supplementary Table 2. Clinical information on the *TTN* c.59926+1G>A carriers. (*continued*)

Subject	Sex	Risk factors	SVT (Age, yrs)	DCM (Age, yrs)	Age echo, yrs	Left atrial description^	LAVI, ml/m2	Other left atrial measurements	Diastolic function^	Measurements
					69	Normal		PLAX: 41 mm 4chamber Major: 56 mm		IVS thickness 6 mm LVPW thickness 7 mm LV mass 87 g/m2
E-V-1†	Male	No	pAF (40)	DCM (40)	37	Normal		PLAX: 36 mm 4chamber Major: 58 mm	Normal	LVPW thickness 7 mm
					39			PLAX: 44 mm 4chamber Major: 57 mm		
					44	Dilated		PLAX: 37 mm 4chamber Major: 72 mm		
E-V-2†	Female	No	pAF (38)	No (46)	46	Normal	28.0		Normal	IVS thickness 7 mm LVPW thickness 6 mm LV mass 63 g/m2
E-V-3†	Male	Ht	No (52)	No (52)	52	Normal	26.0		Normal	IVS thickness 10 mm LVPW thickness 9 mm LV mass 79 g/m2
E-V-4†	Female	No	pAF (52)	DCM (49)	49	Normal		PLAX: 38 mm	Normal	E/A 1.30 DT 240 ms E/e' 6.00 IVS thickness 8 mm LVPW thickness 10 mm
					52 (with AF)	Dilated	39.0	PLAX: 46 mm 4chamber Major: 64 mm		E/e' 8.90

Supplementary Table 2. Clinical information on the *TTN* c.59926+1G>A carriers. (*continued*)

Subject	Sex	Risk factors	SVT (Age, yrs)	DCM (Age, yrs)	Age echo, yrs	Left atrial description^	LAVI, ml/m2	Other left atrial measurements	Diastolic function^	Measurements
					53 (with AF)	Normal		PLAX: 39 mm 4chamber Major: 60 mm		DT 230 ms E/e' 6.10
F-IV-1†	Male	Ht / CAD / SCN5A mutation	Atrial flutter (76)	DCM (76)	76	Normal	28.0		Abnormal relaxation	E/A 0.77 DT 268 ms e' 5 cm/s E/e' 7.80 Long venous flow S<D IVS thickness 9 mm LVPW thickness 10 mm LV mass 113 g/m2

*Families B, C and I could be genealogically linked, going back 3 and 5 generations, respectively. #Families D and I could be genealogically linked, going back 5 generations. †Families E, F and H could be genealogically linked going back 3 and 9 generations, respectively. ^Reported as it was stated in echocardiography report. Mild DCM was noted when fulfilling one of the two criteria (reduced ejection fraction < 45% or widened ventricleof >117% of predicted). 4ch = four chamber, Anthr = Anthracycline, AVNRT = AV-nodal re-entry tachycardia, cm = centimetre, CAD = Coronary artery disease, DCM = Dilated cardiomyopathy, DM = Diabetes Mellitus, DT = E-wave Deceleration time, Dyslip = Dyslipidaemia, g = grams, Ht = Hypertension, IVS = Interventricular septum, LVPW = Left ventricular posterior wall , m2 = square meter, MI = Mitral valve insufficiency, ml = millimetre, ms = milliseconds, (p)AF = (paroxysmal) atrial fibrillation, PLAX = Parasternal Long Axis View, RT = Radiotherapy, s = seconds, SLE = Systemic lupus erythematosus, SVT = Supraventricular tachycardia, yrs = years

Supplementary Table 3. Clinical characteristics of male and female *TTN* c.59926+1G>A carriers.

	Males (N=15)	Females (N=15)	P-value
Age presentation/evaluation, years	48 ±(16)	42 ±(14)	ns
DCM	14 (93%)	6 (40%)	0.005
Risk factors	7 (47%)	10 (67%)	ns
Hypertension	5 (33%)	4 (27%)	ns
Diabetes	0 (0%)	2 (13%)	ns
Dyslipidemia	2 (13 %)	1 (7%)	ns
Coronary artery disease	2 (13 %)	1 (7%)	ns
Obesitas	0 (0%)	3 (20%)	ns
Other[b]	1 (7%)	3 (20%)	ns

Values are median ±(standard deviation) or n (%); DCM = Dilated cardiomyopathy, ns = non-significant
b Composition of chemotherapy, history of excess alcohol consumption, and severe mitral valve insufficiency

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Chapter 4

Variant location is a novel risk factor for individuals with arrhythmogenic cardiomyopathy due to a desmoplakin (*DSP*) truncating variant

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ABSTRACT

Background: Truncating variants in desmoplakin (*DSP*tv) are an important cause of arrhythmogenic cardiomyopathy (ACM), however the genetic architecture and genotype-specific risk factors are incompletely understood. We evaluated phenotype, risk factors for ventricular arrhythmias, and underlying genetics of *DSP*tv cardiomyopathy.

Methods: Individuals with *DSP*tv and any cardiac phenotype, and their gene-positive family members were included from multiple international centers. Clinical data and family history information were collected. Event-free survival from ventricular arrhythmia was assessed. Variant location was compared between cases and controls, and literature review of reported *DSP*tv performed.

Results: There were 98 probands and 72 family members (mean age at diagnosis 43 ± 18 years, 59% female) with a *DSP*tv, of which 146 were considered clinically affected. Ventricular arrhythmia (sudden cardiac arrest, sustained ventricular tachycardia, appropriate implantable cardioverter defibrillator therapy) occurred in 56 (33%) individuals. *DSP*tv location and proband status were independent risk factors for ventricular arrhythmia. Further, gene region was important with variants in cases (cohort n=98, Clinvar n=167) more likely to occur in the regions resulting in nonsense mediated decay of both major *DSP* isoforms, compared to n=124 gnomAD control variants (148 [83.6%] versus 29 [16.4%], $p<0.0001$).

Conclusions: In the largest series of individuals with *DSP*tv, we demonstrate variant location is a novel risk factor for ventricular arrhythmia, can inform variant interpretation, and provide critical insights to allow precision-based clinical management.

INTRODUCTION

Desmoplakin is a plakin family protein that anchors the desmosome to intermediate filaments and is abundant in tissues with greater mechanical stress such as the epidermis and myocardium.[1, 2] Genetic variants in the gene encoding desmoplakin (*DSP*) cause a range of cardio-cutaneous phenotypes including arrhythmogenic cardiomyopathy (ACM), striate palmoplantar keratoderma and lethal acantholytic epidermolysis bullosa in more severe cases.[3] Truncating variants (*DSP*tv) that lead to putative loss of function (LOF) via haploinsufficiency of the protein have been previously reported as causative of disease.[2] *DSP*-null mice show extensive disruption of the cytoarchitecture and cell resilience in skin and heart tissue, with death in early development.[4]

Arrhythmogenic right ventricular cardiomyopathy (ARVC), the right dominant sub-form of ACM,[2, 5] is characterised by progressive loss and fibrofatty replacement of the ventricular myocardium.[6] Diagnosis of ARVC can be challenging and 2010 Task Force Criteria consider electrical, structural (imaging and histological) and genetic characteristics.[7] Historically, clinical descriptions of *DSP*tv were often based on ARVC cohorts, though growing recognition of left ventricular (LV) involvement has necessitated a shift to a broader phenotype description, ACM,[8] encompassing left dominant arrhythmogenic cardiomyopathy (LDAC) and biventricular disease, with new Padua criteria proposed.[9] Dilated cardiomyopathy (DCM) and LDAC lie on a spectrum, with overlap in molecular causes. More recently, *DSP* has been definitely associated with both ARVC and DCM by international gene curation expert panels[10, 11] In one of the largest studies to date, clinical characteristics of *DSP* variants in a population of 44 probands and 63 family members were reported as a distinct ACM characterised by LV fibrosis, myocardial inflammation and high incidence of ventricular arrhythmias.[12] Biallelic *DSP* variants can give rise to Carvajal syndrome, characterised by woolly hair, palmoplantar keratoderma and development of ACM in childhood, and often due to homozygous or compound heterozygous *DSP*tv affecting the C-terminus.[13]

The N-terminal globular head of *DSP* is important in desmosome organisation by binding plaque proteins such as plakophilin and plakoglobin, while the central rod domain contains a coiled-coil region.[14] The C-terminal contains three plakin repeat domains, required for alignment and binding of intermediate filaments.[15] Two predominant isoforms exist due to alternate splicing, *DSPI* which is the longest isoform and *DSPII* which has a shortened central rod domain. DSPI and DSPII are expressed in equivalent levels in epidermis, however DSPI is more prevalent in myocardium.[16] Differences between the two isoforms relate to the rod domain size, considered important for self-association and formation of homo-dimers.[17]

Here we report an international cohort of individuals with a *DSP*tv. We describe the phenotype spectrum of *DSP*tv cardiomyopathy, family history characteristics, and provide insights into the genetic architecture of *DSP*tv cardiomyopathy and its relation to clinical phenotype.

METHODS

Data are available by request to the corresponding author and adhering to site ethical approval. All aspects of the study were performed according to institutional human research ethics committee approval according to the local sites. Detailed methods are available in the supplement.

RESULTS

Study population

Overall there were 98 probands (mean age at diagnosis 42 ± 18 years, 59% female) and 72 family members identified (mean age at diagnosis 45 ± 19 years, 61% female; **Table 1**). There were 95 probands with a cardiomyopathy and 3 with a primary cutaneous phenotype. Among family members, 48/72 were deemed affected, including 5 with a predominantly cutaneous phenotype. In total, 146 individuals were considered affected including cardiomyopathy, ventricular arrhythmia and cutaneous phenotypes.

Sex differences

Females were over-represented compared to males among affected individuals (86 [59%] versus 60 [41%]; **Table 1**). There was no difference in mean age at diagnosis between females and males (40 ± 17 years versus 46 ± 19 years, p=0.07). Myocarditis was more frequent in males (2/43 [5%] versus 6/25 [24%], p=0.046) but this was not always reliably reported. Women had reduced LV ejection fraction (LVEF) on transthoracic echocardiography, but not cardiac magnetic resonance imaging (CMR) derived LVEF. Men had greater indexed right ventricular (RV) end diastolic volume (84 ± 20 versus 100 ± 27, p=0.01). No difference in clinical outcomes were reported between sexes. There was a comparable distribution of variants by gene region for men and women, as well as probands and affected relatives.

Electrophysiological characteristics

There was a high rate of ventricular arrhythmia occurring in 56 (33%) individuals, including 46 (47%) probands and 10 (14%) family members. Ventricular arrhythmia included sudden cardiac death (SCD) in 13 (8%; 10 probands), resuscitated cardiac arrest in 15 (9%; 12 probands), appropriate implantable cardioverter defibrillator (ICD) therapy in 16 (10%; 16

probands) and sustained ventricular tachycardia in 19 patients (11%; 15 probands); including 10 (14%) family members, and with some experiencing multiple events. Six probands experienced two ventricular arrhythmia episodes, initially having sustained ventricular tachycardia (n=4) or resuscitated cardiac arrest (n=2), followed by appropriate ICD therapy. SCD or resuscitated cardiac arrest was the presenting symptom in 24 (14%; 20 probands) patients. T wave inversion beyond V3 occurred in 33 (24%), low voltages in 47 (35%) and premature ventricular contractions in 56 (33%).

Imaging characteristics

Echocardiographic and CMR characteristics are shown in **Table 1**. Signs of LV noncompaction (LVNC) were reported (n=22), with 6 having a ratio of noncompacted to compacted layer >2.3 on CMR. Four probands were reported to have hypertrophic cardiomyopathy (HCM) with ages at diagnosis ranging from 58-83 years, and LV hypertrophy measuring 26mm, 16mm, and an apical pattern in two. *DSP*tv are not established as associated with HCM, and we consider it unlikely that these variants are causal for HCM for these 4 cases, but are reported as they met the pre-specified eligibility criteria. Late gadolinium enhancement (LGE) was reported in 59 (61%), and end-stage heart failure was reported in 10 (8%) patients. Two females developed disease while pregnant, one showed impaired LV function (LVEF <45%) at 32 weeks of gestation, while the other developed narrow complex tachycardia at 38 weeks of gestation with subsequent echocardiogram showing a dilated and impaired LV. A further two women developed disease during the postpartum period. Finally, another patient who died suddenly during pregnancy was identified to be positive for Parvovirus B19 on postmortem Parvo-polymerase chain reaction in myocardial tissue. Myocarditis was reported in 7 individuals on CMR (and another on postmortem investigation).

Genetic analysis

A total of 69 distinct *DSP*tv were identified in the 98 probands (**Supplementary Table 1**). Among the 69 *DSP*tv, there were 31 small insertions or deletions leading to a frameshift and downstream premature termination codon, 25 nonsense variants, 12 canonical splice-site altering variants and a large deletion of exons 5-24. Eleven (16%) variants were classified as pathogenic, 57 (83%) were classified as likely pathogenic and 1 (1%) was classified as a variant of uncertain significance. Two probands had a diagnosis of cardiomyopathy, with woolly hair and keratoderma (OMIM 605676) and were compound heterozygous, each carrying a *DSP*tv (p.Arg2229Serfs*32 or p.Tyr28Alafs*66) and a *DSP* splice site variant (the same c.273+5G>A in both). This splice site variant has an allele count of 79 in gnomAD, with an allele frequency of 0.028% and considered a variant of uncertain significance under a recessive inheritance model.

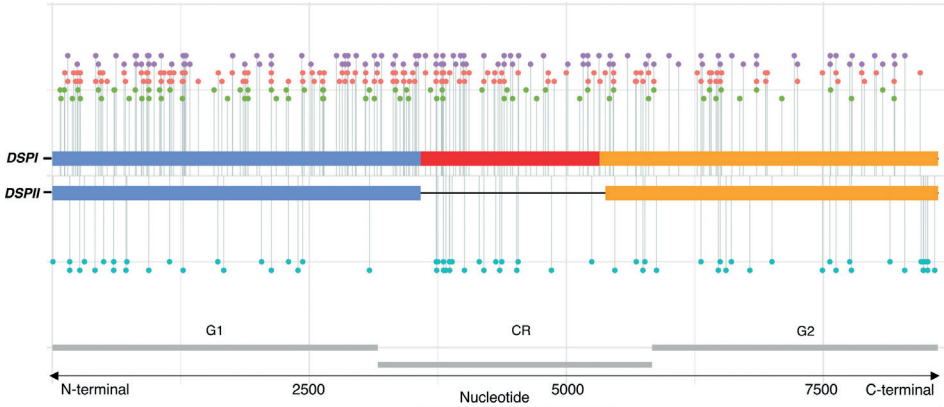
Table 1. Clinical characteristics by proband status and sex.

Diagnosis	Proband status			Sex (Affected only)		
	Total n=178	Probands n=98	Family members n=72	Female n=86	Male n=60	p-value
Affected	146	98 (100)	48 (67)	86 (100)	60 (100)	NA
Mean age at diagnosis ± SD, years	43 ± 18	42 ± 18	45 ± 19	40 ± 17	46 ± 19	0.07
Female sex	101 (59)	57 (58)	44 (61)	NA	NA	NA
T wave inversion beyond V3	33 (24)	25 (32)	8 (13)	22 (33)	9 (18)	0.07
Low voltages	47 (35)	30 (39)	17 (29)	28 (42)	13 (27)	0.09
Premature ventricular contractions	35 (25)	23 (29)	12 (20)	25 (35)	10 (21)	0.09
Ventricular arrhythmia	56 (33)	46 (47)	10 (14)	37 (44)	19 (32)	0.17
Sudden cardiac death	13 (8)	10 (10)	3 (4)	7 (8)	6 (10)	0.71
Resuscitated cardiac arrest	15 (9)	12 (12)	3 (20)	8 (10)	7 (12)	0.65
Sustained VT	19 (11)	15 (15)	4 (6)	14 (17)	5 (8)	0.24
Appropriate ICD therapy	16 (10)	16 (16)	0 (0)	13 (15)	3 (5)	0.09
End-stage heart failure	10 (8)	7 (8)	3 (5)	6 (8)	4 (8)	0.94
Heart transplant / LVAD	6 (6)	6 (6)	0 (0)	4 (5)	2 (3)	0.64
Combined endpoint	64 (38)	52 (53)	12 (17)	41 (48)	23 (39)	0.27
Myocarditis	8 (10)	6 (13)	2 (6)	2 (5)	6 (24)	0.046
Transthoracic echocardiogram						
LV ejection fraction	40 ± 16	35 ± 15	48 ± 13	35 ± 14	42 ± 15	0.03
LV end diastolic diameter	57 ± 9	59 ± 9	53 ± 8	58 ± 8	59 ± 10	0.56
LV end systolic diameter	44 ± 12	47 ± 12	37 ± 9	47 ± 12	44 ± 12	0.29
CMR imaging						
LV ejection fraction, %	42 ± 12	38 ± 12	48 ± 12	39 ± 11	43 ± 13	0.14
LV end diastolic volume, indexed	111 ± 33	117 ± 35	100 ± 23	119 ± 34	105 ± 29	0.09
LV end systolic volume, indexed	67 ± 32	73 ± 35	54 ± 20	74 ± 34	60 ± 27	0.07
RV ejection fraction, %	47 ± 12	44 ± 12	53 ± 8	47 ± 11	45 ± 12	0.46
RV end diastolic volume, indexed	89 ± 24	93 ± 25	83 ± 21	84 ± 20	100 ± 27	0.01
RV end systolic volume, indexed	48 ± 19	52 ± 19	41 ± 16	46 ± 17	56 ± 20	0.05
Regional wall motion abnormalities	44 (45)	35 (56)	9 (26)	25 (46)	19 (51)	0.64
Late gadolinium enhancement	59 (61)	40 (63)	19 (58)	31 (57)	28 (76)	0.07
LV noncompaction	22 (22)	17 (27)	5 (13)	14 (29)	8 (21)	0.42
Gene region						
Constitutive NMD competent	83 (57)	57 (58)	26 (54)	49 (57)	34 (57)	0.84
Non-constitutive NMD competent	27 (18)	20 (20)	7 (15)	17 (20)	10 (17)	
Constitutive NMD incompetent	36 (25)	21 (21)	15 (31)	20 (23)	16 (27)	

Data were analysed using students t-test or chi-square test for continuous and categorial variables, respectively. Data shown are n (%) or mean ± standard deviation. Abbreviations: ICD, implantable cardioverter defibrillator; LVAD, left ventricular assist device; VT, ventricular tachycardia; LV, left ventricular; CMR, cardiac magnetic resonance; RV, right ventricle.

*DSP*tv location

We investigated whether case and control variants localised to the specified gene regions, constitutive NMD-competent, non-constitutive NMD-competent and constitutive NMD-incompetent (**Figure 1**). Pathogenic and likely pathogenic *DSP*tv submitted to ClinVar, as well as variants described above in the international cohort were included, giving a total of 265 cases. This included 69 unique *DSP*tv identified in 98 individuals in the international cohort and 134 unique *DSP*tv from 167 cases reported in ClinVar (**Supplementary Table 2**). One variant reported in ClinVar was excluded from analysis given it resided in the small overlap region of exon 23 which is both non-constitutive and predicted NMD-incompetent due to being <55 bp upstream of the last exon junction (*DSP*: c.5327_5330del, p.Glu1776Glyfs). Another was excluded after it was identified as a ClinVar entry for one of the cohort cases. Literature cases are shown in **Figure 1** but not included in the analysis due to unquantified sample overlap. Variants observed in cases were compared to 72 unique *DSP*tv observed as 124 alleles in gnomAD controls (**Supplementary Table 3**). Case variants were more frequently seen in the constitutive NMD-competent region compared to controls. Across the 3 gene regions (constitutive NMD-competent, non-constitutive NMD-competent and constitutive NMD-incompetent, respectively), *DSP*tv were seen in 148 (56%), 59 (22%) and 58 (22%) cases compared to *DSP*tv observed in controls 29 (23%), 28 (23%) and 67 (54%), overall p<0.0001.



	Constitutive NMD competent region c.1-3582	Non-constitutive NMD competent region c.3583-5379	Constitutive NMD incompetent region c.5380-8616
Literature	50 (46.7)	27 (25.2)	30 (28.0)
Clinvar	74 (56.0)	27 (20.5)	31 (23.5)
Cohort	41 (59.4)	13 (18.8)	15 (21.7)
gnomAD	24 (33.3)	19 (26.4)	29 (40.3)

Figure 1. Linear topology schematic showing distribution of *DSP*tv across key gene regions of major isoforms *DSPI* and *DSPII*. Case variants (Cohort, Clinvar, and Literature) are shown above and control variants below the line. The number of unique variants (per proband) from each source are shown in the table. CR indicates central fibrous rod domain; DSPtv, desmoplakin truncating variant; G1, globular 1; G2, globular 2; gnomAD, gnome aggregation database; and NMD, nonsense mediated decay.

Clinical characteristics of patients with *DSP*tv in the three gene regions are shown in **Table 2**. Overall there were few significant differences between the patient groups based on gene region. Age at diagnosis was significantly younger in those with *DSP*tv in both NMD-competent regions (constitutive and non-constitutive). Further, there was a greater risk of ventricular arrhythmia and risk of the combined endpoint in those with *DSP*tv in the constitutive and non-constitutive NMD-competent regions.

Event-free survival from ventricular arrhythmia based on gene region

Information with regard to occurrence of ventricular arrhythmia or censoring was available for 167 individuals. There were 56 probands and family members who experienced a ventricular arrhythmia during their lifetime. Univariable Cox proportional hazards models showed gene region and proband status as significantly associated with worse survival from ventricular arrhythmias (**Table 3; Figure 2**). Adjusting for other variables, variants in the constitutive NMD competent region (HR 2.8, 95% CI 1.3-6.0, p=0.01), non-constitutive NMD-competent region (HR 3.2, 95%CI 1.3-7.9, p=0.009) and proband status (HR 3.3, 95%CI 1.7-6.6, p=0.0006) remained significant independent life-time risk factors for ventricular arrhythmia (**Table 3**).

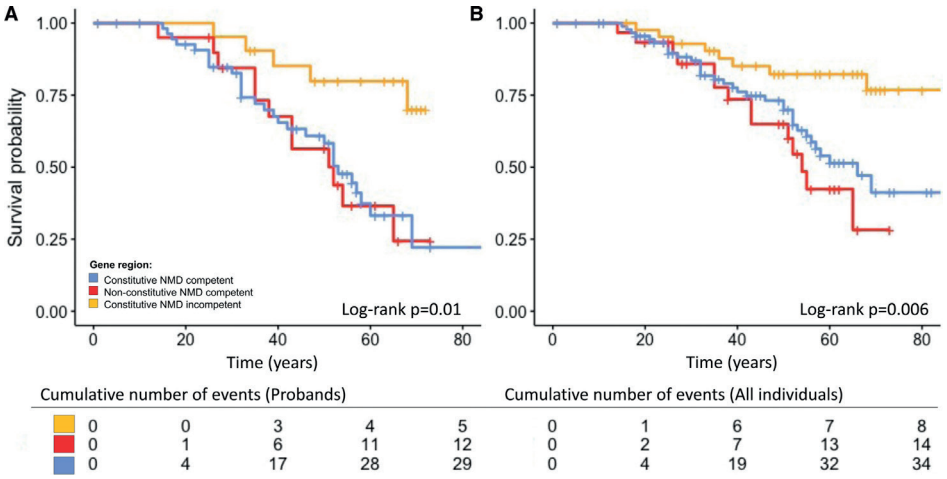


Figure 2. Independent life-time risk factor for ventricular arrhythmia. (A) Gene region including probands only. (**B**) Gene region including probands and affected family members. Time is given in years. NMD indicates nonsense mediated decay.

Table 2. Cardiac investigation of affected individuals with *DSP*tv by gene region.

	N	Constitutive NMD-competent	Non-constitutive NMD-competent	Constitutive NMD-incompetent	p-value
n	146	83 (57)	36 (25)	27 (18)	-
Age at diagnosis, years	143	40 ± 18	37 ± 16	52 ± 16	0.002
Female sex	146	49 (59)	17 (63)	20 (56)	0.84
Family history of disease	107	36 (57)	15 (68)	9 (43)	0.25
Ventricular arrhythmia	144	34 (41)	14 (54)	8 (22)	0.03
SCD	143	7 (9)	4 (15)	2 (6)	0.40
Resuscitated cardiac arrest	143	7 (9)	5 (23)	2 (6)	0.06
Sustained VT	143	14 (17)	1 (4)	4 (11)	0.19
Appropriate ICD therapy	143	13 (16)	3 (12)	0 (0)	0.04
Combined endpoint	144	39 (48)	15 (58)	10 (28)	0.04
Myocarditis	68	6 (18)	1 (5)	1 (7)	0.32
LV noncompaction	87	14 (26)	6 (40)	2 (11)	0.16
PVCs	119	21 (33)	4 (18)	10 (30)	0.43
Transthoracic echocardiogram					
LV ejection fraction	100	41 ± 16	28 ± 11	38 ± 13	0.008
LV end diastolic diameter	98	59 ± 9	57 ± 10	57 ± 7	0.71
LV end systolic diameter	77	45 ± 12	47 ± 14	46 ± 9	0.95
CMR imaging					
LV ejection fraction	88	41 ± 12	38 ± 13	41 ± 11	0.55
LV end diastolic volume, indexed	75	121 ± 39	114 ± 28	104 ± 22	0.13
LV end systolic volume, indexed	76	75 ± 37	69 ± 30	61 ± 24	0.27
RV ejection fraction	62	49 ± 10	45 ± 11	44 ± 14	0.35
RV end diastolic volume, indexed	56	92 ± 26	81 ± 14	93 ± 25	0.35
RV end systolic volume, indexed	55	51 ± 19	48 ± 13	51 ± 21	0.87
Late gadolinium enhancement	91	29 (64)	12 (63)	18 (67)	0.97
Regional wall motion abnormalities	91	22 (47)	6 (35)	16 (59)	0.29
Electrocardiogram					
Sinus rhythm	81	43 (98)	16 (100)	21 (100)	0.65
PR interval	110	167 ± 43	155 ± 27	172 ± 50	0.35
QRS	108	102 ± 17	95 ± 12	97 ± 16	0.21
QTc	105	424 ± 40	417 ± 32	421 ± 39	0.76
T wave inversion beyond V3	117	21 (33)	3 (13)	7 (23)	0.14
Low voltages	114	23 (37)	8 (38)	10 (33)	0.93

Data shown are mean ± standard deviation (n = number of persons with available data) or n (%). Abbreviations: LV, left ventricle; RV, right ventricle; NMD, nonsense mediated decay; SCD, sudden cardiac death; ICD, implantable cardioverter defibrillator; PVCs, premature ventricular contractions (>500 per 24 hours). Data shown are mean ± standard deviation or n (%)

Table 3. Life time risk factors for ventricular arrhythmia for individuals with a *DSP*tv.

		Univariable			Multivariable		
	N	**HR**	**95% CI**	***p* value**	**HR**	**95% CI**	***p* value**
Female sex	167	1.5	0.9-2.6	0.14			
Proband status	167	3.5	1.8-7.0	0.0003	3.3	1.7-6.6	0.0006
Gene region:	167						
Constitutive NMD competent		2.8	1.3-6.1	0.009	2.8	1.3-6.0	0.01
Non-constitutive NMD competent		3.8	1.6-9.2	0.003	3.2	1.3-7.9	0.009
Constitutive NMD incompetent		REF	-	-	REF	-	-
Family history of sudden cardiac death	110	0.7	0.3-1.7	0.44			

Abbreviations: *DSP*tv, desmoplakin truncating variant; NMD, nonsense mediated decay; REF, reference category. For the adjusted analysis, there were total n=167 individuals included with n=56 events.

Cutaneous phenotype

Cutaneous abnormalities were not systematically reported, however notably one family with a *DSP*tv in the non-constitutive NMD-competent region (cardiac isoform, *DSPI*) had an affected relative with hyperkeratosis and cardiomyopathy. An additional 13 individuals with *DSP*tv in the constitutive NMD-competent region and 5 in the constitutive NMD-incompetent region were reported with overt cardio-cutaneous features noted at clinical review. In eight patients (5%; 3 probands) only cutaneous abnormalities were reported, and were the sole finding in one family following an autosomal dominant inheritance pattern.

Postmortem findings and cardiac transplant histology

Thirteen patients (8%; 10 probands) presented with SCD (**Supplementary Table 4**). In all 13, a postmortem investigation was performed. The mean age at death was 26 ± 11 years. Where recorded, the activity at time of death varied from exercise through to sleep. No decedent had a pre-morbid diagnosis of a cardiac condition. Nine decedents received a postmortem diagnosis of ARVC or probable ARVC. There was LV involvement in all cases and fibrosis and fatty infiltration commonly reported.

Two patients underwent a heart transplant due to end stage heart failure. Biventricular involvement was observed in both hearts, as were signs of LVNC. One heart showed ARVC with septal involvement and replacement fibrosis in both ventricles and septum. The other heart showed LVNC with notable RV involvement consisting of fatty changes and atrophy.

Family history characteristics

Among the probands, 49 (51%) had a documented family history of cardiomyopathy, while 16 (17%) had a family history of a suspicious SCD under the age of 40 years. Of 72 family members with positive gene results included, 48 (67%) had overt disease, while 24 (33%) remained asymptomatic (mean age of 49 ± 22 years and 15 [63%] were female). There were

8 family members aged 60 years or older (60-86 years; 5 females) with no clinical evidence of disease, suggesting incomplete penetrance. By gene region, there was no statistical difference in the proportion of probands with a positive family history (constitutive NMD-competent 27 [49%], non-constitutive NMD-competent 13 [65%], constitutive NMD-incompetent 9 [43%], p=0.33).

Literature review of previously reported *DSP*tv

Three hundred and fifteen studies were identified, 240 were screened and 185 full texts were assessed for eligibility (85 were excluded from the final qualitative synthesis, including 66 that did not report any *DSP* variant, 2 where phenotype was not provided, 2 with no full text article available, and 1 review; **Supplementary Figure 1**). Of 98 studies (describing both disease and genotype-first cohorts) included in the final selection, a total of 105 *DSP*tv in 143 probands from apparently unrelated families were reported, including 57 nonsense, 42 frameshift, and 6 splice site variants (**Supplementary Tables 5-7**). All reported variants were absent or very rare (allele count ≤ 2) in gnomAD and were classified as pathogenic or likely pathogenic. One variant (p.Thr2104fs*12) was present 13 times in gnomAD however has strong evidence of pathogenicity and reported in a compound heterozygous state.

Both dominant and recessive patterns of inheritance of *DSP*tv were reported. Cascade genetic testing to confirm autosomal dominant inheritance was reported for only 19 *DSP*tv (dominant *DSP*tv) in 22 families. Families reported with autosomal dominant inheritance commonly demonstrated adult age of onset, incomplete penetrance and variable clinical expression. Of 105 reported *DSP*tv, 26 were only identified in affected individuals with homozygous or compound heterozygous inheritance. Four *DSP*tv co-occurred in trans with one of three missense DSP variants (p.Ala2655Asp, p.Arg2366Cys and p.Asn287Lys), each of which involved highly conserved residues within globular heads, are absent in gnomAD, and classified as likely pathogenic. There were 16 individuals with 23 *DSP*tv identified to have autosomal recessive disease, either homozygous (n=9) or compound heterozygous (n=7). In just those variants identified in a homozygous state there was only 1 (11%) in the constitutive NMD-competent region, 5 (56%) in the non-constitutive NMD-competent region and 3 (33%) in the constitutive NMD-incompetent region.

DISCUSSION

*DSP*tv lead to a distinct cardiomyopathy characterised by LV involvement and a high-risk of ventricular arrhythmia and SCD. We present a large international series of cases with *DSP*tv and demonstrate that the location of the *DSP*tv is a novel risk factor for ventricular arrhythmia (**Figure 3**). Truncating variants in the constitutive NMD-competent region were

enriched in cases compared to controls, and predicted to result in NMD and haploinsufficiency of both DSPI and DSPII. Our findings highlight the importance of personalized medicine and the move towards gene-guided management of patients in the future.

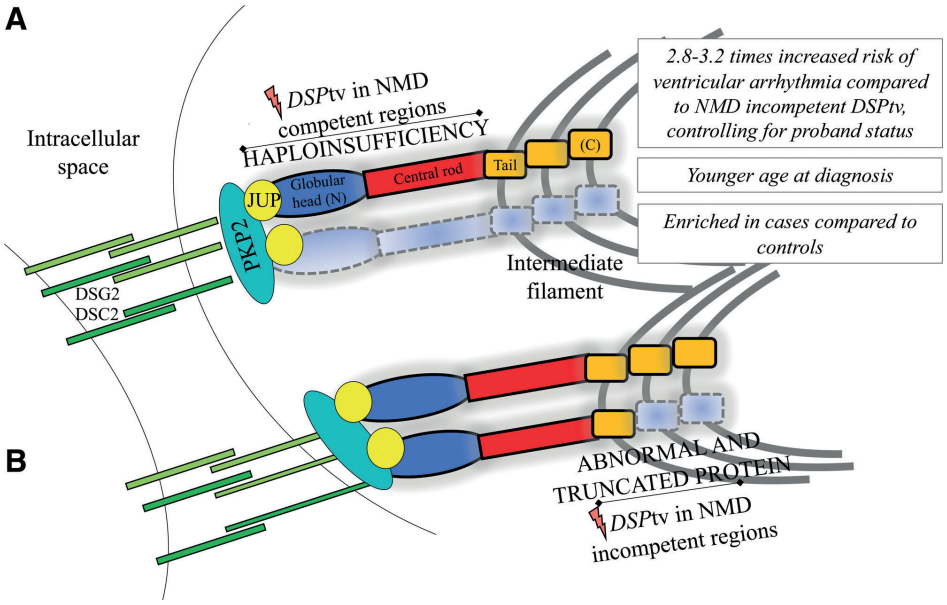


Figure 3. Summary of the key findings and illustration of the impact of DSPtv location on protein expression (for DSPI). DSC2 indicates desmocollin-2; DSG2, desmoglein-2; JUP, plakoglobin; PKP2, plakophilin-2; and NMD, nonsense mediated decay.

Ventricular arrhythmias occur frequently in patients with *DSP*tv cardiomyopathy, with one previous study reporting 23% presenting with SCD events.[18] In our cohort, 47% of probands had ventricular arrhythmia either at presentation or during follow-up. In addition, 14% of relatives experienced ventricular arrhythmia, including 7% as their initial presenting symptom. This included two probands who presented with resuscitated cardiac arrest without any overt structural abnormalities of the heart, supporting the notion that life-threatening electrical phenotype can precede overt cardiac structural disease.[19-21] While we were unable to robustly ascertain clinical risk factors due to the large proportion of cases who presented with ventricular arrhythmia, i.e. without necessary pre-event clinical data, a recent series of 107 patients with any *DSP* variants (n=30 events) showed ventricular arrhythmias were associated with reduced LVEF, while premature ventricular contractions (>500 beats in 24 hours), LGE and RV dysfunction were not shown to be associated with ventricular arrhythmia.[12] Family history of SCD has not previously been evaluated in this group, and we showed it is not associated with ventricular arrhythmia in our population.

Prior observation that *DSP*tv are predominantly associated with a left-dominant form of ACM[2, 5, 22] is in line with our findings. Recent examples of *DSP*tv presenting as recurrent myocarditis and acute myocardial infarction-like events have also been reported.[23, 24] Females were overrepresented in our population, but otherwise shared similar clinical characteristics compared to males, except reduced indexed RV end diastolic diameter on CMR. This finding is in contrast to other reported inherited cardiomyopathy patient cohorts, where a higher prevalence of males is often reported.[25-27] Of note, a recent report of ARVC presenting as clinical myocarditis showed disproportionately more women, with 10/11 having *DSP*tv.[28] *DSP*tv cardiomyopathy patients frequently had low QRS voltage and negative T waves beyond V3. Low QRS voltage in limb leads have previously been shown to be associated with the presence and amount of LGE in a study of patients with ARVC.[8] Regional wall motion abnormalities on CMR and epicardial to mid wall LGE patterns in the LV were frequently seen in our cohort. Septal LGE frequently occurs in patients with LDAC,[15] and recent work has shown patients with *DSP* and *FLNC* ACM are more likely to have LGE, often with a ring-like pattern, compared to other DCM genotypes.[29] Four probands were reported to have HCM, however it should be noted that all 4 probands were male, presenting in older age and 3 had mild LV hypertrophy, all characteristics previously described in the non-familial sub-group of HCM.[30] Previous assessment of the clinical validity of *DSP* variants causing HCM failed to identify sufficient evidence of gene-disease association.[31] While our finding remains unclear, it seems reasonable to consider these clinical diagnoses as unrelated to the *DSP*tv.

A recent systematic evaluation of cutaneous abnormalities among *DSP*tv showed all patients expressed some degree of skin or hair abnormalities, except those with *DSP*tv in the non-constitutive NMD-competent region (cardiac isoform, DSPI).[32] Interestingly, we report one proband and their affected relative with palmoplantar keratoderma, with a *DSP*tv in the non-constitutive NMD-competent region. Another study reported 10% of *DSP*tv had cutaneous disease only, while 12% were reported to have LV dominant ACM and cutaneous disease.[5]

We show *DSP*tv localised to the constitutive NMD-competent region, corresponding to the N-terminal globular head, were enriched in patients compared to controls, and this finding was replicated in the variants identified through literature review. This region plays a critical role in organisation and assembly of the desmosomal complex by binding with plakophilin and plakoglobin. One previous report of *DSP* missense variants in patients with a clinical diagnosis of ARVC suggested a potential 'hotspot' N-terminal region, with 8/17 (47%) missense variants localised to the N-terminal compared to 1/28 (4%) of controls ($p<0.0008$).[33] Further, they concluded *DSP*tv were significantly more prevalent in ARVC cases than controls. Indeed, a recent study also showed clustering of missense variants in the N-terminal, but reported *DSP*tv to be more evenly distributed across the gene,[12] po-

tentially limited by sample size. Another showed enrichment of missense variants in the spectrin repeat domain, which is part of the constitutive NMD competent region.[34] While it seems likely that truncating variants in the NMD-incompetent region escape NMD and have a later onset and less deleterious impact, functional work to date has shown highly variable pattern of protein expression representing both haploinsufficiency and dominant negative effects.[35] Our literature review identified biallelic *DSP*tv localized more often to the constitutive NMD-incompetent region compared to dominant *DSP*tv, suggesting that single heterozygous *DSP*tv are more likely to cause disease when occurring in the NMD-competent regions. Further, very few cases with homozygous variants in the constitutive NMD-competent region have been reported, with one example of a sib pair with severe lethal acantholytic epidermolysis bullosa, who died at 1 and 3 days respectively.[36] It seems unlikely these infants were DSP null, given *DSP* knockout mice show embryonic lethality,[4] suggesting expression of low-level truncated protein may be able to rescue the phenotype to some degree. Taken together, identification of a *DSP*tv in the NMD-competent regions should be considered important and may prompt gene and disease-specific adaptation and use of the ACMG/AMP criteria.[37] We suggest *DSP*tv in this region be allocated very strong level of evidence, PVS1, when considering pathogenicity, when seen in an individual with a well characterised and concordant phenotype.

STUDY LIMITATIONS

This was a large retrospective cohort study, and while it was an international effort, differences in practices and data collection by site meant some variables were incomplete. Furthermore, the event rate and data missingness precluded more detailed risk factor analyses. Diagnosis was made by the referring clinician and most recruitment was from specialised tertiary referral centers and therefore likely represents more severe phenotypes. The literature review was limited by publication bias, and inconsistent reporting of clinical, family and genetic information.

CONCLUSION

We present a large international series of individuals with *DSP*tv and show gene region is a novel risk factor, specifically *DSP*tv leading to predicted NMD of truncated protein and haploinsufficiency of DSPI and/or DSPII is a risk factor for ventricular arrhythmias. By sub-typing disease by genotype there is increasing ability to offer precision medicine-based advice and therapies, and thereby improved outcomes for patients and their families.

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SUPPLEMENTAL DATA

METHODS

International cohort

An international (Australia, United Kingdom, Netherlands and United States of America) retrospective cohort of unrelated patients and family members with a *DSP*tv was assembled, comprising patients or their relatives seen in specialised cardiac genetic clinics, outpatient cardiology clinics or clinical genetics services. Cases were submitted between July 2016 and August 2018, and cross-sectional clinical data collected from the medical record at each site.

The proband was defined as the first affected in the family who underwent genetic testing with a *DSP*tv identified. Cases seen in the specialised multidisciplinary Genetic Heart Disease clinic at Royal Prince Alfred Hospital between 2002-2018 were included. International centers were included via existing collaborative networks. These included the Department of Medical Genetics, University Medical Center Groningen, Groningen, The Netherlands; Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands; Department of Medical Genetics, Maastricht University Medical Center, Maastricht, The Netherlands; Department of Medical Genetics, Amsterdam University Medical Center, Amsterdam, The Netherlands; Department of Medical Genetics, Leiden University Medical Center, Leiden, The Netherlands; Department of Medical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands; Department of Medical Genetics, Radboud University Medical Center, Nijmegen, The Netherlands; Stanford Inherited Cardiovascular Diseases Group, Stanford University, California USA; and Cardiovascular Research center, Royal Brompton & Harefield Hospitals London UK. Members of the Australian Cardiac Genetic Testing network (ACGT) were invited to contribute probands. The ACGT network includes cardiac genetic professionals throughout Australia, including >90 clinicians, scientists and genetic counsellors working towards a standardised cardiac genetic testing pathway. Members were contacted from July 2016 and invited to contribute cases until August 2018. All aspects of the study were performed according to institutional human research ethics committee approval.

Eligibility criteria

*DSP*tv were those affecting a canonical splice-site, nonsense variants, or insertion or deletion variants leading to a frameshift. In-frame insertions and deletions were not included. All variant nomenclature adhered to the Human Genome Variation Society sequence variant nomenclature recommendations, using reference transcript NM_004415.3. Probands and family members were included irrespective of cardiac phenotype, if they carried one of the variants described above. Both deceased and living patients were included, regardless of their age. Diagnosis was recorded by the referring institution and participants were classified as probands or family members, or as clinically affected (including any cardiac phenotype such as cardiomyopathy or ventricular arrhythmias, or cutaneous phenotype) and unaffected.

Genetic testing was performed by the referring institution. More detailed information is available in the supplement.

Variant location analyses

Genetic variant data were sought from three sources; (1) Variants identified in patients included in this study; (2) Variants submitted to ClinVar with a review status of one-star and above, that were classified as pathogenic or likely pathogenic[38] (Data downloaded on 5th October 2018); and (3) *DSP*tv listed in gnomAD v2.1.[39] ClinVar assertions were only those reporting cases, were evaluated to remove duplication with cohort cases and only used in the gene region analyses.

Variants were grouped in to different gene regions based on (i) whether the region is included in one or both of the 2 major isoforms *DSPI* and *DSPII* (**Figure 1**), and (ii) whether a variant in that location would be expected to trigger nonsense mediated decay (NMD). This results in three regions (**Figure 2**), a constitutive (incorporated in to both major isoforms) and NMD-competent region at the N-terminal (exons 1-22, part of exon 23, c.1-c.3582), a region that is non-constitutive (incorporated in to *DSPI* only) and NMD-competent located in the central rod domain (*DSPI/DSPIa* c.3583-c.4050 and *DSPI* c.4051-c.5379), and a region that is constitutive but NMD-incompetent at the C-terminus (exon 24; c.5324-8616).

Genetic variant classification

Disease variants were classified using the American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG/AMP) standards for variant classification.[37] In brief, the criterion pathogenic moderate (PM2) were allocated to variants with a frequency in the Genome Aggregation Database (gnomAD) of <0.04%,[37] and pathogenic very strong (PVS1) was used for variants in the NMD competent regions. PVS1_strong was allocated to *DSP*tv in the constitutive NMD incompetent region. Use of PVS1 or PVS1_strong was used based on:

1. *DSP* is intolerant to LOF variation, with only 18% of expected LOF variants observed (LOF observed/expected score; oe 0.18), and LOF observed/expected upper bound fraction (LOEUF) of 0.26 based on gnomAD v2.1.[39]
2. *DSP*tv variants are enriched in DCM cases compared to controls, with significant enrichment in two DCM cohorts.[40]
3. *DSP*tv in the constitutive NMD incompetent region are predicted to escape NMD, however functional evidence supports truncation or alteration of this domain as having a critical impact on the protein function, hence PVS1_strong was applied.[41] Further, re-analysis of variants reported by Mazzarotto et al.[40] was performed based on gene region, with significant excess seen for all three regions: constitutive NMD competent (OR 37, 95%CI 16-90, p<0.0001), non-constitutive NMD competent (OR 25, 95%CI

9-73, p<0.0001) and constitutive NMD incompetent (OR 11, 95% CI 4-31, p=0.0006) compared to gnomAD v2.1 controls. There was a high etiologic fraction for all regions: 0.97, 0.96 and 0.91 respectively.

Clinical assessment

Clinical data from all patients (probands and family members) with a pathogenic or likely pathogenic *DSP*tv were collected retrospectively. Clinical information was obtained by review of the medical record and cardiac investigations from the referring institution. Review of the genetic result, ECG, transthoracic echocardiogram, 24-hour ambulatory ECG (Holter) monitoring, cardiac magnetic resonance (CMR) imaging, three-generation pedigree, post-mortem report and correspondence from the treating geneticist and/or cardiologist were reviewed by the study team where possible.

Diagnosis and clinical definitions

The LV was considered to be involved when one or more of the following was present: LV ejection fraction <55%, presence of LV late gadolinium enhancement (LGE) or intramyocardial fat (including septum) on CMR imaging or pathologic abnormalities found on autopsy after sudden cardiac death (SCD). Cutaneous abnormalities were defined as palmoplantar keratoderma and/or woolly hair. Cutaneous abnormalities were not systematically investigated, but included if noted in the medical record. Regional wall motion abnormalities included those reported in either the right or left ventricle. Premature ventricular contractions were defined as >500/24 hours. Those with a primary ventricular arrhythmia phenotype had a high burden of premature ventricular complexes on Holter monitoring (>10% over 24 hours) or resuscitated cardiac arrest in the absence of structural and functional abnormalities of the myocardium assessed at cardiac evaluation. A composite outcome of ventricular arrhythmia included SCD, resuscitated cardiac arrest, appropriate implantable cardioverter-defibrillator (ICD) therapy, or sustained ventricular tachycardia. Appropriate ICD therapy was defined as anti-tachycardia pacing, or an ICD discharge for termination of ventricular tachycardia or fibrillation. SCD in probands was defined as sudden death in an otherwise healthy individual of any age within 1 hour after the onset of symptoms, or when unwitnessed, within 24 hours after the individual was last seen in good health. Family history of SCD included those with a suspicious death of a first-degree relative aged less than 40 years.

Literature review

We conducted a literature review of all *DSP*tv reported in PubMed (accessed on June 3, 2019). We selected *DSP*tv reported in publications through a search using the terms "*DSP*" or "desmoplakin" in combination with "mutation" or "variant" in title and/or abstract. No limitations were placed on language, type, or date of publications. This selection was matched

with variants reported in HGMD Pro and the ARVC database.[42] We selected all variants in patients with available clinical information. Particular care was taken in reviewing the cardiologic and ectodermal manifestations, age of patients at evaluation and diagnosis, additional performed genetic tests, family history and cascade screening, immunohistochemical, ultrastructural or functional analysis.

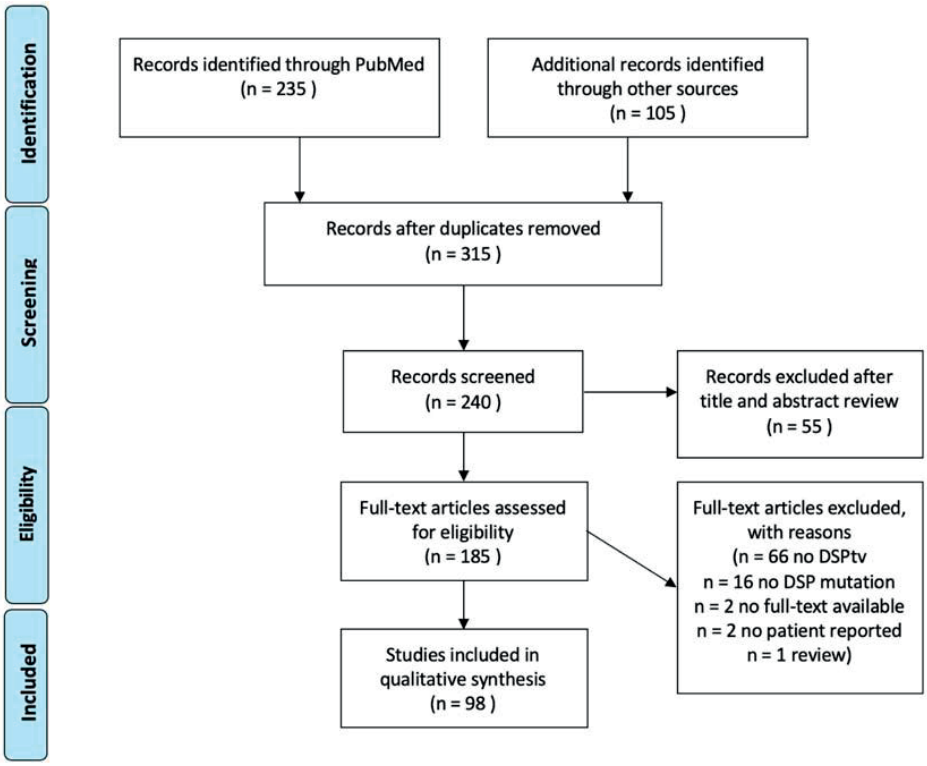
Statistical analysis

Data were analysed using RStudio (version 1.2). One-way analysis of variance or *t*-test was used for comparing continuous variables and chi-square or Fisher exact test for categorical variables. Multivariable Cox proportional hazards models were used to assess freedom from ventricular arrhythmia, using time since birth as the time variable. For patients with ventricular arrhythmia, time to first event was used. Where there was no ventricular arrhythmia, the date of the last known cardiac evaluation was used for censoring. Hazard ratios and 95% confidence interval (CI) were calculated. To account for family-clustering in the data, analyses were also repeated in a proband only dataset (data not shown). Variables reaching $p<0.05$ in univariate Cox regression were included in the multivariate Cox regression model, using a backward step-wise approach.

RESULTS

Literature review of reported *DSP*tv

Isolated cardiomyopathy, cardiocutaneous disease, and isolated cutaneous disorders were associated with both mono- and bi-allelic *DSP*tv. Cardiac manifestations reported included DCM, ACM (either right, left or biventricular involvement), SCD, early onset cardiac failure, and peripartum cardiomyopathy. Cutaneous abnormalities were congenital or appeared later, most commonly in the first year of life, and included palmoplantar keratoderma, woolly hair, hypotrichosis, alopecia, abnormally shaped teeth, enamel defects in both the deciduous and permanent dentition, tong erosion, nail dystrophy, and lethal acantholytic epidermolysis bullosa, with more severe manifestations seen in bi-allelic disease. Affected patients harboring homozygous or compound heterozygous *DSP*tv always presented with skin abnormalities, more often in association with an early onset cardiomyopathy (15 [75%] families) than as an isolated trait (5 [25%] families). As many patients without reported cardiomyopathy were evaluated in their first decade, a later-onset cardiomyopathy could not be ruled out in these families. When families were ascertained via a proband <10 years of age, early onset cardiomyopathy associated with cutaneous disease was the most prevalent phenotype, and disease was caused by biallelic *DSP*tv.



Supplementary Figure 1. Overview of the systematic review search criteria.

Supplementary table 1. *DSP*tv identified in the patient cohort.

Genomic location (GRCh37/hg19)	Likely coding effect	HGVS nomenclature (DNA)	HGVS nomenclature (Protein)	Domain	Region
g.7542225_7542228dup	Frameshift	c.77_80dup	p.(Tyr28Alafs*66)	G1	C-NMDc
g.7542233G>Tv	Nonsense	c.85G>T	p.(Glu29*)	G1	C-NMDc
g.7542266del	Frameshift	c.118del	p.(Met40Cysfs*8)	G1	C-NMDc
g.7555976C>T	Nonsense	c.196C>T	p.(Gln66*)	G1	C-NMDc
g.7556030C>T	Nonsense	c.250C>T	p.(Arg84*)	G1	C-NMDc
g.7556058G>A	? Splicing	c.273+5G>A	r.spl?	G1	C-NMDc
g.7559484C>T	Nonsense	c.448C>T	p.(Arg150*)	G1	C-NMDc
g.7559514C>T	Nonsense	c.478C>T	p.(Arg160*)	G1	C-NMDc
g.7562888del	Deletion	c.601-?_8559+?	(deletion of exon 5-24)	G1	C-NMDc
g.7562894_7562895insT	Frameshift	c.607_608insT	p.(Asp203Valfs*17)	G1	C-NMDc
g.7563990dup	Frameshift	c.748dup	p.(Gln250Profs*7)	G1	C-NMDc
g.7565622C>T	Nonsense	c.808C>T	p.(Arg270*)	G1	C-NMDc
g.7565686_7565687dup	Frameshift	c.872_873dup	p.(Glu292Argfs*26)	G1	C-NMDc
g.7565754G>A	? Splicing	c.939+1G>A	r.(spl?)	G1	C-NMDc
g.7566609G>C	? Splicing	c.940-1G>C	r.(spl?)	G1	C-NMDc
g.7566715G>A	? Splicing	c.1044+1G>T	r.(spl?)	G1	C-NMDc
g.7567602_7567603del	Frameshift	c.1060_1061del	p.(Leu354Alafs*15)	G1	C-NMDc
g.7568019dup	Nonsense	c.1146dup	p.(Glu383*)	G1	C-NMDc
g.7568140G>T	? Splicing	c.1266+1G>T	r.(spl?)	G1	C-NMDc
g.7569576_7569579del	? Splicing	c.1574+3_1574+6del	r.(spl?)	G1	C-NMDc
g.7571619A>T	Nonsense	c.1705A>T	p.(Lys569*)	G1	C-NMDc
g.7571742_7571754del	Frameshift	c.1828_1840del	p.(Ser610Metfs*22)	G1	C-NMDc
g.7571787C>T	Nonsense	c.1873C>T	p.(Gln625*)	G1	C-NMDc
g.7571818G>T	? Splicing	c.1903+1G>T	r.(spl?)	G1	C-NMDc
g.7572073A>G	? Splicing	c.1904-2A>G	r.(spl?)	G1	C-NMDc

Published	Probands in cohort	gnomADv2.1.1 allele count	Referring clinical diagnosis	ACMG/ AMP criteria	Classification
N	1	0	Carvajal syndrome (heterozygous) Also carries the *DSP* c.273+5G>A variant	PVS1, PM2	Likely pathogenic
N	1	0	HCM	PVS1, PM2	Likely pathogenic
N	2	0	Case 1:DCM,Case 2:HCM	PVS1, PM2,PS4_supporting	Pathogenic
N	1	0	CM unspecified	PVS1, PM2	Likely pathogenic
Y	1	0	CM unspecified	PVS1, PM2	Likely pathogenic
Y	2	78	Case 1/2: Carvajal syndrome (heterozygous)	Autosomal Recessive variant	Uncertain significance
N	1	0	DCM	PVS1, PM2	Likely pathogenic
Y	2	0	Case 1: DCM, Case 2: ARVC	PVS1, PM2, PS4_supporting	Pathogenic
N	1	0	ARVC	PVS1, PM2	Likely pathogenic
N	1	0	ARVC	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	DCM, PPK	PVS1, PM2	Likely pathogenic
Y	3	1	Case 1/2: DCM, Case 3: DCM unspecified	PVS1, PM2, PS4_supporting	Pathogenic
N	1	0	Cutaneous	PVS1, PM2	Likely pathogenic
N	4	0	Case 1,2,3,4:DCM	PVS1, PM2, PS4_moderate	Pathogenic
Y	4	0	Case 1/2: ARVC (PPK, woolly hair), Case 3: CM unspecified, Case 4: OHCA	PVS1, PM2, PS4_moderate	Pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	3	0	Case 1/2: DCM,Case 3: ARVC	PVS1, PM2, PS4_supporting	Pathogenic
N	1	0	ACM	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	ARVC	PVS1, PM2	Likely pathogenic
Y	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	CM Unspecified	PVS1, PM2	Likely pathogenic

Supplementary table 1. *DSP*tv identified in the patient cohort. (*continued*)

Genomic location (GRCh37/hg19)	Likely coding effect	HGVS nomenclature (DNA)	HGVS nomenclature (Protein)	Domain	Region
g.7574319_7574320del	Frameshift	c.2131_2132del	p.(Ser711Cysfs*4)	G1	C-NMDc
g.7574367_7574368insTT	Frameshift	c.2179_2180insTT	p.(Lys727Ilefs*39)	G1	C-NMDc
g.7574464_7574465insA	Frameshift	c.2276_2277insA	p.(Thr760Tyrfs*7)	G1	C-NMDc
g.7574487T>A	? Splicing	c.2297+2T>A	r.(spl?)	G1	C-NMDc
g.7575542_7575548del	Frameshift	c.2451_2455del	p.(Asp81Glufs*32)	G1	C-NMDc
g.7576525A>C	? Splicing	c.2631-2A>C	r.(spl?)	G1	C-NMDc
g.7576526G>C	? Splicing	c.2631-1G>C	r.(spl?)	G1	C-NMDc
g. 7576533G>A	Nonsense	c.2637G>A	p.(Trp879*)	G1	C-NMDc
g.7576534dup	Frameshift	c.2638dup	p.(Asp880Glyfs*14)	G1	C-NMDc
g.7578760_7578761dup	Frameshift	c.3049_3050dup	p.(Leu1017Phefs*2)	G1	C-NMDc
g.7578796G>A	? Splicing	c.3084+1G>A	r.(spl?)	G1	C-NMDc
g.7579556C>T	Nonsense	c.3133C>T	p.(Arg1045*)	G1	C-NMDc
g.7579760C>T	Nonsense	c.3337C>T	p.(Arg1113*)	CR	C-NMDc
g.7579806_7579807del	Frameshift	c.3383_3384del	p.(Val1128Glyfs*5)	CR	C-NMDc
g.7579868del	Frameshift	c.3445del	p.(Glu1149Lysfs*10)	CR	C-NMDc
g.7579888G>A	Nonsense	c.3465G>A	p.(Trp1155*)	CR	C-NMDc
g.7580102C>T	Nonsense	c.3679C>T	p.(Gln1227*)	CR	NC-NMDc
g.7580162C>T	Nonsense	c.3739C>T	p.(Arg1247*)	CR	NC-NMDc
g.7580216G>T	Nonsense	c.3793G>T	p.(Glu1265*)	CR	NC-NMDc
g.7580222C>T	Nonsense	c.3799C>T	p.(Arg1267*)	CR	NC-NMDc
g.7580603C>T	Nonsense	c.4180C>T	p.(Gln1394*)	CR	NC-NMDc
g.7580818T>G	Nonsense	c.4395T>G	p.(Tyr1465*)	CR	NC-NMDc
g.7580846del	Frameshift	c.4423delA	p.(Thr1475Profs*9)	CR	NC-NMDc
g.7580900G>T	Nonsense	c.4477G>T	p.(Glu1493*)	CR	NC-NMDc
g.7581031_7581035del	Frameshift	c.4608_4612del	p.(Arg1537Glufs*5)	CR	NC-NMDc
g.7581134C>T	Nonsense	c.4711C>T	p.(Gln1571*)	CR	NC-NMDc
g.7581220del	Frameshift	c.4797delA	p.(Gly1600Alafs*2)	CR	NC-NMDc
g.7581553dup	Frameshift	c.5130dup	p.(Glu1711Argfs*23)	CR	NC-NMDc
g.7581631_7581632del	Frameshift	c.5208_5209del	p.(Gly1737Thrfs*7)	CR	NC-NMDc

Published	Probands in cohort	gnomADv2.1.1 allele count	Referring clinical diagnosis	ACMG/ AMP criteria	Classification
Y	1	0	CM unspecified	PVS1, PM2	Likely pathogenic
N	1	0	Cutaneous	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	4	0	Case 1/2: CM Unspecified,Case 3/4: DCM	PVS1, PM2, PS4_moderate	Pathogenic
N	1	0	DCM, PPK, skin fragility, alopecia	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	ARVC	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
Y	1	0	ARVC	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
Y	3	0	Case 1: ARVC,Case 2/3: DCM	PVS1, PM2, PS4_supporting	Pathogenic
Y	1	0	DCM, PPK, woolly hair	PVS1, PM2	Likely pathogenic
N	1	0	HCM	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	3	0	Case 1/2: ARVC, Case 3: DCM	PVS1, PM2, PS4_supporting	Pathogenic
N	1	1	DCM	PVS1, PM2	Likely pathogenic
Y	1	0	ARVC	PVS1, PM2	Likely pathogenic
Y	1	0	VA	PVS1, PM2	Likely pathogenic
Y	1	1	DCM	PVS1, PM2	Likely pathogenic
Y	1	0	ARVC	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	2	0	Case1: ARVC, Case 2: DCM	PVS1, PM2, PS4_supporting	Pathogenic
N	5	0	Case 1/2/3/4: DCM,Case 5: CM Unspecified	PVS1, PM2, PS4_moderate	Pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	DCM	PVS1, PM2	Likely pathogenic
N	1	0	ARVC	PVS1, PM2	Likely pathogenic
Y	1	0	DCM, woolly hair	PVS1, PM2	Likely pathogenic

Supplementary table 1. *DSP*tv identified in the patient cohort. (*continued*)

Genomic location (GRCh37/hg19)	Likely coding effect	HGVS nomenclature (DNA)	HGVS nomenclature (Protein)	Domain	Region
g.7582914C>T	Nonsense	c.5419C>T	p.(Gln1807*)	CR	C_NMDi
g.7582955dup	Frameshift	c.5460dup	p.(Val1821Serfs*15)	CR	C_NMDi
g.7583295C>T	Nonsense	c.5800C>T	p.(Arg1934*)	CR	C_NMDi
g.7583346C>T	Nonsense	c.5851C>T	p.(Arg1951*)	G2	C_NMDi
g.7583831del	Frameshift	c.6336del	p.(Asn2114Lefs*2)	G2	C_NMDi
g.7583888del	Frameshift	c.6393del	p.(Gly2133Valfs*2)	G2	C_NMDi
g.7583951dup	Frameshift	c.6456dup	p.(Leu2153Alafs*3)	G2	C_NMDi
g.7584001_7584002ins TAGATAGTCA	Frameshift	c.6506_6507ins TAGATAGTCA	p.(Gln2169Hisfs*3)	G2	C_NMDi
g.7584182del	Frameshift	c.6687del	p.(Arg2229Serfs*32)	G2	C_NMDi
g.7584345C>T	Nonsense	c.6850C>T	p.(Arg2284*)	G2	C_NMDi
g.7584591_7584598del	Frameshift	c.7096_7103del	p.(Leu2367Serfs*10)	G2	C_NMDi
g.7585065_7585068del	Frameshift	c.7570_7573del	p.(Thr2524Argfs*11)	G2	C_NMDi
g.7585268_7585271del	Frameshift	c.7773_7776del	p.(Ser2591Argfs*11)	G2	C_NMDi
g.7585572_7585575del	Frameshift	c.8077_8080del	p.(Lys2693Profs*3)	G2	C_NMDi
g.7585683del	Frameshift	c.8188del	p.(Gln2730Serfs*16)	G2	C_NMDi

ACM = arrhythmogenic cardiomyopathie, ARVC = arrhythmogenic right ventricular cardiomyopathy, CM = cardiomyopathy, DCM = dilated cardiomyopathy, HCM = hypertrophic cardiomyopathy, OHCA = out of hospital cardiac arrest, PPK = palmoplantar keratoderma

Published	Probands in cohort	gnomADv2.1.1 allele count	Referring clinical diagnosis	ACMG/ AMP criteria	Classification
N	2	0	Case 1/2: ARVC	PVS1_strong, PM2, PS4_ supporting	Likely pathogenic
Y	1	0	DCM	PVS1_strong, PM2	Likely pathogenic
Y	1	0	DCM	PVS1_strong, PM2	Likely pathogenic
Y	2	0	Case 1/2: ARVC	PVS1_strong, PM2, PS4_ supporting	Likely pathogenic
N	3	0	Case 1/2/3: DCM	PVS1_strong, PM2, PS4_ supporting	Likely pathogenic
N	2	0	Case 1: DCM, Case 2: DCM	PVS1_strong, PM2, PS4_ supporting	Likely pathogenic
Y	1	0	ARVC	PVS1_strong, PM2	Likely pathogenic
N	1	0	DCM	PVS1_strong, PM2	Likely pathogenic
N	1	0	DCM, PPK, woolly hair (heterozygous) Also has DSP c.273+5G>A variant	PVS1_strong, PM2	Likely pathogenic
Y	1	0	ARVC	PVS1_strong, PM2	Likely pathogenic
N	1	0	DCM	PVS1_strong, PM2	Likely pathogenic
Y	1	0	HCM	PVS1_strong, PM2	Likely pathogenic
N	1	3	DCM	PVS1_strong, PM2	Likely pathogenic
Y	2	0	Case 1: DCM, Case 2: ventricular arrhythmia	PVS1_strong, PM2, PS4_ supporting	Likely pathogenic
Y	1	0	DCM	PVS1_strong, PM2	Likely pathogenic

Supplementary Table 2. ClinVar variants included as cases in the case-control analysis.

Source	DNA_effect	protein_effect	Alternate Regions	Region
Clinvar	c.1054_1059delGACACTinsCA	p.Asp352Hisfs	G1	C-NMDc
Clinvar	c.1060_1061delCT	p.Leu354Alafs	G1	C-NMDc
Clinvar	c.1087C>T	p.Gln363Ter	G1	C-NMDc
Clinvar	c.1140+2T>G		G1	C-NMDc
Clinvar	c.1141-2A>G		G1	C-NMDc
Clinvar	c.1141-2A>T		G1	C-NMDc
Clinvar	c.1146delT	p.Phe382Leufs	G1	C-NMDc
Clinvar	c.1162delA	p.Thr388Leufs	G1	C-NMDc
Clinvar	c.1182delG	p.Leu395Serfs	G1	C-NMDc
Clinvar	c.123C>G	p.Tyr41Ter	G1	C-NMDc
Clinvar	c.1266+2T>C		G1	C-NMDc
Clinvar	c.1267-2A>G		G1	C-NMDc
Clinvar	c.1273C>T	p.Arg425Ter	G1	C-NMDc
Clinvar	c.1292dupA	p.Tyr431Terfs	G1	C-NMDc
Clinvar	c.1420-1G>T		G1	C-NMDc
Clinvar	c.151C>T	p.Gln51Ter	G1	C-NMDc
Clinvar	c.1615C>T	p.Gln539Ter	G1	C-NMDc
Clinvar	c.1650G>A	p.Trp550Ter	G1	C-NMDc
Clinvar	c.1751delA	p.Glu584Glyfs	G1	C-NMDc
Clinvar	c.1857C>A	p.Tyr619Ter	G1	C-NMDc
Clinvar	c.1873C>T	p.Gln625Ter	G1	C-NMDc
Clinvar	c.1883delG	p.Gly628Alafs	G1	C-NMDc
Clinvar	c.1903+1G>C		G1	C-NMDc
Clinvar	c.208_209dup	p.Ile71Profs	G1	C-NMDc
Clinvar	c.2130+1G>A		G1	C-NMDc
Clinvar	c.2131_2132delAG	p.Ser711Cysfs	G1	C-NMDc
Clinvar	c.2131_2132delAG	p.Ser711Cysfs	G1	C-NMDc
Clinvar	c.2131_2132delAG	p.Ser711Cysfs	G1	C-NMDc
Clinvar	c.226C>T	p.Gln76Ter	G1	C-NMDc
Clinvar	c.2297+1G>A		G1	C-NMDc
Clinvar	c.243_251delCTTGATGCG	p.Cys81_Ile418delinsTer	G1	C-NMDc
Clinvar	c.2436+2T>C		G1	C-NMDc
Clinvar	c.2436+2T>C		G1	C-NMDc
Clinvar	c.2437-1G>C		G1	C-NMDc
Clinvar	c.250C>T	p.Arg84Ter	G1	C-NMDc
Clinvar	c.2528C>A	p.Ser843Ter	G1	C-NMDc
Clinvar	c.2547T>A	p.Tyr849Ter	G1	C-NMDc

Supplementary Table 2. ClinVar variants included as cases in the case-control analysis. (*continued*)

Source	DNA_effect	protein_effect	Alternate Regions	Region
Clinvar	c.2602C>T	p.Gln868Ter	G1	C-NMDc
Clinvar	c.2630+1delG		G1	C-NMDc
Clinvar	c.2644G>T	p.Glu882Ter	G1	C-NMDc
Clinvar	c.268C>T	p.Gln90Ter	G1	C-NMDc
Clinvar	c.268C>T	p.Gln90Ter	G1	C-NMDc
Clinvar	c.273+1G>A		G1	C-NMDc
Clinvar	c.273delT	p.Glu92Asnfs	G1	C-NMDc
Clinvar	c.273delT	p.Glu92Asnfs	G1	C-NMDc
Clinvar	c.2793+1G>T		G1	C-NMDc
Clinvar	c.2821C>T	p.Arg941Ter	G1	C-NMDc
Clinvar	c.2821C>T	p.Arg941Ter	G1	C-NMDc
Clinvar	c.2821C>T	p.Arg941Ter	G1	C-NMDc
Clinvar	c.2848dupA	p.Ile950Asnfs	G1	C-NMDc
Clinvar	c.2870_2874delCAATT	p.Ser957Terfs	G1	C-NMDc
Clinvar	c.2920delA	p.Thr974Leufs	G1	C-NMDc
Clinvar	c.2947_2948delAC	p.Thr983Hisfs	G1	C-NMDc
Clinvar	c.3044_3047dup	p.Phe1016Leufs	G1	C-NMDc
Clinvar	c.3049_3050dupTT	p.Leu1017Phefs	G1	C-NMDc
Clinvar	c.3133C>T	p.Arg1045Ter	G1	C-NMDc
Clinvar	c.3133C>T	p.Arg1045Ter	G1	C-NMDc
Clinvar	c.3160_3169delAAGAACAAAT	(p.Lys1054Serfs	G1	C-NMDc
Clinvar	c.3160_3169delAAGAACAAAT	p.Lys1054Serfs	G1	C-NMDc
Clinvar	c.3195C>G	p.Tyr1065Ter	CR	C-NMDc
Clinvar	c.3241G>T	p.Glu1081Ter	CR	C-NMDc
Clinvar	c.3316G>T	p.Glu1106Ter	CR	C-NMDc
Clinvar	c.3337C>T	p.Arg1113Ter	CR	C-NMDc
Clinvar	c.3337C>T	p.Arg1113Ter	CR	C-NMDc
Clinvar	c.3337C>T	p.Arg1113Ter	CR	C-NMDc
Clinvar	c.3348T>A	p.Tyr1116Ter	CR	C-NMDc
Clinvar	c.3415_3417delTATinsG	p.Tyr1139Glyfs	CR	C-NMDc
Clinvar	c.3419delA	p.Asp1140Alafs	CR	C-NMDc
Clinvar	c.3426dupG	p.Gln1143Alafs	CR	C-NMDc
Clinvar	c.3474dup	p.Glu1159Argfs	CR	C-NMDc
Clinvar	c.3507C>A	p.Tyr1169Ter	CR	C-NMDc
Clinvar	c.3526delG	p.Val1176Phefs	CR	C-NMDc
Clinvar	c.423-1G>T		G1	C-NMDc
Clinvar	c.423-1G>T		G1	C-NMDc

Supplementary Table 2. ClinVar variants included as cases in the case-control analysis. (*continued*)

Source	DNA_effect	protein_effect	Alternate Regions	Region
Clinvar	c.491_492delCCins15		G1	C-NMDc
Clinvar	c.491_492delCCinsAGCTCGAGTCCCTCG	p.Ala164Glufs	G1	C-NMDc
Clinvar	c.534_535insA	p.Gly179Argfs	G1	C-NMDc
Clinvar	c.699G>A	p.Trp233Ter	G1	C-NMDc
Clinvar	c.699G>A	p.Trp233Ter	G1	C-NMDc
Clinvar	c.699G>A	p.Trp233Ter	G1	C-NMDc
Clinvar	c.712dupA	p.Ile238Asnfs	G1	C-NMDc
Clinvar	c.867C>A	p.Cys289Ter	G1	C-NMDc
Clinvar	c.867C>A	p.Cys289Ter	G1	C-NMDc
Clinvar	c.888C>G	p.Tyr296Ter	G1	C-NMDc
Clinvar	c.919C>T	p.Gln307Ter	G1	C-NMDc
Clinvar	c.928dup	p.Glu310Glyfs	G1	C-NMDc
Clinvar	c.928dup	p.Glu310Glyfs	G1	C-NMDc
Clinvar	c.939+1G>A		G1	C-NMDc
Clinvar	c.939+1G>A		G1	C-NMDc
Clinvar	c.939+1G>A		G1	C-NMDc
Clinvar	c.939+1G>A		G1	C-NMDc
Clinvar	c.465delC	p.Ile156Serfs	G1	C-NMDi
Clinvar	c.478C>T	p.Arg160Ter	G1	C-NMDi
Clinvar	c.478C>T	p.Arg160Ter	G1	C-NMDi
Clinvar	c.5460_5466delAGTCCTG	p.Val1821Serfs	CR	C-NMDi
Clinvar	c.5673_5674delGA	p.Lys1892Glufs	CR	C-NMDi
Clinvar	c.5680_5683delAGTC	p.Ser1894Leufs	CR	C-NMDi
Clinvar	c.5680_5683delAGTC	p.Ser1894Leufs	CR	C-NMDi
Clinvar	c.5725delA	p.Ile1909Leufs	CR	C-NMDi
Clinvar	c.5745dup	p.Lys1916Terfs	CR	C-NMDi
Clinvar	c.5800C>T	p.Arg1934Ter	CR	C-NMDi
Clinvar	c.5851C>T	p.Arg1951Ter	G2	C-NMDi
Clinvar	c.5851C>T	p.Arg1951Ter	G2	C-NMDi
Clinvar	c.5851C>T	p.Arg1951Ter	G2	C-NMDi
Clinvar	c.6273delA	p.Ala2092Leufs	G2	C-NMDi
Clinvar	c.6310delA	p.Thr2104Glnfs	G2	C-NMDi
Clinvar	c.6393delA	p.Gly2133Valfs	G2	C-NMDi
Clinvar	c.6398dupG	p.Val2134Cysfs	G2	C-NMDi
Clinvar	c.6410dupC	p.Val2138Cysfs	G2	C-NMDi
Clinvar	c.6456dupG	p.Leu2153Alafs	G2	C-NMDi
Clinvar	c.6466dupA	p.Arg2156Lysfs	G2	C-NMDi

Supplementary Table 2. ClinVar variants included as cases in the case-control analysis. (*continued*)

Source	DNA_effect	protein_effect	Alternate Regions	Region
Clinvar	c.6496C>T	p.Arg2166Ter	G2	C-NMDi
Clinvar	c.6496C>T	p.Arg2166Ter	G2	C-NMDi
Clinvar	c.6496C>T	p.Arg2166Ter	G2	C-NMDi
Clinvar	c.6510_6511insCT	p.Asn2171Leufs	G2	C-NMDi
Clinvar	c.6850C>T	p.Arg2284Ter	G2	C-NMDi
Clinvar	c.6937delG	p.Glu2313Argfs	G2	C-NMDi
Clinvar	c.6940G>T	p.Glu2314Ter	G2	C-NMDi
Clinvar	c.6954_6955delAG	p.Gly2319Serfs	G2	C-NMDi
Clinvar	c.7248delT	p.Phe2416Leufs	G2	C-NMDi
Clinvar	c.7491_7492delTG	p.Cys2497Terfs	G2	C-NMDi
Clinvar	c.7623delG	p.Lys2542Serfs	G2	C-NMDi
Clinvar	c.7641C>G	p.Tyr2547Ter	G2	C-NMDi
Clinvar	c.7873dupA	p.Thr2625Asnfs	G2	C-NMDi
Clinvar	c.7899dup	p.Thr2634Tyrfs	G2	C-NMDi
Clinvar	c.8014C>T	p.Gln2672Ter	G2	C-NMDi
Clinvar	c.8188delC	p.Gln2730Serfs	G2	C-NMDi
Clinvar	c.8442dupC	p.Ser2815Glnfs	G2	C-NMDi
Clinvar	c.3630T>A	p.Tyr1210Ter	CR	NC-NMDc
Clinvar	c.3630T>A	p.Tyr1210Ter	CR	NC-NMDc
Clinvar	c.3733delG	p.Glu1245Lysfs	CR	NC-NMDc
Clinvar	c.3735_3741dup	p.Asp1248Lysfs	CR	NC-NMDc
Clinvar	c.3735_3741dup	p.Asp1248Lysfs	CR	NC-NMDc
Clinvar	c.3735_3741dup	p.Asp1248Lysfs	CR	NC-NMDc
Clinvar	c.3735_3741dup	p.Asp1248Lysfs	CR	NC-NMDc
Clinvar	c.3788_3789dup	p.Thr1264Profs	CR	NC-NMDc
Clinvar	c.3793G>T	p.Glu1265*	CR	NC-NMDc
Clinvar	c.3799C>T	p.Arg1267Ter	CR	NC-NMDc
Clinvar	c.3805C>T	p.Arg1269Ter	CR	NC-NMDc
Clinvar	c.3829C>T	p.Gln1277Ter	CR	NC-NMDc
Clinvar	c.3865C>T	p.Gln1289Ter	CR	NC-NMDc
Clinvar	c.3865C>T	p.Gln1289Ter	CR	NC-NMDc
Clinvar	c.3961C>T	p.Gln1321Ter	CR	NC-NMDc
Clinvar	c.4003C>T	p.Gln1335Ter	CR	NC-NMDc
Clinvar	c.4009delG	p.Glu1337Argfs	CR	NC-NMDc
Clinvar	c.4009delG	p.Glu1337Argfs	CR	NC-NMDc
Clinvar	c.4054A>T	p.Arg1352Ter	CR	NC-NMDc
Clinvar	c.4198C>T	p.Arg1400Ter	CR	NC-NMDc

Supplementary Table 2. ClinVar variants included as cases in the case-control analysis. (*continued*)

Source	DNA_effect	protein_effect	Alternate Regions	Region
Clinvar	c.4235_4237delTGAinsG	p.Leu1412Argfs	CR	NC-NMDc
Clinvar	c.4297C>T	p.Gln1433Ter	CR	NC-NMDc
Clinvar	c.4305_4309delCACTG	p.Thr1436Leufs	CR	NC-NMDc
Clinvar	c.4305_4309delCACTG	p.Thr1436Leufs	CR	NC-NMDc
Clinvar	c.4353_4357delGAGAC	p.Arg1452Serfs	CR	NC-NMDc
Clinvar	c.4372C>T	p.Arg1458Ter	CR	NC-NMDc
Clinvar	c.4395T>G	p.(Tyr1465X)	CR	NC-NMDc
Clinvar	c.4531C>T	p.Gln1511Ter	CR	NC-NMDc
Clinvar	c.4531C>T	p.Gln1511Ter	CR	NC-NMDc
Clinvar	c.4822C>T	p.Gln1608Ter	CR	NC-NMDc
Clinvar	c.4824dupA	p.Ala1609Serfs	CR	NC-NMDc
Clinvar	c.4882_4886delAGGAGinsTTCT	p.Arg1628Phefs	CR	NC-NMDc
Clinvar	c.4882_4886delAGGAGinsTTCT	p.Arg1628Phefs	CR	NC-NMDc
Clinvar	c.4999C>T	p.Gln1667Ter	CR	NC-NMDc
Clinvar	c.4999C>T	p.Gln1667Ter	CR	NC-NMDc
Clinvar	c.5212C>T	p.Arg1738Ter	CR	NC-NMDc
Clinvar	c.5269C>T	p.Gln1757Ter	CR	NC-NMDc
Clinvar	c.5327_5330del	p.Glu1776Glyfs	CR	NC-NMDc
Clinvar	c.5378_5379dup	p.Ala1794Argfs	CR	NC-NMDc

Supplementary Table 3. gnomAD variants used as controls in the case control analysis.
See attachment 'Chapter4_S3_Table.xlsx'
Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhPFfi-KMH8qT-kEmNw?e=yFj7ym

Supplementary Table 4. Sudden cardiac death cohort postmortem findings.

Sex	Age at Death	Activity at Time of Death	Summary of Significant Post Mortem Findings	Cause of Death noted on postmortem report
F	32	Exercise	Suspected ARVC, wrong appearance for healed myocarditis.	Probable ARVC
F	35	Normal	Extensive fibrosis and fatty infiltration in the upper septum, RVOT and LV.	ARVC
F	18	Unknown	Areas of myocardial fibrosis associated with fatty change/infiltration.	ARVC
M	32	Exercise	Cardiomegaly with biventricular dilatation. Patchy fibrosis shown throughout right and left ventricle. The heart showed some features of ARVC with LV predominance.	DCM- subsequent ARVC diagnosis
M	14	Sleep	Sub-epicardial fibrosis and fatty change within the left and right ventricular myocardium, showing left ventricular predominance.	ARVC
M	51	Light activity (gardening)	The microscopic examination of the LV showed pathological features ARVC.	ARVC
F	22	Normal	Mild right ventricular dilatation associated with focal fat and fibrosis. Changes most consistent in the LV.	ARVC
M	16	Unknown		ARVC
F	15	Unknown		Chronic myocarditis
M	32	Exercise		ARVC
M	18	Unknown		ARVC with LV involvement
F	18	Unknown	Severely thickened mitral valve with evident myoxid degeneration. No signs of DCM or ARVC	Myxoid mitral valve

Supplementary Table 4. Sudden cardiac death cohort postmortem findings. (*continued*)

Sex	Age at Death	Activity at Time of Death	Summary of Significant Post Mortem Findings	Cause of Death noted on postmortem report
F	36	Unknown	ARVC with lymphocytic myocarditis, with degenerative changes LV, or DCM due to chronic acute lymphocytic (ongoing) myocarditis of viral origin with secondary changes to RV	ARVC or DCM

ARVC = arrhythmogenic right ventricular cardiomyopathy, DCM = dilated cardiomyopathy, LV = left ventricle, RV = right ventricle, RVOT = right ventricular outflow tract

Supplementary Table 5: Literature review frameshift variants.
See attachment 'Chapter4_S5_Table.xlsx'
Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhPFgWUj_ZLbNw7ZW_g?e=gtCcp2

Supplementary Table 6. Literature review splice site variants.
See attachment 'Chapter4_S6_Table.xlsx'
Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhPFheUXbHxWm43a6Q?e=ayF2CV

Supplementary Table 7. Literature review nonsense variants.
See attachment 'Chapter4_S7_Table.xlsx'
Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhPFifFq3I1nwT0tWXw?e=zw2S5j

Chapter 5

A systematic analysis of the clinical outcome associated with multiple reclassified desmosomal gene variants in arrhythmogenic right ventricular cardiomyopathy patients

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ABSTRACT

The presence of multiple pathogenic variants in desmosomal genes (*DSC2, DSG2, DSP, JUP,* and *PKP2*) in patients with arrhythmogenic right ventricular cardiomyopathy (ARVC) has been linked to a severe phenotype. However, the pathogenicity of variants is reclassified frequently, which may result in a changed clinical risk prediction. Here, we present the collection, reclassification, and clinical outcome correlation for the largest series of ARVC patients carrying multiple desmosomal pathogenic variants to date (n=331). After reclassification, only 29% of patients remained carriers of two (likely) pathogenic variants. They reached the composite endpoint (ventricular arrhythmias, heart failure, and death) significantly earlier than patients with one or no remaining reclassified variant (hazard ratios of 1.9 and 1.8, respectively). Periodic reclassification of variants contributes to more accurate risk stratification and subsequent clinical management strategy.

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC), the main subform of arrhythmogenic cardiomyopathy, is a rare inherited heart disease typically manifesting with ventricular arrhythmias (VA) and gradual fibro-fatty replacement of cardiomyocytes, predominantly in the right ventricle.[1,2] The phenotype of ARVC is highly variable, and clinical diagnosis requires fulfilling a combination of widely accepted task force criteria related to ventricular structure and function abnormalities, tissue characterization, repolarization, depolarization/conduction, arrhythmias, and family history, including the results of DNA testing.[3]. Nearly 50% of ARVC patients are carriers of a pathogenic/likely pathogenic (P/LP) variant in genes encoding desmosomal proteins mainly responsible for cell binding, including desmocollin-2 (*DSC2*), desmoglein-2 (*DSG2*), desmoplakin (*DSP*), junction plakoglobin (*JUP*), and plakophilin-2 (*PKP2*). P/LP variants in non-desmosomal genes are rare and include genes such as desmin (*DES*), phospholamban (*PLN*), and transmembrane protein 43 (*TMEM43*).[4–7]

The phenotypic variability of ARVC is high and still poorly understood, even amongst carriers of an identical P/LP variant. It is assumed that both environmental factors and different genetic backgrounds are involved.[1, 8] Participation in competitive or endurance sports is associated with a worse ARVC prognosis, including earlier presentation of symptoms, worsening of structural abnormalities, higher likelihood of heart failure, and a greater risk of arrhythmias.[8] Worse prognosis, including a higher occurrence and earlier onset of malignant VA, sudden cardiac death, and increased risk of developing left ventricular (LV) dysfunction or heart failure, has also been observed in individuals with more than one P/LP variant.[9] This data suggest a cumulative effect of carrying more than one P/LP variant in the desmosomal genes in ARVC.[10]

Previous studies indicate that 2-25% of patients harbour more than one P/LP variant in a desmosomal gene.[10–13] However, since the publication of these studies, variant adjudication criteria have become more strict and large databases like the Genome Aggregation Database (gnomAD) showed that variants previously associated with ARVC occur at a much higher frequency in the population than what would be expected based on disease prevalence. Several studies have been published with additional evidence for or against the pathogenicity of specific variants.[14] Therefore, the true effect of multiple P/LP variants on clinical outcome remains to be established. After thorough reclassification, we aimed to relate the presence of updated multiple P/LP desmosomal gene variants with clinical outcomes in patients. We hypothesised that after reclassification, patients still having multiple P/LP variants in the five major desmosomal genes have a poorer outcome and prognosis than those with a single P/LP variant or with no P/LP. Therefore, we: a) collected data from published and unpublished patients with more than one desmosomal gene variant underlying ARVC, b) reclassified

these variants, and c) updated clinical follow-up data to determine the outcome. We aim to contribute to more accurate risk stratification in ARVC patients with more than one P/LP variant.

METHODS

Inclusion/exclusion criteria of the systematic literature and database search

We searched PubMed (https://www.ncbi.nlm.nih.gov/pubmed/; 2018) and the ARVC Genetic Variants Database (http://www.arvcdatabase.info/)[15] to gather publications containing genetic and clinical information on patients carrying two or more desmosomal gene variants associated with ARVC, including patients having both compound and/or digenic heterozygous variants, as well as at least one homozygous variant. The PubMed search consisted of three combinations of keywords (**Supplementary Table 1**). We searched for selected terms in the titles and abstracts of the publications. In the ARVC Genetic Variants Database.[15] we selected variants co-occurring with other desmosomal variants and collected the respective publications. The search was restricted to English-language literature. To enrich for relevant publications, we screened the full text of the pre-selected publications and collected data from publications in which carriers with more than one variant were mentioned. Furthermore, we checked the literature references in these selected manuscripts for carriers of more than one variant. While the term "variant carrier" historically refers to an individual who carries a heterozygous genetic variant without showing any symptoms of the associated recessive condition, here we use it for ARVC patients who carry a variant that can also be associated with an autosomal dominant inheritance.

ARVC multiple variant database compilation

Using the selected publications (n=67, **Figure 1**), a database of patients with multiple ARVC variants was created. Moreover, unpublished patients with multiple ARVC variants were added, including those from the Dutch ARVC registry (n=87).[16] Only patients carrying multiple variants (in the same gene or different genes) in *DSC2, DSG2, DSP, JUP,* and *PKP2* genes were included. A predefined extraction sheet was used for data gathering, which included the PubMed identification number of the publication, the number and names of genes tested, and the name of the first author. Whenever available, the following information was extracted from each study: the gene(s) involved, the type of variant, its original classification, cDNA position and change, amino acid position and change, the composition of multiple variants (digenic/compound heterozygous or homozygous), and subject ID. In case genomic coordinates were missing, the TransVar online tool was used to add this information.[17] The following clinical information was collected: sex, the family status of a subject (proband or

family member), the age at presentation or diagnosis of disease, the age of the first occurrence of the primary composite endpoint, which consisted of death of any cause, sudden cardiac death, death due to end-stage heart failure, heart transplant and/or left ventricular assist device (LVAD), sustained ventricular tachycardia, ventricular fibrillation, out of hospital cardiac arrest (OHCA), appropriate ICD-therapy, and appropriate anti-tachycardia pacing (ATP). If data were missing in publications with five or more carriers of multiple variants, we asked the corresponding authors for follow-up data after the initial publication (**Supplementary Table 2**). In addition to providing the missing and follow-up data, we asked the authors of the publications with five or more carriers of multiple variants to verify the data collected and to provide data of newly identified yet still unpublished multiple variant carriers when available. The study conforms to the Declaration of Helsinki, was approved by local ethics and/or institutional review boards, and informed consent has been obtained from subjects

Variant reclassification

The variants in patients carrying ostensible multiple P/LP variants were reclassified as: pathogenic (P; class 5), likely pathogenic (LP; class 4), uncertain clinical significance (VUS; class 3), likely benign (LB; class 2), or benign (B; class 1).[18] We used the annotation and visualization software, Alissa Interpret (Agilent, Santa Clara, CA) and Alamut (Interactive Biosoftware, Rouen, France), to reclassify the variants according to the American College of Medical Genetics (ACMG) and Genomics and the Association for Molecular Pathology criteria.[18] The following summarized classification tree was used. Each variant was first checked if there was already information available in Alissa Interpret Managed Variant List (Agilent, Santa Clara, CA) and Alamut (Interactive Biosoftware, Rouen France), and other publicly available databases (e.g. ClinVar, HGMD), and if so what kind of data. Secondly, consensus rules were followed with respect to truncated variants in desmosomal genes. All truncating variants in DSC2 and DSG2 and heterozygous truncating variants in *JUP* were assigned VUS if no other supportive information was available. All truncating variants in *DSP and PKP2* and recessively inherited truncating variants in *JUP* were considered LP if no other supportive information was available. The last step consisted of the reassessment based on the ACMG criteria,[18] thus including *in silico* prediction data, population data, and, when available: segregation/clinical data, functional data, gene-specific information including variant-type and location.

Correlation of severity of the disease and type of multiple variants

We hypothesised that after reclassification of variants, patients with multiple P/LP variants in the five major desmosomal genes would have poorer outcomes and prognoses than those with a single or no causal P/LP variant remaining. To confirm or reject our hypothesis, we performed a Kaplan–Meier survival analysis based on genotype status. Three groups were made based on the pathogenicity of reclassified ARVC variants (**Figure 1**): patients with two

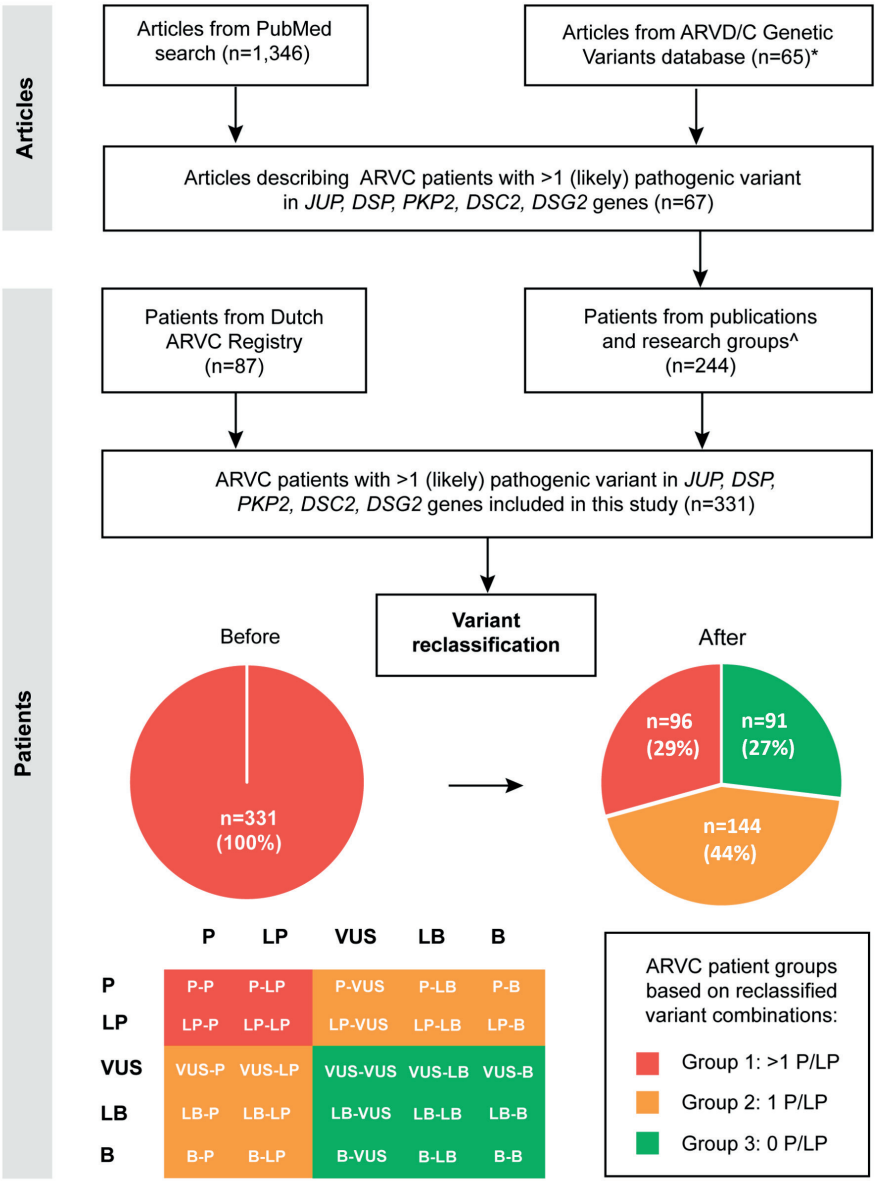

Figure 1. Study workflow and scheme of publication selection with information on patients with two or more ARVC-related desmosomal variants and subsequent patient selection for the database. *61 out of the 65 articles overlapped with the systematic PubMed search, ^number of patients after including information from contacted authors. Patient groups are based on the number of (likely) pathogenic ARVC variants after reclassification; P - Pathogenic variant, LP - Likely Pathogenic variant, VUS - Variant with Uncertain Significance, LB - Likely Benign variant, B - Benign variant.

P/LP variants (Group 1), patients with one P/LP variant and a variant with another classification (VUS/LB/B, Group 2), and patients without P/LP variants but with a combination of two VUS/LB/B variants (Group 3). If a patient had three or more ARVC variants, the two variants with the highest pathogenicity class were selected. Homozygous carriers of the Hutterite (*DSC2* c.1660C>T)[19] or Naxos (*JUP* c.2040_2041delGT)[20] founder variants were excluded from Group 1 and were regarded as separate groups. We performed the analysis using Microsoft Excel 2007 (Microsoft, Redmont, WA, USA) and IBM SPSS Statistics 25 (IBM Analytics, Armonk, New York, USA). To compare the outcomes, defined as the age of the first occurrence of one of the events listed in the composite endpoint, in different groups, we created Kaplan-Meier graphs and performed log-rank pairwise comparisons to determine different event-free survival distributions. Variables were included in a multivariable Cox regression model, and correction for proband status and sex was performed.

RESULTS

Systematic literature and database search

The systematic PubMed literature search yielded 1,346 publications; the search performed in the ARVD/C Genetic Variants Database yielded 65 articles, of which 61 overlapped with the PubMed literature search. From a total of 1,350 articles, we identified 67 studies (**Supplementary Table 2**) describing patients with more than one variant believed to be associated with ARVC, which we used to create our study database (**Figure 1**).

ARVC multiple variants database

Retrieving cases from the selected 67 publications and adding additional cases after contacting the related research groups, including those from the Dutch ARVC registry, resulted in a database containing 500 patients of interest. After de-duplicating patients published in more than one publication, 331 different multiple ARVC variant carriers (135 women and 196 men) were included. Most patients (n=134; 40.5%) were carriers of two heterozygous variants in two different genes (digenic form), 29.3% (n=97) were carriers of a homozygous variant (including 33 homozygous for the Naxos variant and 12 for the Hutterite variant), 24.8% (n=82) were compound heterozygotes with two different variants in one gene, 4.8% (n=16) had a combination of digenic form and compound or homozygous form, while 0.6% (n=2) were carriers of trigenic ARVC variant combinations, i.e. a variant in three different genes (**Supplementary Table 3**). Before reclassification and excluding the Naxos and Hutterite founder variants, the most frequent combinations of gene variants were *PKP2/DSP* (n=38), *PKP2/DSG2* (n=38), *DSG2/DSG2* (n=33), and *PKP2/PKP2* (n=31, **Supplementary Table 4**).

After reclassification, 29% of patients (n=96, **Supplementary Table 3**) were confirmed to have at least two P/LP variants. Of these 96 patients, 33 were homozygous carriers of the Naxos founder variant, 12 were homozygous carriers of the Hutterite variant, and the other 51 carriers of at least two P/LP desmosomal variants were classified as Group 1. Most patients (44%; n=144) had one P/LP variant in combination with a VUS/LB/B variant (Group 2), while 91 patients (27%) had no P/LP variant identified (Group 3, **Figure 1**). After reclassification, the most frequent combination was *PKP2/DSC2* (n=11) followed by *PKP2/DSG2* (n=8); **Supplementary Table 4**). For an overview of the reclassification of the desmosomal genetic variants, see **Supplementary Table 5**.

Correlation between severity of the disease and type of multiple variants

The median event-free survival age for Group 1 (double P/LP variants carriers) was 38 years (95% CI, 30 to 46 years). This was significantly lower than that of Groups 2 (one P/LP variant); 51 years (95% CI, 46 to 56 years) and 3 (no P/LP variant); 49 years (95% CI, 39 to 59 years) (**Figure 2A**, p = 0.004 and P = 0.021 respectively). An overview of the Kaplan-Meier curves stratified for sex and proband status per group is provided in **Figure 2B-E**. In a multivariable Cox model, after correcting for proband status and male sex, carrying two P/LP variants remained significantly associated with the composite endpoint with a hazard ratio of 1.9 (95% CI, 1.2 to 2.9) for Group 1 as compared to Group 2 and a hazard ratio of 1.8 (95% CI, 1.1 to 2.8) for Group 1 as compared to Group 3 (**Supplementary Table 6**). For a description of the occurrence of each endpoint, see **Supplementary Table 7**. An overview of the mean age at presentation or diagnosis, proband status, the occurrence of a composite endpoint, and mean age of occurrence of composite endpoint per group and sex is provided in **Supplementary Table 8.**

Naxos and Hutterite founder variants carriers

The median event-free survival age for the patient populations homozygous for Naxos or Hutterite founder variants was 50 years (95% CI, 37 to 63 years) and 44 years (95% CI, NA), respectively, while the median age for patients from Group 2 and 3 was 51 years (95% CI, 46 to 56 years) and 49 years (95%, CI, 39 to 59 years, **Figure 3A/B**). There were no significant differences in outcome between both homozygous founder variant carriers versus Group 2 or 3. In a multivariable Cox model, after correcting for proband status and male sex, being a homozygous carrier of the Naxos variant was significantly associated with the composite endpoint with a hazard ratio of 1.8 (95% CI, 1.03 to 2.99) compared to Group 2 (carriers of 1 P/LP), but was not associated compared to Group 3 (no P/LP carriers) with a hazard ratio of 1.6 (95% CI, 0.9 to 2.9, **Supplementary Table 9**).

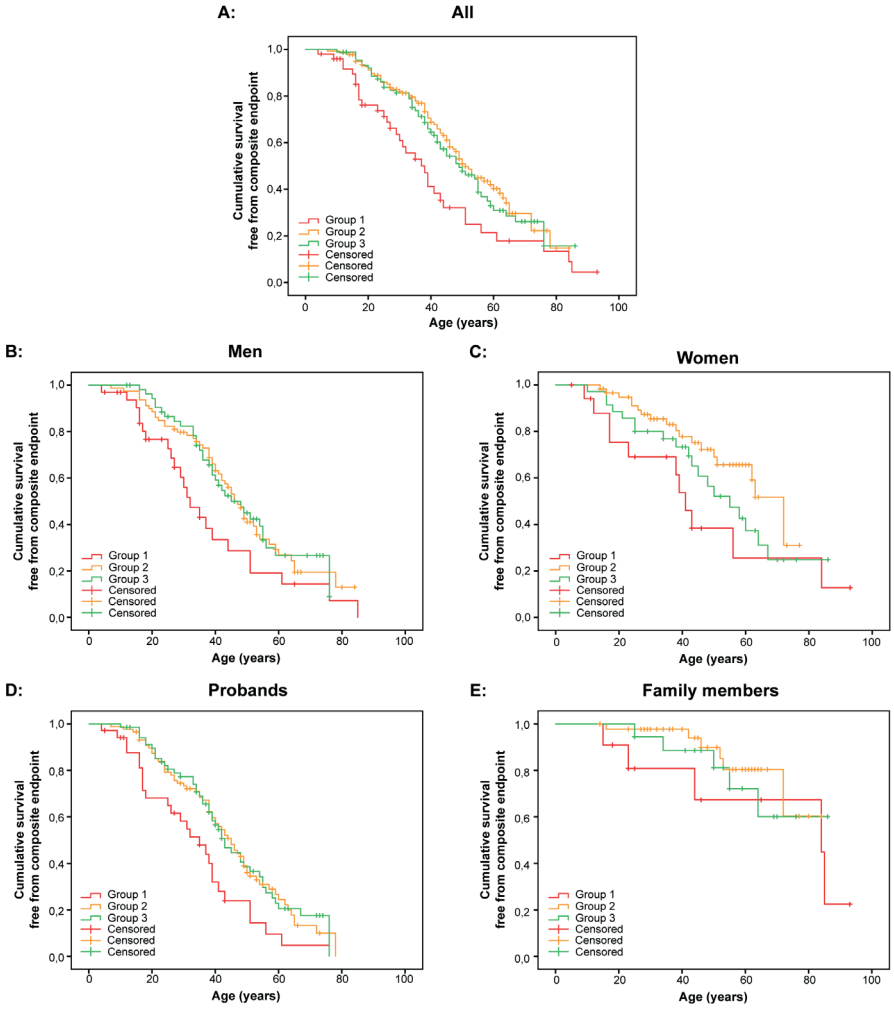

Figure 2. Kaplan–Meier Survival Analysis. Group 1 (double P/LP variant carriers), Group 2 (one P/LP variant carrier), and Group 3 (no P/LP variant carrier). A) All, B-C) All, stratified for sex, D-E) All, stratified for proband status.

Being a homozygous carrier of the Hutterite variant was significantly associated with the composite outcome in a multivariable Cox model, after correcting for proband status and male sex, compared to Group 2 with a hazard ratio of 6.9 (95% CI, 2.3 to 20.2) as well as compared to Group 3 with a hazard ratio of 5.5 (95% CI, 1.7 to 17.4, **Supplementary Table 10**).

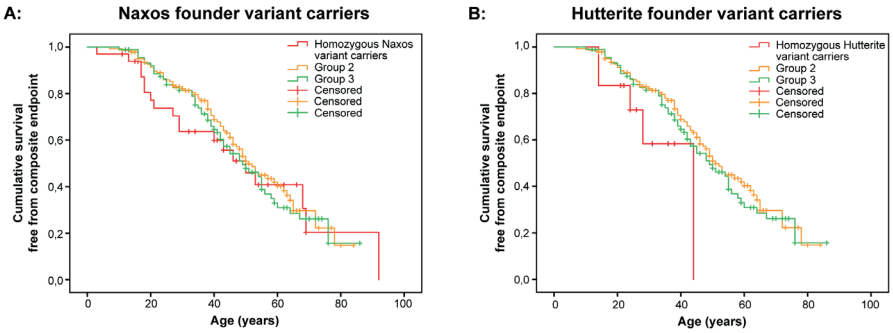

Figure 3. Kaplan-Meier Survival Analyses of homozygous Naxos and homozygous Hutterite founder variant carriers. A) Kaplan-Meier curves of homozygous Naxos variant carriers, Group 2 (one P/LP variant carrier) and Group 3 (no P/LP carrier). B) Kaplan-Meier curves of homozygous Hutterite variant carriers, Group 2 (one or none P/LP variant carrier), and Group 3 (no P/LP variant carrier).

DISCUSSION

This study aimed to establish a reliable estimate of the effect of multiple reclassified P/LP desmosomal gene variants on the severity of ARVC. Published series often have a limited size, and variant classification rules have evolved and became more stringent over the last years.[9,12,13] Therefore, we pooled the results of patients reported to have multiple P/LP variants from a large number of studies in a systematic quantitative analysis. Importantly, we performed the analysis after uniform reclassification of all variants identified and an update of clinical follow-up.

In this series of patients with multiple (reclassified) P/LP variants, the largest such study to our knowledge, we showed that this group reached the composite endpoint, consisting of death of any cause, sudden cardiac death, death due to end-stage heart failure, heart transplant and/or left ventricular assist device (LVAD), sustained ventricular tachycardia, ventricular fibrillation, out of hospital cardiac arrest (OHCA), appropriate ICD-therapy, and appropriate anti-tachycardia pacing (ATP), significantly earlier than those with one or no P/LP variant: at median age 38 years as compared to 51 and 49 years, respectively. Also, after correcting for proband status and male sex, carrying two P/LP variants remained significantly associated with reaching the composite endpoint with hazard ratios of 1.9 and 1.8 as compared to those with one and no P/LP variants, respectively. Putting it simply, the presence of multiple P/LP variants portends earlier and more severe disease.

Previous studies also showed that endpoints were reached earlier in patients with multiple P/LP variants. Bhonsale et al. described 22 patients with multiple pathogenic variants in ARVC-associated genes. They had significantly earlier occurrence of sustained VT/VF, lower

VT-/VF-free survival, a five-fold increase in the risk of developing left ventricular dysfunction, and more frequent cardiac transplantation when compared with those with only a single (likely) pathogenic variant.[13] In a study by Rigato et al., in 7 compound and 14 digenic heterozygous patients, compound/digenic heterozygosity was an independent predictor of lifetime arrhythmic events with a hazard ratio of 3.71 (95% CI=1.548.92; P = 0.003).[9] Moreover, Bauce et al. noted a higher extent of disease phenotype in terms of LV (P = 0.025) and RV dilatation (trend toward statistical significance P = 0.051) in three index cases and seven family members with multiple (likely) pathogenic variants.[12].

Reclassification revealed that only 29% of published cases with ostensibly two or more "mutations" actually had two P/LP variants after reclassification. Reclassifications were based on several observations or their combinations: 1) improved population frequency information as a result of a higher number of control exomes/genomes available (e.g. ExAc database of ~60.000 exomes vs gnomAD database of ~140.000 exomes and genomes), 2) the use of improved computational tools, 3) newly available patient information either published or in databases (e.g. HMD), such as the identification of more independent probands with similar phenotypes, 4) the availability of (more) co-segregation data (underscoring pathogenicity), or lack of co-segregation (disputing pathogenicity), or 5) novel functional data supporting pathogenicity. In a recent study, rare variants associated with arrhythmogenic cardiomyopathy were reclassified in 31% of cases after 5 years of follow-up since 1996.[21] The authors suggest a periodic genetic reanalysis of rare variants every 5 years. A recent study by Costa et al. showed that 59% of 80 ARVC-related variants were reclassified with a presumed clinically relevant reclassification in 33 variants (41%). This led to 10% of patients being downgraded from a definite diagnosis of ARVC to borderline/possible disease.[14] It is recommended to exercise caution, particularly when considering variants that were classified prior to 2015/2017 (ExAC/gnomAD).

This modified classification is relevant not only for patient diagnosis, prognosis, and treatment but also for genetic cascade screening of family members and, potentially, prenatal or pre-implantation diagnostics even though many aspects of penetrance of desmosomal gene variants are still poorly understood[22]; i.e. what is the penetrance of a single P/LP desmosomal gene variant in a family with ARVC, in an individual identified in a population study, or a heterozygous relative of an index-patient with multiple P/LP variants. The impact and importance of correct classification of genetic variants have recently also been demonstrated in Brugada Syndrome patients, where functional characterisation/validation helped correctly classify sodium voltage-gated channel alpha subunit 5 (*SCN5A*) variants. Loss of function (LOF) variants were associated with earlier onset of lethal arrhythmic events and, thus, a worse prognosis as compared to non-LOF *SCN5A* variants.[23] This was further corroborated by Ciconte et al., where proven LP/P *SCN5A* variant carriers had a higher prevalence

of aborted cardiac arrest or spontaneous ventricular tachycardia/fibrillation requiring ICD therapy.[24]

In our dataset of patients carrying multiple desmosomal variants, we used guidelines based on the current ACMG criteria to identify patients carrying two or more reclassified P/LP variants in the desmosomal genes. This refinement showed that genotype, i.e. carrying multiple desmosomal P/LP variants has an additional effect on the outcome. The control groups consisted of a group of patients (Group 2) carrying one desmosomal P/LP variant combined with a VUS or B/LB and another group of patients (Group 3) with a combination of two variants classified as either VUS or B/LB. Of note, no difference in outcome was found between these two latter groups. This could be because both groups are still too heterogeneous, and the effect of these variants remains unknown, emphasising the need for additional functional and co-segregational analyses.[14] Additionally, some VUS could have a high or low suspicion of pathogenicity. Combining this group with B/LB or P/LP variants could affect the outcome. To assess a possible effect, we divided the group that carried a VUS into subgroups where patients carry an additional P/LP variant (n=99) or additional VUS (n=63) or B/LB variant (n=16). If there are any possible suspicious VUS in those groups, the outcome would be expected to be more severe in the group with a P/LP. However, as shown in the Kaplan–Meier survival curves (**Supplementary Figure 1**), there were no differences observed. After correcting for sex and proband status, an HR of 1.1 (95% CI, 0.7 and 1.7) for the group carrying a VUS and a P/LP variant compared to the group of carriers with two VUS. An HR of 1.1 (95% CI, 0.4 and 2.6) was found for the group carrying a VUS and B/LB variant compared to the group with two VUS, and when comparing the group carrying a VUS and a P/LP variant to the group carriers with a VUS and a B/LB, the HR was 0.9 (95% CI, 0.4 and 2.2). Therefore, although we cannot exclude that there is an effect of grouping these VUS with B/LB variants or P/LP variants, we did not find an effect of possible highly suspicious VUS in these groups.

We did not include carriers of multiple variants in genes other than the five desmosomal genes. The reason is that ARVC is considered a desmosomal disease, and the majority of the (likely) pathogenic variants are found in the desmosomal genes.[25] A recent international ARVC gene-curation effort reported that, next to *TMEM43*, only the five desmosomal genes had definite evidence for an association with ARVC.[6]

Finally, the recessive *DSC2* c.1660C>T (p.Q554*) Hutterite and *JUP* c.2040_2041delGT (p.W680Gfs*11) Naxos founder variants also showed to be associated with a worse outcome after correcting for male sex and proband status, although this was not significant for the homozygous Naxos variant carriers versus Group 3 (no P/LP variants).

Limitations and strengths

Our analyses are limited by the incompleteness of reported data in the original studies. In addition, not all relevant genes were tested in all studies included. Therefore, the possibility cannot be excluded that a small number of patients included in our study had additional P/LP variants in those unanalysed genes. This may have led to an overestimation of the severity of phenotypes in subjects with one or no P/LP variants. Furthermore, the patients included in this cohort are from several large studies and registries describing ARVC patients and their family members. Unfortunately, we lack individual data regarding definite ARVC diagnosis or which task force criteria are fulfilled. Especially for family members, it is possible that they did not meet the diagnostic criteria or were asymptomatic. However, by analysing probands and family members separately, we believe this is of limited consequence since probands carrying two or more P/LP variants significantly affected the outcome (**Figure 2D)**. Also, data regarding ethnicity, and specific clinical data, like the history of exercise, cardiac function (ejection fraction), or the presence of late gadolinium enhancement on MRI, was generally unavailable. Additionally, segregation data might have become available in unknown centers for us, which could have played a role in the correct classification. Another potential limitation could be that data on the localisation of variants (in *trans* or *cis*) was generally unavailable in patients with compound heterozygous variants.

The ultimate strength of this study is the large sample size (multinational cohort) and that we performed our analysis after uniform and stringent reclassification of all published variants that were formerly qualified as "mutations" to overcome differences in variant classification and to establish the real impact of multiple P/LP variants on the ARVC phenotype. This reclassification eventually led to 29% of patients having two P/LP variants. Usually, around 17% of variants have conflicting interpretation, and their real impact on phenotype is unknown.[26] Also, for ARVC, it is known that several variants that were initially published as "mutations" or likely "mutations" did not turn out to be causative after more extensive functional and population studies (for examples, see reference 27-29). It is important to note that our study focused on ARVC patients with multiple variants ("mutations") prior to variant reclassification, and thus the comparisons were derived from a specific subset of patients. Therefore, there could be intrinsic differences in the outcomes of interest for this particular population as compared to the broader ARVC population with a single P/LP desmosomal variant or no P/LP variants.

CONCLUSION

After reclassification of variants identified in 331 patients with two ostensible desmosomal "mutations" from a large multicenter international series, only 29% carried two P/LP vari-

ants in desmosomal genes according to current classification criteria. Event-free survival analysis in 51 individuals of this group revealed these patients had a worse outcome: a median event-free survival at the age of 38 years compared to 51 and 49 years for patients with one or no P/LP variant, respectively. Carrying two P/LP variants is significantly associated with reaching the composite endpoint (ventricular arrhythmias, heart failure, or death). These results corroborate previous findings that carrying more than one P/LP variant contributes to the risk of developing life-threatening cardiac events in ARVC patients and contributes to a more accurate risk assessment in ARVC patients.

These findings underscore the clinical relevance and importance of periodically reclassifying all relevant ARVC genes in patients. Knowledge of correctly classified variants in relevant genes is of great importance to more accurately predict the future development of the disease and the identification of high-risk patients, even in the early stages of disease manifestation.

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SUPPLEMENTAL DATA

Supplementary Figure 1. Kaplan–Meier Survival Analysis on three groups based on the carriership of at least a single VUS and another variant: VUS & VUS, VUS & P/LP, and VUS & B/LB.

Supplementary Table 1. Search parameters used in literature search.

Supplementary Table 2. 67 selected articles.

Supplementary Table 3. Types of multiple variant combinations.

Supplementary Table 4. Combination of genes in the identified multiple desmosomal mutations carriers.

Supplementary Table 5. Genotype status of our included patients and the reclassification of the genetic desmosomal variants.

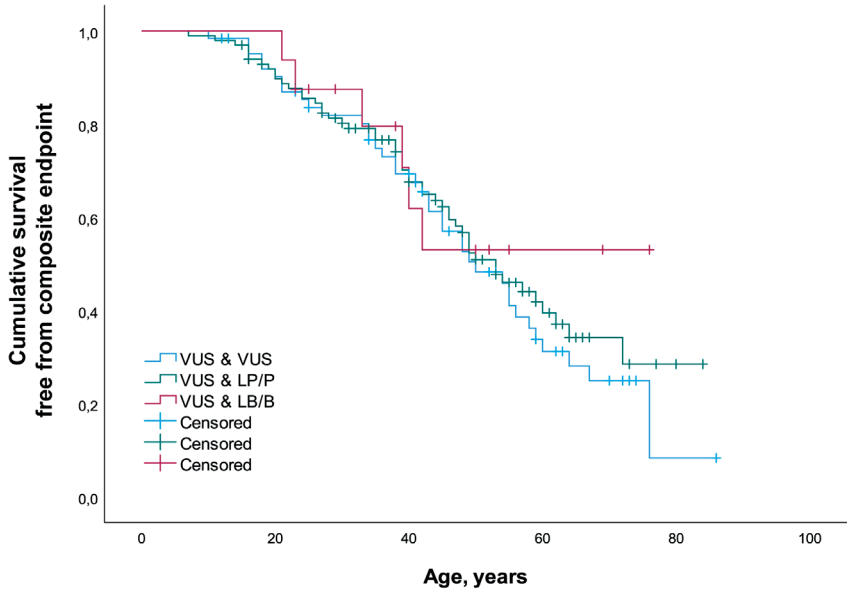
Supplementary Table 6. Regression analyses of genotype and its association with the composite endpoint in group 1 (double P/LP variants carriers) versus group 2 (one P/LP).

Supplementary Table 7. Occurrences and description of the endpoints in the 331 subjects.

Supplementary Table 8. Clinical characteristics.

Supplementary Table 9. Regression analyses of homozygous Naxos variant carriers versus group 2 (one P/LP variant) or versus group 3 (no P/LP variant).

Supplementary Table 10. Regression analyses of homozygous Hutterite variant carriers versus group 2 (one P/LP variant) or versus group 3 (no P/LP variant).



Supplementary Figure 1. Kaplan–Meier Survival Analysis on three groups based on the carriership of at least a single VUS and another variant: VUS & VUS, VUS & P/LP, and VUS & B/LB.

Supplementary Table 1. Search parameters used in literature search.

PubMed	
Source	http://www.ncbi.nlm.nih.gov/pubmed
Inclusion criteria	English
Search key 1	("arrhythmogenic cardiomyopathy"[Title/Abstract] OR "arrhythmogenic right ventricular dysplasia"[Title/Abstract] OR "arrhythmogenic right ventricular cardiomyopathy"[Title/Abstract] OR "ARVD"[Title/Abstract] OR "ARVC"[Title/Abstract] OR "arrhythmogenic right ventricular dysplasia/cardiomyopathy"[Title/Abstract] OR "ARVD/C"[Title/Abstract]) AND ("digenic heterozygosity"[Title/Abstract] OR "compound heterozygosity"[Title/Abstract] OR "multiple mutations"[Title/Abstract]) AND (mutation*[Title/Abstract])
Number of articles (May 13th 2016)	**12**
Search key 2	("arrhythmogenic cardiomyopathy"[Title/Abstract] OR "arrhythmogenic right ventricular dysplasia"[Title/Abstract] OR "arrhythmogenic right ventricular cardiomyopathy"[Title/Abstract] OR "ARVD"[Title/Abstract] OR "ARVC"[Title/Abstract] OR "arrhythmogenic right ventricular dysplasia/cardiomyopathy"[Title/Abstract] OR "ARVD/C"[Title/Abstract]) AND (mutation*[Title/Abstract])
Number of articles (May 13th 2016)	**485**
Search key 3	("arrhythmogenic cardiomyopathy"[Title/Abstract] OR "arrhythmogenic right ventricular dysplasia"[Title/Abstract] OR "arrhythmogenic right ventricular cardiomyopathy"[Title/Abstract] OR "ARVD"[Title/Abstract] OR "ARVC"[Title/Abstract] OR "arrhythmogenic right ventricular dysplasia/cardiomyopathy"[Title/Abstract] OR "ARVD/C"[Title/Abstract]) AND ("gene"[Title/Abstract] OR "genes"[Title/Abstract] OR "genetic"[Title/Abstract] OR mutation*[Title/Abstract] OR "NGS"[Title/Abstract] OR "next- generation sequencing"[Title/Abstract] OR "WES"[Title/Abstract] OR "whole-exome"[Title/Abstract] OR "whole exome"[Title/Abstract] OR "exome sequencing"[Title/Abstract] OR "whole exome sequencing"[Title/Abstract] OR "targeted array"[Title/Abstract] OR "sequencing"[Title/Abstract] OR "DHPLC"[Title/Abstract] OR "Denaturing high performance liquid chromatography"[Title/Abstract] OR "liquid chromatography"[Title/Abstract] OR "gene panel"[Title/Abstract] OR "gene panels"[Title/Abstract])

Supplementary Table 1. Search parameters used in literature search. (*continued*)

PubMed	
Number of articles (May 13th 2016)	**726**
Number of articles (January 17th 2018)	**(123 new articles) 849**
Total	**1346**

Supplementary Table 2. 67 selected articles.

See attachment 'Chapter5_S2_Table.xlsx'

Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhI0N-2H49TPLIx9-Qw?e=mWKdXm

Supplementary Table 3. Types of multiple variant combinations.

	Homozygous carriers of the Naxos or Hutterite founder variant included				**Homozygous carriers of the Naxos or Hutterite founder variant excluded**			
Combination of variants	**Before reclassification**		**After reclassification**		**Before reclassification**		**After reclassification**	
	number of multiple desmosomal variant carriers	**proportion of multiple desmosomal variant carriers, %**	**number of multiple desmosomal variant carriers**	**proportion of multiple desmosomal variant carriers, %**	**number of multiple desmosomal variant carriers**	**proportion of multiple desmosomal variant carriers, %**	**number of multiple desmosomal variant carriers**	**proportion of multiple desmosomal variant carriers, %**
Homozygous	97	29.3	60	62.5	52	18.2	15	29.4
Compound	82	24.8	10	10.4	82	28.7	10	19.6
Digenic	134	40.5	26	27.1	134	46.9	26	51
Compound, digenic	12	3.6	-	-	12	4.2	-	-
Homozygous, digenic	4	1.2	-	-	4	1.4	-	-
Trigenic	2	0.6	-	-	2	0.7	-	-
Total	331	100	96	100	286	100	51	100

Supplementary Table 4. Combination of genes in the identified multiple desmosomal mutations carriers.

	Homozygous carriers of the Naxos or Hutterite founder variant included				Homozygous carriers of the Naxos or Hutterite founder variant excluded			
	Before reclassification		After reclassification		Before reclassification		After reclassification	
Compound and/or digenic	number of multiple desmosomal variant carriers	proportion of multiple desmosomal variant carriers, %	number of multiple desmosomal variant carriers	proportion of multiple desmosomal variant carriers, %	number of multiple desmosomal variant carriers	proportion of multiple desmosomal variant carriers, %	number of multiple desmosomal variant carriers	proportion of multiple desmosomal variant carriers, %
PKP2/PKP2	31	9.4	3	3.1	31	10.8	3	5.9
PKP2/PKP2/DSP	3	0.9	–	–	3	1.0	–	–
PKP2/PKP2/DSG2	6	1.8	–	–	6	2.1	–	–
PKP2/DSP	38	11.5	3	3.1	38	13.3	3	5.9
PKP2/DSG2	38	11.5	8	8.3	38	13.3	8	15.7
PKP2/DSC2	21	6.3	11	11.5	21	7.3	11	21.6
PKP2/JUP	10	3.0	2	2.1	10	3.5	2	3.9
DSP/DSP	13	3.9	2	2.1	13	4.5	2	3.9
DSP/DSP/DSC2	1	0.3	–	–	1	0.3	–	–
DSP/DSG2	9	2.7	–	–	9	3.1	–	–
DSP/DSC2	4	1.2	2	2.1	4	1.4	2	3.9
DSP/JUP	–	–	–	–	–	–	–	–
DSG2/DSG2	33	10.0	5	5.2	33	11.5	5	9.8
DSG2/DSG2/PKP2	2	0.6	–	–	2	0.7	–	–
DSG2/DSC2	10	3.0	–	–	10	3.5	–	–
DSG2/JUP	1	0.3	–	–	1	0.3	–	–
DSC2/DSC2	4	1.2	–	–	4	1.4	–	–
DSC2/JUP	3	0.9	–	–	3	1.0	–	–
JUP/JUP	1	0.3	–	–	1	0.3	–	–
Homozygous								
PKP2	2	0.6	3	3.1	2	0.7	3	5.9
DSP	8	2.4	4	4.2	8	2.8	4	7.8
DSG2	19	5.7	2	2.1	19	6.6	2	3.9
DSC2	23	6.9	15	15.6	11	3.8	3	5.9

Supplementary Table 4. Combination of genes in the identified multiple desmosomal mutations carriers. (*continued*)

	Homozygous carriers of the Naxos or Hutterite founder variant included				Homozygous carriers of the Naxos or Hutterite founder variant excluded			
	Before reclassification		After reclassification		Before reclassification		After reclassification	
Compound and/or digenic	**number of multiple desmosomal variant carriers**	**proportion of multiple desmosomal variant carriers, %**	**number of multiple desmosomal variant carriers**	**proportion of multiple desmosomal variant carriers, %**	**number of multiple desmosomal variant carriers**	**proportion of multiple desmosomal variant carriers, %**	**number of multiple desmosomal variant carriers**	**proportion of multiple desmosomal variant carriers, %**
JUP	45	13.6	36	37.5	12	4.2	3	5.9
Trigenic								
PKP2/DSG2/DSC2	1	0.3	–	–	1	0.3	–	–
DSP/DSG2/DSC2	1	0.3	–	–	1	0.3	–	–
Digenic, homozygous								
PKP2/PKP2/DSP/DSP	1	0.3	–	–	1	0.3	–	–
PKP2/PKP2/JUP	1	0.3	–	–	1	0.3	–	–
DSP/DSP/DSG2	1	0.3	–	–	1	0.3	–	–
DSG2/DSG2/DSP	1	0.3	–	–	1	0.3	–	–
Total	331	100	96	100	286	100	51	100

DSC2 = desmocollin-2 , *DSG2* = desmoglein-2, *DSP* = desmoplakin, *JUP* = plakoglobin, *PKP2* = plakophilin-2

Supplementary Table 5. Genotype status of our included patients and the reclassification of the genetic desmosomal variants.

See attachment 'Chapter5_S5_Table.xlsx'

Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhI0OLSmAeXpjf-0hmg?e=FXSh8l

Supplementary Table 6. Regression analyses of genotype and its association with the composite endpoint in group 1 (double P/LP variants carriers) versus group 2 (one P/LP).

	Univariable Cox regression analyses			Multivariable Cox regression analysis		
Group 1 versus Group 2	HR	95% CI	*P* value	HR	95% CI	*P* value
Proband, yes	7.106	3.552-14.218	<0.001	6.748	3.320-13.716	<0.001
Sex, male	1.870	1.225-2.855	0.004	1.326	0.855-2.056	0.207
Genotype, double (likely) pathogenic	1.833	1.206-2.787	0.005	1.856	1.208-2.853	0.005
Group 1 versus Group 3						
Proband, yes	4.751	2.267-9.959	<0.001	4.938	2.341-10.418	<0.001
Sex, male	1.363	0.873-2.126	0.173	1.151	0.729-1.816	0.547
Genotype, double (likely) pathogenic	1.655	1.069-2.563	0.024	1.780	1.136-2.789	0.012

P/LP = Pathogenic/Likely Pathogenic

Supplementary Table 7. Occurrences and description of the endpoints in the 331 subjects.

Endpoint	**Patients number**	**Patients %**
Missing data	5	1.5
No endpoint reached	140	42.3
Death any cause	4	1.2
Sudden cardiac death	18	5.4
Death due to end-stage heart failure	0	0
Hearttransplant or LVAD, or both	12	3.6
Sustained ventricular tachycardia	104	31.4
Ventricular fibrillation	6	1.8
Out of hospital cardiac arrest	21	6.3
Appropriate ICD treatment	3	0.9
ICD shock	15	4.5
appropriate anti-tachycardia pacing	3	0.9

ICD = implantable cardioverter-defibrillator, LVAD = left ventricular assist device

Supplementary Table 8. Clinical characteristics.

	Group 1 (n=51)		**Group 2 (n=144)**		**Group 3 (n=91)**		**Homozygous Naxos variant carriers (n=33)**		**Homozygous Hutterite variant carriers (n=12)**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Number of subjects	33/51 (65%)	18/51 (35%)	81/144 (56%)	63/144 (44%)	55/91 (60%)	36/91 (40%)	19/33 (58%)	14/33 (42%)	8/12 (67%)	4/12 (33%)
Proband status	22/28 (79%)	13/18 (72%)	63/78 (81%)	27/61 (44%)	44/55 (80%)	26/34 (77%)	11/19 (58%)	5/14 (36%)	1/8 (13%)	0/4 (0%)
Mean age presentation or diagnosis ±SD, years	28 ±18	31 ±21	38 ±16	37 ±17	38 ±16	41 ±17	30 ±19	36 ±15	26 ±11	24 ±4
Endpoint reached, yes	23/33 (70%)	11/18 (61%)	54/79 (68%)	20/61 (33%)	34/54 (63%)	20/36 (56%)	12/19 (63%)	7/14 (50%)	4/8 (50%)	1/4 (25%)
Mean age endpoint, ±SD, years	30 ±20	35 ±24	43 ±17	43 ±17	42 ±17	44 ±19	32 ±20	47 ±20	27 ±11	27 ±7

group 1 = double P/LP variants carriers, group 2 = patients with one P/LP variant, group 3 = patients with no P/LP, P/LP = pathogenic/likely pathogenic, SD = standard deviation

Supplementary Table 9. Regression analyses of homozygous Naxos variant carriers versus group 2 (one P/LP variant) or versus group 3 (no P/LP variant).

	Univariable Cox regression analyses			Multivariable Cox regression analysis		
Homozygous Naxos variant carriers versus group 2	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
Proband, yes	4.761	2.633-8.613	<0.001	4.604	2.453-8.643	<0.001
Sex, male	2.190	1.378-3.482	0.001	1.555	0.948-2.550	0.080
Genotype, double (likely) pathogenic	1.173	0.699-1.969	0.545	1.759	1.035-2.989	0.037
Homozygous Naxos variant carriers versus group 3						
Proband, yes	3.251	1.733-6.100	<0.001	3.626	1.848-7.114	<0.001
Sex, male	1.480	0.909-2.411	0.115	1.176	0.707-1.955	0.532
Genotype, double (likely) pathogenic	1.082	0.633-1.847	0.774	1.647	0.940-2.887	0.081

P/LP = pathogenic/likely pathogenic

Supplementary Table 10. Regression analyses of homozygous Hutterite variant carriers versus group 2 (one P/LP variant) or versus group 3 (no P/LP variant).

	Univariable Cox regression analyses			Multivariable Cox regression analysis		
Homozygous Hutterite variant carriers versus group 2	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
Proband, yes	4.132	2.176-7.845	<0.001	5.126	2.442-10.761	<0.001
Sex, male	2.105	1.262-3.511	0.004	1.551	0.905-2.657	0.110
Genotype, double (likely) pathogenic	2.351	0.920-6.011	0.074	6.868	2.337-20.164	<0.001
Homozygous Hutterite variant carriers versus group 3						
Proband, yes	2.471	1.243-4.915	0.010	3.714	1.623-8.500	0.002
Sex, male	1.324	0.770-2.278	0.310	1.157	0.663-2.019	0.608
Genotype, double (likely) pathogenic	2.091	0.803-5.446	0.131	5.467	1.718-17.402	0.004

P/LP = pathogenic/likely pathogenic

Chapter 6

Identification of sarcomeric variants in probands with a clinical diagnosis of arrhythmogenic right ventricular cardiomyopathy (ARVC)

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ABSTRACT

Aims: Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited cardiomyopathy characterized by ventricular arrhythmias and sudden death. Currently 60% of patients meeting Task Force Criteria (TFC) have an identifiable mutation in one of the desmosomal genes. As much overlap is described between other cardiomyopathies and ARVC, we examined the prevalence of rare, possibly pathogenic sarcomere variants in the ARVC population.

Methods: One hundred and thirty-seven (137) individuals meeting 2010 TFC for a diagnosis of ARVC, negative for pathogenic desmosomal variants, *TMEM43*, *SCN5A*, and *PLN* were screened for variants in the sarcomere genes (*ACTC1*, *MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNC1*, *TNNI3*, *TNNT2*, and *TPM1*) through either clinical or research genetic testing.

Results: Six probands (6/137, 4%) were found to carry rare variants in the sarcomere genes. These variants have low prevalence in controls, are predicted damaging by Polyphen-2, and some of the variants are known pathogenic hypertrophic cardiomyopathy mutations. Sarcomere variant carriers had a phenotype that did not differ significantly from desmosomal mutation carriers. As most of these probands were the only affected individuals in their families, however, segregation data are noninformative.

Conclusion: These data show variants in the sarcomere can be identified in individuals with an ARVC phenotype. Although rare and predicted damaging, proven functional and segregational evidence that these variants can cause ARVC is lacking. Therefore, caution is warranted in interpreting these variants when identified on large next-generation sequencing panels for cardiomyopathies.

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited myocardial disease characterized by fibrofatty replacement of the myocardium. This results in a predisposition to often life-threatening ventricular arrhythmias and functional alterations to the right and left ventricles that can lead to the development of heart failure. A familial pattern of disease can be recognized in up to 50% of cases, predominantly inherited in an autosomal dominant manner.[1] Reduced penetrance, and variable expressivity of presentation have complicated the identification of a genetic cause in some cases.

ARVC is primarily thought of as a disease of the desmosome, or the cell–cell junctions at the intercalated disk. Pathogenic variants in five genes (*DSP*, *PKP2*, *DSG2*, *DSC2*, and *JUP*) are classically thought to play a large role in ARVC pathogenesis.[2,3] In routine clinical practice, however, even in a well phenotyped population, up to 40% of ARVC cases still elude identification of a genetic root cause.[2,3] Because of the importance of identifying those at risk for sudden death, gene finding efforts have continued.

Increasingly, nondesmosomal genes have been implicated in ARVC pathogenesis, such as *CDH2* and *CTNNA3*.[4,5] *PLN* has been identified as a causative factor in a significant portion of individuals with ARVC patients.[3,6] Additionally, recent reports have identified *SCN5A* mutations in a small percentage of ARVC patients.[7] Overlap between ARVC and dilated cardiomyopathy has been well described, and pathogenic variants in sarcomere genes have been associated with DCM.[8] Little data exists, however, on the prevalence of other cardiomyopathy associated genes in the ARVC population.[9] In this study, we sequenced an expanded panel of cardiac genes among individuals with a clinical diagnosis of ARVC and without mutations in the ARVC-associated genes *PKP2*, *DSG2*, *DSP*, *DSC2*, and *JUP,* or nondesmosomal *TMEM43*, *SCN5A*, and *PLN*. We aimed to (1) assess the prevalence and pathogenicity of sarcomere gene mutations in individuals with ARVC without an identified desmosomal pathogenic variant and (2) compare clinical characteristics and course between the two groups.

2. METHODS

1. Study population

Patients were eligible for inclusion if they were (1) diagnosed with ARVC based on the 2010 diagnostic Task Force Criteria (TFC) for ARVC,[10] (2) were the family proband as defined as the first person in the family to come to clinical attention and gain a clinical diagnosis of ARVC, (3) lacked a pathogenic/likely pathogenic variant in any of the 5 desmosomal

genes noted above, or *TMEM43*, *PLN*, or *SCN5A*, (4) underwent broad cardiomyopathy gene screening as described below, and (5) enrolled in the ARVC registries from the Johns Hopkins University and the Netherlands Heart Institute, a cardiovascular research institute with collaborative participation of all eight Dutch University Medical Centers. This study was approved by the JHSOM Institutional Review Board.

2.2 Molecular genetic screening

Sequencing and deletion/duplication analysis of the nine most common sarcomere genes (*ACTC1*, *MYBPC3*, *MYH7*, *MYL2*, *MYL3*, *TNNC1*, *TNNI3*, *TNNT2*, and *TPM1*, hereafter referred to as *sarcomere genes*) were completed through a variety of methods: clinical genetic testing, and research-lab based panel and exome sequencing. Within the JHU registry, 92 patients underwent clinical cardiomyopathy panel sequencing and deletion/duplication analysis, and 18 patients consented to whole-exome sequencing using the Ilumina HiSeq2000 platform. For exome sequencing, the human assembly GRCh37/hg19 was used as reference genome. In the Dutch registry, 27 patients were sequenced using a next-generation sequencing (NGS) panel consisting of 209 genes (candidate genes and genes known to be involved in cardiomyopathy). Therefore, in total 137 probands underwent sequencing and deletion/duplication analysis of the sarcomere genes. Nucleic acid deviations were compared with the reference sequence for presence of variants in the sarcomere genes. All mutations were confirmed by Sanger sequencing after polymerase chain reaction amplification.

Potentially causal variants were identified using standard filtering criteria as follows. Variants were excluded if they had a minor allele frequency (MAF) >0.1% in the Exome Aggregation Consortium Browser (ExAC)[11,12] and/or if they were present in dbSNP 126, 129, and 131. Variants also were included if predicted damaging by Polyphen-2 and below the MAF above.[13] Variants were assessed and classified according to the 2015 American College of Medical Genetics classification criteria, as reported in **Table 1**.[14] All patients and their families gave informed consent for genomic DNA sample collection, storage, and sequencing.

2.3 Phenotype evaluation

All individuals were phenotyped via medical records for diagnostic criteria according to the 2010 TFC.[10] A definite ARVC diagnosis was characterized by the presence of ≥2 major criteria, 1 major and 2 minor criteria, or 4 minor criteria. As indicated in **Supplementary Table 1**, none of the individuals met diagnostic criteria for hypertrophic cardiomyopathy of having a septal thickness of ≥1.5 cm.[15]

2.4 Statistical analysis

Statistical analyses were performed using SPSS (version 22.0). All continuous data were calculated as mean and categorical variables as numbers (percentages). Variables were compared

using the Fisher's exact test for proportions, and chi-square test. A P-value of <0.05 was considered statistically significant.

Table 1. Variants in the sarcomere genes identified upon sequencing of 137 probands with ARVC.

Family	Gene	Exon	Amino acid change	Nucleotide change	% ExAC	Polyphen (2)	ACMG pathogenicity classification
1	**MYH7**	22	p.847_847del	c.2541_2549del	0.0000%	n/a	VUS
2		21	p.Arg1500Gln	c.4499G>A	0.0025%	0.999	VUS
3		37	p.Arg1846Cys	c.5536C>T	0.0017%	1.000	VUS
4‡		20	p.Arg723Cys	c.2167C>T	0.0025%	0.995	P
5★	**MYBPC3**	4	p.Gly148Arg	c.442G>A	0.0042%	0.070	P
6†	**MYL3**	4	p.Arg154His	c.461G>A	0.0025%	0.861	LP

ExAC frequencies as of 4/2017 are listed.[11] Assessment of pathogenicity according to criteria put forth by the American College of Medical Genetics is listed (LP = likely pathogenic; P = pathogenic;VUS = variant of uncertain significance).[14]

‡Segregated with disease in relatives from multiple families. (ClinVar: http://www.ncbi.nlm.nih.gov/clinvar/variation/14095/).

★This variant is a low penetrance variant and frequently observed in the Dutch HCM population. It is a founder mutation and creates a cryptic splice acceptor site (P. Van Tintelen, personal communication). Prediction programs (Polyphen-2) are not valid because of supposed pathogenic splice site effect. Aberrant splicing is demonstrated in two university hospitals. This individual also carried a variant in *LMNA* c.1003C > T; p.Arg335Trp that has been described as likely pathogenic.[19]

†This mutation has been demonstrated to cause a disturbed binding to myosin in vitro.[20]

3 RESULTS

3.1 Genetic screening

In total, 137 individuals meeting diagnostic criteria for ARVC underwent sequencing of the sarcomere genes. Sarcomere variants identified are described in **Table 1**. These variants were rare in ExAC (<0.1% as described above). A total of six variants were identified in 6/137 (4.3%) separate probands. Variants were identified in *MYH7*, *MYBPC3*, and *MYL3*. Variants in *MYH7* included three missense mutations in *MYH7* and one in-frame deletion. There was one missense variant identified in *MYBPC3* and one variant in *MYL3*. For most families, the proband was the only reported affected in the family. In one family (Individual #2 as described in **Table 2**), the variant was identified in her, and also in her mother who had met diagnostic criteria for ARVC with T wave inversions through V3 on ECG and over 500 PVCs on Holter monitoring. The variant identified in *MYH7* was previously published in the literature segregating with HCM in multiple families.[16] For the other four families, family screening was not completed or cardiac screening, including ECG, Holter monitoring, and either echocardiogram and/or cardiac MRI in first degree relatives was within normal

Table 2. Clinical characteristics of the 6 patients identified to have pathogenic sarcomere variants.

		Clinical presentation				ECG or SAECG		Structure	
Family #	Sex	Age at presentation (years)	AA medication	Type of presentation	Cardiac Syncope	Repolarization abnormality	Depolarization abnormality	RV structural abnormality	LV dysfunction
1	M	50	+	Sympt	+	M	n/a	M	+
2	F	14	+	Sympt	+	M	–	M	+
3	M	27	+	Sympt	+	M	–	M	–
4	M	26	+	Sympt	+	m	–	M	+
5	M	28	+	Sympt	–	M	m	M	+
6	M	36	+	Sympt	–	m	–	M	–

	Arrhythmia			Outcome							
Family #	Holter ectopy	EPS inducibility	Sustained VA	VT storm	ICD implant	ICD therapy	VT ablation	Heart failure	Death/cardiac transplant	Follow-up (years)	TFC
1	n/a	+	+	+	+	+	+	+	+	23	6(D)
2	470	+	+ (At presentation)	–	+	+	+	+	–	20	4(D)
3	n/a	+	+ (At presentation)	+	+	+	+	–	–	27	6(D)
4	472	+	+	–	+	+	+	–	–	14	5(D)
5	n/a	+	+ (At presentation)	–	+	+	–	–	–	6	9(D)
6	1181	n/a	+	–	–	–	–	–	–	13	4(D)

AA = antiarrhythmic; B = borderline; D = definite; EP = electrophysiology; F = female; ICD = implantable cardiovertor defibrillator; LV dysfunction = LV EF ≤50%; M = male; m = minor abnormality as per the 2010 ARVC revised Task Force Criteria; M = major abnormality as per the 2010 ARVC Revised Task Force Criteria; Sympt = symptomatic with chest pain, dyspnea, palpitations, presyncope, or syncope; TFC = 2010 revised Task Force Criteria for the diagnosis of ARVC; VA = ventricular arrhythmias including VT and VF; VT /VF = ventricular tachycardia/ventricular fibrillation.

limits. As noted in **Table 1**, many of these variants may not meet pathogenicity criteria for a pathogenic call when classified according to the ACMG criteria; however, would still be reported. Even those that may be classified as pathogenic for a diagnosis of HCM may not be reported as pathogenic for a diagnosis of ARVC given the lack of evidence for disease association.

3.2 Clinical evaluation

All individuals met diagnostic criteria as described in **Table 2**.[10] Compared to previously published ARVC patients carrying desmosomal mutations, individuals with sarcomere variants tended to be slightly older (36.8 years of age vs. 33.2 years of age), though not significant.[2] Clinical characteristics of the study population were compared to previously published values[2] in desmosomal mutations (definitions in **Supplementary Table 2**): gender, type of presentation, syncope, inducibility at electrophysiology study, premature ventricular contraction (PVC) count on 24-hour ambulatory monitoring (Holter), ICD placement, appropriate ICD therapy, ventricular tachycardia (VT) storm,VT ablation, and left ventricular dysfunction (left ventricular ejection fraction below 50%), heart failure, and transplant. Individuals with ARVC carrying sarcomere variants were more likely to have undergone a VT ablation ($P = 0.009$), but otherwise had a similar disease presentation and course to desmosomal mutation carriers. **Figure 1** shows the ECG and cardiac MRI short axis image of individual 3, showing characteristic T wave inversions across the precordium and dyskinetic basal right ventricular (RV) free wall with enhancement, suggestive of a diagnosis of ARVC. **Supplementary Table 1** describes septal thickness; all values were way below the threshold for HCM.

4 DISCUSSION

It has been well documented that approximately 40–60% of individuals meeting diagnostic criteria for ARVC have a mutation mainly in genes encoding the cardiac desmosome.[2,3] The availability of tools such as whole-exome sequencing and expansion of number of genes included on clinical cardiomyopathy panels has opened the doors for further gene discovery, but has also introduced significant challenges in counseling patients for genetic testing, and in interpretation of variants for the clinician. Understanding the pathogenicity of a variant is critical in the identification of those at risk for sudden death and implementing risk-stratifying cascade screening.

In this cohort, we describe that a small but significant percentage (4.3%) of individuals with ARVC may have putative likely pathogenic/pathogenic variants reported in the sarcomere genes. A similar yield has recently also been reported by others.[9] Without systematic functional studies or extensive segregation in affected individuals on each of these rare

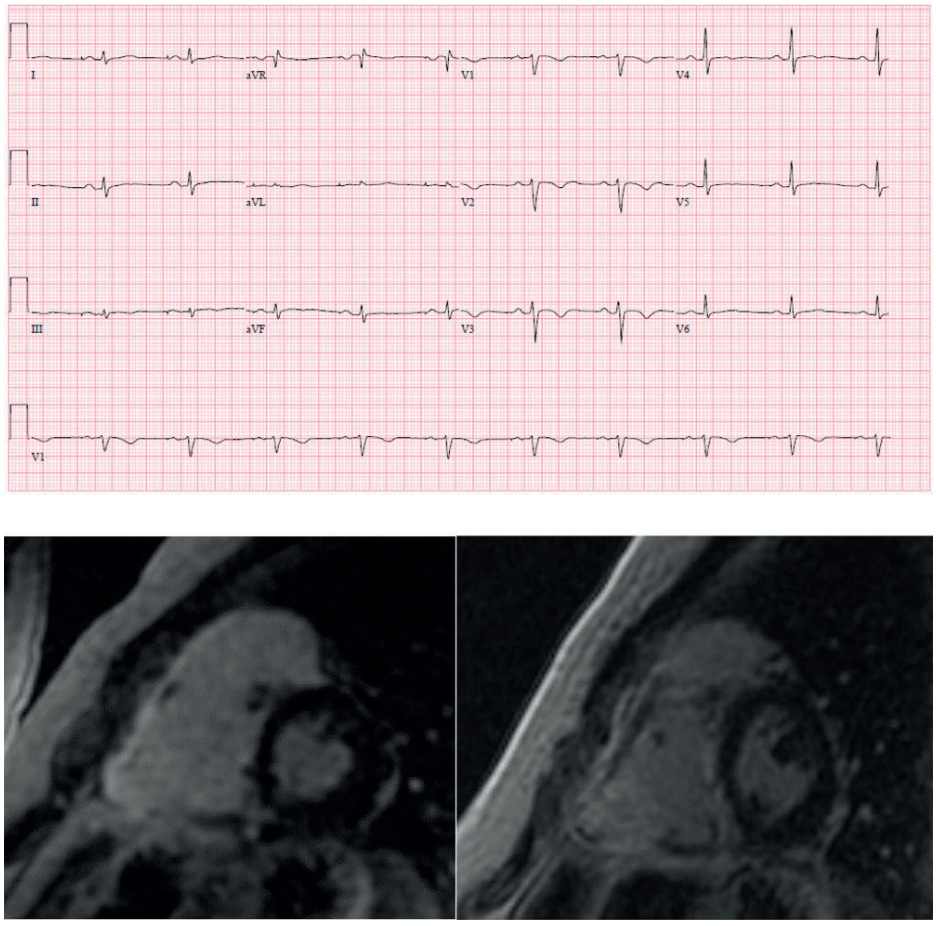

Figure 1. Electrocardiogram and cardiac MRI of proband #3 showing major criterion of T wave inversions across the precordium and dyskinetic base and enhancement of the RV wall.

novel variants, however, it is difficult to determine the role these variants play in the ARVC phenotype of individuals. Integration of segregation analysis in families with ARVC is also challenging given the well described reduced penetrance.[3] In addition, mounting exome population data over the years have led to systematic reclassification of previously described pathogenic variants in the cardiomyopathies as uncertain, or even benign.[17] Therefore, we as well as other centers are hesitant to immediately label these variants as "pathogenic" given the implications for cascade screening in these families. Given this, without further evidence, sarcomere variants should not be used to guide presymptomatic screening in families with ARVC.

Continued genetic overlap between the cardiomyopathies has been described, and it follows that structural heart disease with arrhythmias that primarily affects the RV may produce

a phenotype mimicking ARVC.[4,10] Indeed, these individuals with sarcomere variants meet TFC for ARVC, and do not have significant differences in structural disease than desmosomal variant carriers. The cohort reported here is less likely to have a reported family history of disease than previous reported prevalences in desmosomal mutation carriers.[2] They have no significant differences in phenotype by TFC, do not meet HCM criteria, but also, importantly, they do not have any significant differences in clinical course (other than being more likely to undergo catheter ablation, which is by physician judgment) than previously described individuals with ARVC with desmosomal mutations.[2] These are important findings as it indicates that these individuals are not misdiagnosed, in fact, they have similar phenotype and clinical course, confirming that these individuals do indeed have ARVC.

This study is limited in that most individuals are the only one affected in their family, so familial segregation is not informative. Unfortunately, due to family choice, prospective information on family screening could not be obtained. Negative family history is not unusual in ARVC, as reduced penetrance is well described.[3] Additionally, as with most cardiomyopathy variants, functional data are lacking for the majority of the variants identified here. These sarcomere variants may have a pathogenic role, since there is some (*in silico*) functional evidence, cosegregation with HCM in prior publications, and altered splicing for one of these variants.[14,18] At this time, however, as conclusive data of a role in ARVC are lacking, this analysis provides important information to clinicians who may order large-scale sequencing panels that caution is advised when sarcomere variants are resulted in a patient with an ARVC phenotype. Even if a variant is reported as pathogenic/likely pathogenic due to limited functional data, these datasuggest that sarcomere variants should not be interpreted or used clinically as pathogenic in ARVC families.

5 CONCLUSION

Despite these limitations, the results of our study highlight that a small proportion of patients exhibiting an ARVC phenotype may have variants identified in the sarcomere genes. This provides important additional evidence for clinical practice to recommend caution in interpretation of comprehensive cardiomyopathy gene testing results in genetic screening of ARVC patients. At this point, without further functional studies or strong segregation with disease in multiple families, these variants should not be used for clinical care. In addition, the identification of these variants, yet absence of evidence of a causative role, highlights the importance of cardiac genetics expertise in the interpretation of these large cardiomyopathy panels in families with inherited heart disease.

Further work would be of great interest to investigate the functional role of these variants in the function of the sarcomere and the desmosome, and in the pathogenesis of ARVC. Until then, these variants should not be utilized in clinical decision making, or family screening.

ACKNOWLEDGMENT

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SUPPLEMENTAL DATA

Supplementary Table 1. Hypertrophic cardiomyopathy echocardiogram measurements in the 6 probands.

Supplementary Table 2. Definitions of clinical variables employed in this study.

Supplementary Table 1. Hypertrophic cardiomyopathy echocardiogram measurements in the 6 probands.

Family	Intraventricular Septum Measurement (IVSd)	Meet diagnostic criteria for HCM?
1	0.9 cm	no
2	0.95 cm	no
3	0.9 cm	no
4	1.2 cm	no
5	1.1 cm	no
6	0.99 cm	no

Supplementary table 2. Definitions of clinical variables employed in this studs.

Term	Definition
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
VT storm	Occurrence of ≥ 3 episodes of ventricular tachycardia/ ventricular fibrillation in a 24-h period
Proven or definite ARVD/C (Diagnostic terminology as per 2010 revised Task Force Criteria)	2 major or 1 major and 2 minor criteria or 4 minor 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

Chapter 7

No major role for rare plectin variants in arrhythmogenic right ventricular cardiomyopathy

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ABSTRACT

Aims: Likely pathogenic/pathogenic variants in genes encoding desmosomal proteins play an important role in the pathophysiology of arrhythmogenic right ventricular cardiomyopathy (ARVC). However, for a substantial proportion of ARVC patients, the genetic substrate remains unknown. We hypothesized that plectin, a cytolinker protein encoded by the *PLEC* gene, could play a role in ARVC because it has been proposed to link the desmosomal protein desmoplakin to the cytoskeleton and therefore has a potential function in the desmosomal structure.

Methods: We screened *PLEC* in 359 ARVC patients and compared the frequency of rare coding *PLEC* variants (minor allele frequency [MAF] <0.001) between patients and controls. To assess the frequency of rare variants in the control population, we evaluated the rare coding variants (MAF <0.001) found in the European cohort of the Exome Aggregation Database. We further evaluated plectin localization by immunofluorescence in a subset of patients with and without a *PLEC* variant.

Results: Forty ARVC patients carried one or more rare *PLEC* variants (11%, 40/359). However, rare variants also seem to occur frequently in the control population (18%, 4754/26197 individuals). Nor did we find a difference in the prevalence of rare *PLEC* variants in ARVC patients with or without a desmosomal likely pathogenic/pathogenic variant (14% versus 8%, respectively). However, immunofluorescence analysis did show decreased plectin junctional localization in myocardial tissue from 5 ARVC patients with *PLEC* variants.

Conclusions: Although *PLEC* has been hypothesized as a promising candidate gene for ARVC, our current study did not show an enrichment of rare *PLEC* variants in ARVC patients compared to controls and therefore does not support a major role for *PLEC* in this disorder. Although rare *PLEC* variants were associated with abnormal localization in cardiac tissue, the confluence of data does not support a role for plectin abnormalities in ARVC development.

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a heritable progressive heart condition characterized by fibro-fatty replacement of the ventricular myocardium.[1] ARVC is most commonly transmitted as an autosomal dominant trait[2] and has an estimated prevalence of ~1:2500.[3] The majority of the likely pathogenic or pathogenic variants variants (>50%) are found in the five genes coding for the major desmosomal proteins: plakophilin-2 (PKP2), desmoplakin (DSP), desmoglein-2 (DSG2), desmocollin-2 (DSC2), and plakoglobin (JUP).[4] Clinically, ARVC patients suffer from ventricular arrhythmias, syncope, and sudden cardiac death as early as young adulthood,[5] with the majority of cases diagnosed before the age of 40 years.[6] Identification of ARVC-associated variants greatly facilitates the identification of family members at risk and provides a better understanding of the underlying pathophysiological mechanisms.[1] However, while more ARVC-associated genes are known,[7] they only contribute to a small proportion of cases of this predominantly desmosomal disease, and roughly half of ARVC cases remain gene elusive.[8]

We wanted to explore whether the candidate gene *PLEC*, which encodes for plectin, underlies ARVC pathogenesis. Plectin is a large cytolinker protein that belongs to the plakin family of proteins. It is believed to connect the cardiac desmosome to the cytoskeletal intermediate filament desmin via linking DSP within cardiomyocytes.[9] In cardiac tissue, plectin is mainly localized at the intercalated disk and the sarcomeric Z-line, whereas in skin it is located at desmosomes and hemi-desmosomes.[10] This means that plectin potentially has a general and fundamental role in junctional complexes.[9] *PLEC* is a well-known player in the skin disease epidermolysis bullosa simplex with muscular dystrophy (EBS-MD). It is caused by compound heterozygous or homozygous *PLEC* variants, and research on these conditions has led to observations that implicated it in various forms of cardiomyopathy other than ARVC. When fully knocked out in mice, plectin deficiency causes severe skin blistering, generalized skeletal myopathies and ultra-structural abnormalities in the heart.[11] *S*triated-muscle-specific conditional *Plec* knock-out mice showed a decline in endurance performance and, by the age of 16 months, an increase in connective tissue formation in the heart.[12] Sporadic cases of cardiac involvement have also been reported in people with likely pathogenic or pathogenic variants in *PLEC*. These include an EBS-MD patient with ventricular hypertrophy[13]; an EBS-MD patient who, by the age of 30, was discovered to have asymptomatic dilated cardiomyopathy (DCM) that later progressed to right ventricular involvement including (septal) fibrosis[14]; an EBS-MD patient who had a left ventricular non-compaction cardiomyopathy[15]; and a compound heterozygous carrier for two truncating *PLEC* variants who had DCM and episodes of malignant ventricular arrhythmias.[16]

Combining PLEC's potential role in the desmosome with the experimental data and the clinical reports from literature linking EBS-MD and cardiac conditions, led us to hypothesize that plectin may play a role in the pathophysiology of, or increase the susceptibility to, ARVC. We therefore analysed *PLEC* in a large group of ARVC patients, described in previous studies,[17-19] and compared this to *PLEC* variants found in the Exome Aggregation Consortium dataset (ExAC).[20] Cluster analysis was performed to identify possible hotspot regions in *PLEC*. In addition, immunofluorescence analysis was performed on endomyocardial biopsies.

RESULTS

ARVC cohort

We identified 47 'rare' (population frequency <0.001) or novel coding variants in Plectin isoform 1 (NM_201380) and three rare variants in two other isoforms (isoform 1a [NM_201384] and 1g [NM_201383]). For an overview of the identified rare or novel coding *PLEC* variants see **S1 Table**. Plectin has eight isoforms that differ only in the N-terminal sequences encoded by alternatively spliced first exons.[21] The three variants we identified in isoform 1a and 1g were not in included in the subsequent analyses because isoform 1a is not expressed in the heart[22] and isoform 1g does not belong to the muscle-specific set of major isoforms.[9] Of the 359 patients, 40 (11%) carried one or more rare variants: 35 carried one rare variant, four carried two rare variants, and one carried four rare variants. Almost all were missense variants (96%, 45/47), but one was an in frame insertion and one a nonsense variant. There was no significant difference in the percentage of ARVC patients who carried one or more rare *PLEC* variants between those with and without a previously identified ARVC-associated variant (14% versus 8%, respectively [**Table 1**]).

Table 1. Overview of proportion of individuals with one or more *PLEC* variant.

Cohort	ARVC-NL (n=79)		ARVC-UK (n=84)		ARVC-US (n=196)		**Total (n=359)**		**Fisher's exact**
Rare *PLEC* variant carriers	23% (18/79)		8% (7/84)		7% (15/196)		11% (40/359)		
ARVC mutation	Yes	No	Yes	No	Yes	No	Yes	No	
	n=42	n=37	n=46	n=38	n=98	n=98	n=186	n=173	
Rare *PLEC* variant carriers	24% (10/42)	22% (8/37)	13% (6/46)	3% (1/38)	9% (10/98)	5% (5/98)	14% (26/186)	8% (14/173)	P = 0.093

ARVC = Arrhythmogenic right ventricular cardiomyopathy, n = number of subjects, *PLEC* = plectin

ExAC Eu

The median coverage of *PLEC* (NM_2013830) in the ExAC Eu cohort was 40x. In total, 4761 rare coding *PLEC* variants were found. **S2 Table** provides an overview of the identified variants in the ExAC Eu dataset. Seven rare missense variants were found in the homozygous state. Of the 4761 rare variants, 4689 were missense variants (98.5%), 42 were in frame insertions/duplication or deletions (0.9%), and 30 were truncating variants (0.6%). The estimated proportion of individuals with a rare *PLEC* variant (after taking homozygous variants [n = 7] into account) is 18% ([4761–7]/26197).

Frequency of rare *PLEC* variants in ARVC versus ExAC Eu

The proportion of individuals in the ARVC cohorts with one or more rare *PLEC* variants is 0.11 (**Table 1**) as compared to 0.18 in ExAC. The case excess of rare *PLEC* variants in the ARVC cohort would therefore be -0.07, showing that there is no enrichment of rare *PLEC* variants in ARVC patients (**Table 2**). Thirty-three of the 47 rare *PLEC* variants (70%) in the ARVC cohort had a scaled Combined Annotation Dependent Depletion (CADD) score of 20 or more, compared to 3448 of the 4761 *PLEC* variants (72%) in the ExAC Eu dataset,[23] indicating no differences in impact of these variants between patients and controls. Ten patients (3%, 10/359) in the ARVC cohort carried one or more unique variant(s) in

PLEC. Of these 10, six also carried a pathogenic or likely pathogenic variant associated with ARVC. In the ExAC Eu cohort, 500 unique variants were identified, and the estimation

Table 2. Odds ratio and Chi-Square test results for the proportion of carriers with one or more rare *PLEC* variant in ARVC cases versus ExAC European controls.

ARVC cases			**Controls***							
with a rare *PLEC variant*	**without** a rare *PLEC* variant	**Proportion** of individuals with a rare *PLEC* variant	**with** a rare *PLEC* variant	**without a** rare *PLEC* variant	**Proportion** of individuals with a rare *PLEC* variant	Case Excess	Odds ratio	CI lower	CI upper	Chi-Square
40	319	0.11	4754	21433	0.18	-0.07	0.55	0.39	0.77	0.0002

*ExAC Eu cohort. ARVC = Arrhythmogenic right ventricular cardiomyopathy, CI = Confidence Interval, *PLEC* = plectin

of the frequency of unique variants in the European general population would then be approximately 2% (500/26197).

To evaluate the possibility that having a rare *PLEC* variant might negatively modify disease expression, we compared the clinical characteristics of patients who carried an ARVC-associated variant to those of patients who carried an ARVC-associated variant and a rare *PLEC* variant. However, the only difference we found was that patients with an ARVC-associated variant and a rare *PLEC* variant were older at first presentation/evaluation compared to patients carrying only an ARVC-associated variant (**S3 Table**). There were no differences in other markers of clinical severity, suggesting that the presence of a *PLEC* variant was unlikely to be a negative modifier of disease expression.

Nonrandom mutation cluster analysis

Of the 47 rare *PLEC* variants, 43 were distinct and four variants were found twice. Mapping these 43 rare variants along the protein sequence and running the non-random mutation cluster analysis with Bonferroni correction revealed no cluster formation at P value <0.1.

Immunofluorescence analysis

Analysis of immunostained myocardial tissue

Immunostained samples demonstrated predominant junctional N-cadherin localization in myocardium, in both the *PLEC* negative (group 1) and *PLEC* positive (group 2) ARVC patients (*PLEC* negative TFC+ patients, diagnostic score 3.3 ± 0.2 [normal]; *PLEC* positive TFC+ patients, diagnostic score 2.9 ± 0.4[normal]). *PLEC* negative TFC+ patients displayed robust junctional localization for plectin (diagnostic score 3.3 ± 0.2 [normal]), whereas *PLEC* positive TFC+ patients displayed aberrant junctional plectin localization (diagnostic score 0.7 ± 0.1 [abnormal], **Fig 1A** and **1B**).

Inter-observer reliability analysis (%Agreement) among observers demonstrated an overall inter-observer agreement of 91.7 ± 4.7% and 86.7 ± 9.1% for ARVC TFC+ patients without and with a *PLEC* variant, respectively, for normal N-cadherin junctional localization (diagnostic scores ranging from 2–4). Observers demonstrated an overall inter-observer agreement of 87.5 ± 5.2% for normal plectin junctional localization (diagnostic scores of 2–4) for ARVC patients without an additional rare *PLEC* variant. Whereas, inter-observer agreement was 100.0 ± 0.0% for abnormal junctional plectin localization (diagnostic scores of 0–1) in myocardium for ARVC patients carrying an additional *PLEC* variant (**Fig 1C, S4 Table**), indicating that the observation of abnormal localization is not due to variation between the observers.

DISCUSSION

Plectin is a cytolinker protein thought to link the cytoskeleton to the cardiac desmosome. Since the desmosomes play a key role in the pathophysiology of ARVC, we hypothesized that ARVC patients might carry genetic *PLEC* variants that contribute to susceptibility to ARVC. Based on our findings of similar frequency and location of rare *PLEC* variants in both patients and controls and no striking difference in phenotypes in patients with and without *PLEC* variants, we conclude that *PLEC* variants do not play a major role in ARVC pathogenesis. Our immunofluorescence study, however, appeared to show different plectin localization in cases with *PLEC* variants, which may suggest that some variants have an effect in the heart on a molecular level.

Rare variant frequency in patients and controls

We identified 47 rare or novel heterozygous *PLEC* variants in isoform 1 in TFC+ ARVC patients, and the majority of these were missense variants. Forty (11%) of all patients carried a rare protein-altering variant. However, based on the frequency of rare variants in controls (ExAC Eu), it appears that many individuals in the control population also carry a rare variant (~18%). In the context of these population data, it does not seem likely that *PLEC* plays a major role in ARVC pathophysiology. This is further underscored by the fact that our cluster analysis did not reveal any regions with rare variants that cluster in patients. We also saw no differences in the predicted impact of protein alterations between variants identified in patients or in controls, as comparable percentages of variants with CADD scores >20 were found in both. This is consistent with recent work by Walsh et al showing that some cardiomyopathy-related genes (*MYBPC3*, *MYH6*, and *SCN5A*) show little or no excess burden of variants in DCM patients.[24] This suggests that most variants in these genes are not associated with DCM, although we know that some specific variants are definitely involved.[24] We therefore cannot exclude that some identified *PLEC* variants may have an effect on the development of ARVC or another type of cardiomyopathy. This was, for example, suggested in a patient with skin blistering and DCM, who was compound heterozygous for a truncating and missense variant in *PLEC*.[14] As skin blistering in these patients is generally related to homozygous or compound heterozygous *PLEC* variants, this suggests that in this case the missense variant does play a role and contributes to the phenotype. Moreover, a missense variant in exon 31 of *PLEC* has been described to cause an autosomal dominant form of skin disease, EBS-Ogna,[25] which manifests exclusively as skin fragility. This missense variant leads to selective proteolysis of isoform 1a, and thus deficiency in this isoform, resulting in reduced levels and dysfunction of hemi-desmosomes.[26] Similar to what occurs in EBS-Ogna, it could be that some other rare *PLEC* missense variants increase the proteolysis of plectin in the heart and thereby diminish the mechanical coupling. Moreover, heterozygous missense

variants were also recently shown to play a role in EBS.[27] Our patients, however, exhibited no striking skin blistering.

Clinical characteristics

The older age at presentation/evaluation and similar occurrence of life-threatening arrhythmias during presentation/evaluation in the cohort of patients who carry an ARVC-associated variant and a rare *PLEC* variant compared to that of the cohort of patients carrying only an ARVC-associated variant does also not support a major role for *PLEC* as a negative disease modifier in ARVC. In fact, a possible protective effect of carrying a rare *PLEC* variant cannot yet be ruled out.

Immunofluorescence: evidence for mechanistic effects

In comparison to our other results, our immunofluorescence analysis showed abnormal localization of plectin in hearts of patients with a *PLEC* variant. As localization of plectin was normal in ARVC patients with or without a desmosomal likely pathogenic/pathogenic variant (*PLEC*-variant negative), abnormal localization of this protein is not a general phenomenon in ARVC patients. Moreover, the fact that the *PKP2* pathogenic variants, c.2146-1G>C and c.148_151delACAG, p.Thr50Serfs*61, were present in both ARVC subgroups (those with and without a rare *PLEC* variant) studied with immunohistochemistry suggests that abnormal plectin localization is also not specifically related to desmosomal variants. In a previous report on EBS patients carrying heterozygous missense *PLEC* variants, immunofluorescence analyses of skin biopsies also showed a decreased signal for plectin.[27] Taken together, these results suggest that the presence of rare *PLEC* variants is accountable for abnormal plectin expression and localization, although this mislocalization may not be sufficient to directly cause ARVC.

In the skin, plectin is a crucial component of the hemidesmosomes (HDs), and HDs connect the epidermis to the extracellular matrix.[9] Plectin insufficiency in the skin results in reduced number of HDs and skin fragility.[28] In the cardiomyocyte, the binding partners of plectin are less well known. *In vivo* studies have shown that plectin is closely associated with desmosomes, while *in vitro* analyses have shown that plectin molecules are biochemically connected to desmoplakin.[29] In addition, the loss of co-localization of plectin and desmin at the Z-discs and intercalated disc, together with desmin and plectin aggregate formation, observed in the heart of the patient with EBS-MD and DCM indicates that plectin plays an important role in the structural organization of the desmin network and in providing mechanical support.[14] We know that plectin contains a number of plakin repeat domains, typical of desmosomal proteins and responsible for binding intermediate filaments, as well as a highly variable N-terminal actin-binding domain.[30] By binding both intermediate filaments and myofilaments, it is possible that plectin attracts the structurally robust desmosomes around the more fragile

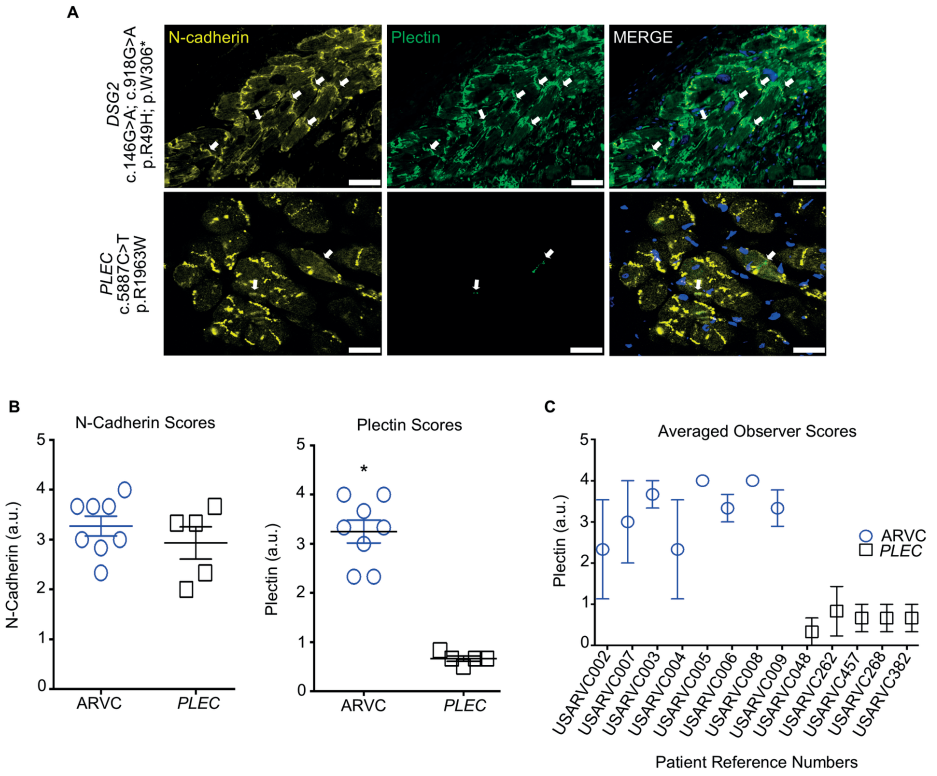

Fig 1. Immunohistochemical analysis of myocardium from TFC+ patients with and without a *PLEC* variant. (A) Representative images of immunostained myocardium from patients from group 1 (n = 8) and group 2 (n = 5) (see also **S7 Table**). Of note, there is a reduced junctional signal for plectin (white arrows) in myocardium from patients with a *PLEC* variant compared to patients without a *PLEC* variant, even though there is a normal junctional signal for N-cadherin between groups. Scale bar, 25μm. (B) N-cadherin and plectin immunostain scores between groups (n = 3 individual scores were recorded per data point per patient by 3 independent observers). (C) Each patient's averaged observer scores for plectin. Averaged scores presented as mean±SEM. * P<0.0005 deemed significant for plectin-immunostained myocardium from group 1 versus group 2 using 2-tailed unpaired t-test with equal variances.

adherens junctions at the cardiac intercalated disks and thus provides the continuity between myofibrils of neighbouring cardiomyocytes. The abnormal localization of plectin seen in our immunofluorescence analysis could reflect the decrease of its interjunctional linking capacity, which could mean the adherens junctions are less supported by desmosomes and thus more sensitive to stress and damage. One can theorize that this could be a factor that lowers the threshold for developing ARVC. However it is unlikely that these changes on their own are responsible for the development of ARVC, since some of the variants we analysed with immunofluorescence also occurred relatively frequently in the general population (**S7 Table**), and two occurred even in homozygous state. This, however, does not mean these variants do not have an effect. A heterozygous missense variant that is found relatively often

in the European population (MAF = 0.081%) demonstrated a decreased signal for plectin in the skin of a patient with EBS.[27] Additionally, the *PLEC* missense variant (c.1298G>A) described in the EBS-MD patient with DCM has a relatively high MAF (1.9% in ExAC Eu).[14] This suggests that, in combination with another variant, even a relatively common *PLEC* variant could function as a genetic modifier. In line with this, a recent genome-wide association study identified a missense variant in *PLEC* (MAF 1.2% in Iceland) associated with atrial fibrillation, albeit with a small effect size.[31] However, in order to investigate a putative risk factor/modifier role of *PLEC* variants in ARVC, a much larger sample size is needed. Thus, while the presence of *PLEC* variants in the general population suggest it is not a major player in the pathogenesis of ARVC, it seems that a small sub-set of variants do appear have a mechanistic role that may be additive. Future research on ARVC should keep this in mind when looking at cases with a *PLEC* variant.

CONCLUSION

In a large cohort of patients with ARVC, we could not confirm a major role for *PLEC*, a promising candidate gene due to its cytolinking connection to the cardiac desmosome. Some *PLEC* variants, however, are associated with plectin mislocalization, and the association of these changes with ARVC requires further investigation.

LIMITATIONS

We used the ExAC database as a control cohort. However, the ExAC database contains aggregated data, which means it is not possible to evaluate data at an individual level. The frequency of rare variants in *PLEC* that we estimated from ExAC is therefore an approximation based on the assumption that each rare variant is carried by only one individual. This likely an overestimation as we found 88% of our ARVC cohort carried one rare variant while 12% carried more than one rare variant (4 patients carried two variants and 1 patient carried four). However, if we make an estimation assuming that in the ExAC cohort 50% of the individuals carry one rare variant, 25% carry two rare variants and 25% carry three rare variants, the proportion of individuals in ExAC Eu with rare variants would still be 13% (compared to 11% in the ARVC cohort).

The proportion of individuals in the Dutch ARVC cohort with a rare *PLEC* variant is significantly higher compared to the US and UK cohort. This is likely due to the fact that the ExAC Eu cohort for a significant proportion consists of data of individuals from the US and UK. Identified *PLEC* variants in the UK and US ARVC cohorts are therefore more

likely to be filtered out, resulting in a lower proportion of these individuals with a rare *PLEC* variant compared to Dutch individuals. Although we also used the Genome of the Netherlands database (n = ~500 individuals) to filter out common variants specific for the Dutch population, the relative small size of this database is likely to lead to an overestimation of rare *PLEC* variants in the Dutch ARVC cohort compared to the US and UK cohort.

As also mentioned above, absence of enrichment of rare variants in patients versus controls does not exclude the possibility that some specific variants may have an effect. To evaluate a possible effect one should assess each variant separately. This would, however, require a high workload of segregation analyses and functional studies in which the chance of finding a significant result would still be low. The immunofluorescence results however are interesting to follow-up. The limitations of these results currently cannot distinguish between abnormal localization due to a general underlying mechanism or abnormal localization due to the specific *PLEC* variant (or combination of variants). To truly obtain more insight we would have to analyse healthy hearts of carriers with rare *PLEC* variants, samples which are not available to us.

MATERIALS AND METHODS

Patients

Dutch (NL) cohort

Seventy-nine patients were included of whom 75 were diagnosed with definite ARVC according to the revised 2010 Task Force Criteria (TFC+) for ARVC.[32] In three patients, a borderline diagnosis of ARVC was made. A biopsy from another patient showed fibrosis and adipocyte accumulation with atrophy of the myocytes fitting the diagnosis of ARVC (major TFC criterion). The majority of patients were previously screened for variants in desmosomal genes. Some individuals were not screened for all desmosomal genes, either because of the identification of a pathogenic variant in an ARVC-associated gene or because family members with ARVC were shown to be variant-negative for those genes. For a detailed description of the number of patients analysed for the different desmosomal genes see **S5 Table**. In 42 patients (53%) a likely pathogenic or pathogenic variant associated with ARVC was identified, classified following the guidelines from the American College of Medical Genetics[33]: 29 in *PKP2*, 10 in *PLN*, two in *DSG2*, and one in *SCN5A*.

United Kingdom (UK) cohort

The British cohort included 84 TFC+ patients. Forty-six patients (55%) carried a likely pathogenic or pathogenic variant associated with ARVC: 26 in *PKP2*, nine in *DSP*, eight in *DSG2*, one in *DSC2*, one in *LMNA*, and one digenic in *DSC2* and *SCN5A*.

United States (US) cohort

In total 196 TFC+ US patients were included. Ninety-eight patients (50%) carried a pathogenic or likely pathogenic desmosomal variant: 76 in *PKP2*, five in *DSP*, seven in *DSG2* (three compound heterozygous), three in *DSC2* (one compound heterozygous), two in *PLN*, two in *SCN5A*, one in *TMEM43*, and two digenic (one in *SCN5A* and *LMNA* and one in *PKP2* and *DSG2*).

ExAC European cohort (ExAC Eu)

Genetic variants of *PLEC* (transcript ID ENST00000322810.8, RefSeq NM_201380) were downloaded from the ExAC database (http:// exac.broadinstitute.org, version 0.3.1). Since our cohort mainly consisted of patients of European descent (**S6 Table**), we used the genetic data from individuals of European descent (non-Finnish) in the ExAC cohort (ExAC Eu).

Genetic analysis

Genomic DNA was isolated from blood samples using standardized procedures. Written informed consent was obtained from all participants following local medical ethics committee guidelines. The study was approved by the relevant National Research Ethics Service (NRES) committee of the NHS Health Research Authority, the Johns Hopkins School of Medicine Institutional Review Board, and the METc boards of the University Medial Centers of Groningen, Utrecht, and Amsterdam. Our study and all experiments conformed with the principles of the Declaration of Helsinki. In 66 Dutch patients, *PLEC* was analysed by Sanger sequencing. Primers for PCR amplification of the coding regions of the *PLEC* gene were designed to encompass the coding exons as well as adjacent intronic sequences as described previously.[27] Amplifications were conducted following a standard PCR protocol and PCR products were confirmed by direct Sanger sequencing. The remainder of the Dutch patients, the UK and the US patients were all screened with targeted gene panel sequencing (*PLEC* included—list of genes included in panels available on request) or by whole exome sequencing with results then confirmed via Sanger sequencing.

Data analysis

Variant analysis: ARVC cohorts

Chromosomal positions of variants of the *PLEC* gene identified in the three ARVC cohorts (NL, UK and US) were annotated with information from the ExAC database using an in-house developed script and frequency information on these variants were collected. Variant annotation is according to the NM_201380 isoform unless otherwise indicated. All variants found in the ARVC cohorts with a MAF <0.001 in the ExAC Eu dataset were included for further analysis. These included missense, in-frame insertions/ deletions, frame-shift, nonsense variants, and variants affecting the consensus RNA splice donor and acceptor sites (first and last two bases of each intron). Additionally *PLEC* variants identified in the Dutch cohort

with a MAF <0.001 in the Genome of the Netherlands database (http://www.nlgenome.nl/) were excluded. The NL, UK, and US ARVC cohorts were combined and analysed as one cohort (ARVC cohort, n = 359).

Variant analysis: ExAC Eu

Only high-quality (Pass filter) *PLEC* variants found in the ExAC Eu subpopulation were used. All variants (variant types as indicated above) in the designated canonical transcript (NM_201380) with a MAF <0.001 were included for further analysis.

*Proportion of individuals with a rare **PLEC** variant*

The proportion of individuals with rare *PLEC* variants in the ExAC Eu dataset was calculated by dividing the sum of the adjusted allele count by the mean of the total adjusted alleles divided by two, as previously described.[24] The frequency of carriers of rare variants in the ARVC cohort was calculated by dividing the sum of patients with one or more rare *PLEC* variants by the total number of ARVC patients analysed for *PLEC*.

Comparison of rare variants between ARVC and ExAC Eu

The proportion of carriers of rare variants in the ARVC cohort was compared with that in ExAC Eu. Case excess was defined by subtracting the proportion of individuals in ExAC Eu with a rare variant from the proportion of individuals carrying a rare *PLEC* variant in the ARVC cohort. We calculated the odds ratio (OR) with 95% confidence intervals.

Cluster analysis

To identify putative clustering of rare missense variants in *PLEC*, distinct rare variants were mapped along the protein sequence. Nonrandom mutation cluster, implemented in the iPAC Bioconductor R package, was used to identify clusters of variants in the ARVC cohort.[34]

Immunofluorescence analysis

Cohort selection and patient myocardial samples

Endomyocardial biopsies were obtained from 13 US patients. Group 1 (n = 8) consisted of TFC+ ARVC patients with no *PLEC* variant (five with *PKP2* variants, one with compound heterozygous variants in *DSG2*, and two with no pathogenic/likely pathogenic variant identified). Group 2 included TFC+ ARVC patients who either had a variant in *PLEC* alone (n = 3) or a variant in *PLEC* and a likely pathogenic/pathogenic variant in *PKP2* (n = 2). The specific desmosomal variants and *PLEC* variants are documented in **S7 Table**.

Immunostaining myocardial samples

Patient myocardial samples were formalin-fixed, paraffin-embedded, cut at a 5μm thickness and mounted on clear, plus microscope slides.

Slides were deparaffinized, rehydrated, underwent antigen retrieval, then blocked at room temperature for one hour as previously described.[35] Slides were incubated overnight at 4°C with mouse anti-N-cadherin (Santa Cruz, sc-59987; 1:500) or rabbit anti-Plectin (Cell Signalling, cs-D6A11; 1:400). The following day, slides were washed and incubated with secondary antibodies (donkey anti-mouse Alexa Fluor-647 [Invitrogen, A31571; 1:500] and goat anti- rabbit Alexa Fluor-488 [Invitrogen, A11070; 1:500]), washed and cover-slipped with mounting media (Fluoroshield with DAPI, Sigma F6057). Immunoreactive signal was visualized using a Leica TCS SPE RGBV confocal microscope (Leica Microsystems) at 40X magnification. Slides were coded and analysed in a blinded fashion by three independent observers.

Analysis of immunostained myocardial samples

Slides were imaged, coded, and distributed to three independent, blinded observers and graded as previously described.[35,36]

Observers were requested to score samples into one of five diagnostic classes: (0) no myocyte junction plectin staining; (1) rare junction, predominantly cytoplasmic plectin staining; (2) even mix of junction and cytoplasmic plectin staining (odds 50:50); (3) mildly reduced junction, rare cytoplasmic plectin staining; or (4) robust plectin staining, only at cell-cell junctions. Observers were additionally requested to classify patient samples for junctional distribution of N-cadherin using the five diagnostic classes described above. All three observer scores were recorded and averaged for each individual patient (n = 3 different observer scores/patient/ immunostain), then averaged by immunohistochemical stain and by group. In addition, observer scores were compared between observers and percent agreement was determined, as described previously.[37]

Statistical Analysis

Data are presented as mean ± SEM, and a P value <0.05 was considered significant. Associations between continuous dependent variables were analysed using 2-tailed t-test (binary independent variables) or 2-way ANOVA (2 or more variables). Fisher's exact test was used for comparing frequencies.

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SUPPLEMENTAL DATA

S1 Table. Rare or novel *PLEC* variants identified in the ARVC cohort (n=359).

S2 Table. Rare *PLEC* variants identified in the ExAC Eu cohort.

S3 Table. *PLEC* + ARVC-associated variant versus only ARVC-associated variant.

S4 Table. Interobserver analysis of immunostained myocardium and percent agreement between observers.

S5 Table. Dutch cohort (n=79): genes analysed.

S6 Table. ARVC cohort (n=359): ethnicities.

S7 Table. Information regarding the variant status of the patients in which immunofluorescence stainings on heart tissue were performed.

S1 Table. Rare or novel *PLEC* variants identified in the ARVC cohort (n=359).
See attachment 'Chapter7_S1_Table.xlsx'
Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhIw2vpMys4ctE7JkMw?e=R8otcU

S2 Table. Rare *PLEC* variants identified in the ExAC Eu cohort.
See attachment 'Chapter7_S2_Table.xlsx'
Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhIw4fx3fNkogbyhjAw?e=O40Za0

S3 Table. *PLEC* + ARVC-associated variant versus only ARVC-associated variant.

	PLEC + ARVC mutation	Only ARVC mutation	P-value
	n=26	n=159	
Probands	77% (20/26)	90% (143/159)	0,09
Male	65% (13/20)	53% (64/120)	0,23
Age first presentation / evaluation (years)	41 ± 13	34 ± 14 (n=158)	0,02
Symptomatic at presentation	77% (20/26)	90% (143/159)	0,09
malignant ventricular event	42% (11/26)	44% (70/159)	1,00

ARVC = Arrhythmogenic right ventricular cardiomyopathy, *PLEC* = plectin, Proband = the person through whom the family is ascertained

S4 Table. Interobserver analysis of immunostained myocardium and percent agreement between observers.

	ARVC, TFC+ Patients (n = 8; Group 1)		*PLEC* Patients (n = 5; Group 2)	
Individual Observer	Individual Scores	Averaged Score	Individual Scores	Averaged Score
OB1	3.6 ± 0.2		3.0 ± 0.5	
OB2	3.8 ± 0.1	3.3 ± 0.2	3.5 ± 0.3	2.9 ± 0.4
OB3	2.5 ± 0.4		2.3 ± 0.7	
N-cadherin				
Between Observers	% Agreement	Averaged Score	% Agreement	Averaged Score
OB1 & OB2	100.0 ± 0.0%		100.0 ± 0.0%	
OB1 & OB3	87.5 ± 6.8%	91.7 ± 4.7%	80.0 ± 13.7%	86.7 ± 9.1%
OB2 & OB3	87.5 ± 6.8%		80.0 ± 13.7%	
Individual Observer	Individual Scores	Averaged Score	Individual Scores	Averaged Score
OB1	3.6 ± 0.2***		0.9 ± 0.1	
OB2	3.4 ± 0.4**	3.3 ± 0.2‡	1.3 ± 0.3	0.7 ± 0.1
OB3	2.7 ± 0.5**		0.0 ± 0.0	
Plectin				
Between Observers	% Agreement	Averaged Score	% Agreement	Averaged Score
OB1 & OB2	93.8 ± 5.3%		100.0 ± 0.0%	
OB1 & OB3	87.5 ± 7.0%	87.5 ± 5.2%	100.0 ± 0.0%	100.0 ± 0.0%
OB2 & OB3	81.3 ± 7.8%		100.0 ± 0.0%	

Data presented as mean±SEM, n-values inset. ***P<0.0005, **P<0.005, *P<0.05 deemed significant for individual observer scored plectin-immunostained myocardium from ARVC, Group 1 versus Group 2. ‡P<0.0005 deemed significant for averaged inter-observer scored plectin-immunostained myocardium from ARVC, Group 1 versus Group 2. Individual observer and averaged inter-observer scores were analyzed via 2-way ANOVA with Sidak's multiple comparison and 2-tailed unpaired t-test with equal variances, respectively. OB1-3, Observers 1-3

S5 Table. Dutch cohort (n=79): genes analysed.

Plakophilin-2	Desmocollin-2	Desmoglein-2	Desmoplakin	Plakoglobin	Phospholamban
76/79 (1)	76/79 (2)	77/79 (3)	70/79 (4)	71/79 (5)	74/79 (6)
Plakophilin-2	Desmocollin-2	Desmoglein-2	Desmoplakin	Plakoglobin	Phospholamban
76/79 (1)	76/79 (2)	77/79 (3)	70/79 (4)	71/79 (5)	74/79 (6)

(1) Three patients not analyzed for plakophilin-2 (*PKP2)*. One patient, not tested for *PKP2*, had a brother with ARVC who was tested genotype negative for *PKP2*. Another
patient carried the pathogenic *PLN* p.(Arg14del).
(2) Three patients not analyzed for desmocollin-2 *(DSC2)*. One patient had a pathogenic *PKP2* variant and a sister with ARVC, who tested negative for *DSC2*. One patient had a brother with ARVC who tested negative for *DSC2*.
(3) Two patients not analyzed for desmoglein-2 *(DSG2)*. One patient had a pathogenic *PKP2* variant and a sister with ARVC, who tested negative for *DSG2*.
(4) Nine patients not analyzed for desmoplakin *(DSP)*. One patient had a pathogenic PKP2 variant and a sister with ARVC, who tested negative for DSP. In five patient already a pathogenic variant was found in PKP2 or PLN.
(5) Eight patients not analyzed for plakoglobin *(JUP)*. One patient had a pathogenic PKP2 variant and a sister with ARVC, who tested negative for JUP. In four patient already a pathogenic variant was found in PKP2 or PLN.
(6) Five patients not screened for phospholamban *(PLN)*. One patient had a pathogenic PKP2 variant and a sister with ARVC, who tested negative for PLN.

S6 Table. ARVC cohort (n=359): ethnicities.

Ethnicity	Number of subject
Caucasian	346 (96.4%)
African-American	5 (1.4%)
Hispanic	4 (1.1%)
Asian	4 (1.1%)

ARVC = arrthythmogenic right ventricular cardiomyopathy

S7 Table. Information regarding the variant status of the patients in which immunofluorescence stainings on heart tissue were performed.
See attachment 'Chapter7_S7_Table.xlsx'
Or visit the following webpage to open the table: https://1drv.ms/x/s!AhacIYasuBuZhIw34uHXlh6nwNd7rg?e=LK66LV

Summary and Future Perspectives

SUMMARY

This thesis focuses on the genetic basis of arrhythmogenic cardiomyopathy (ACM). A brief history of the evolving concepts and classifications of cardiomyopathies is given in the General introduction, and in more depth regarding ACM in **Chapter 1**. This chapter also provides an overview of the pathological features, genetic basis and mechanisms underlying ACM. The hallmark of ACM is fibrofatty replacement of the myocardium in the ventricles. Although previously used, the term ACM has recently been defined in more detail in an expert consensus statement.[1] Under this umbrella term different subforms are being recognized. One could classify these subforms according to the predominantly involved ventricle, while -by definition- not excluding involvement of the other ventricle. In case of predominant right ventricular involvement one could suggest to classify it as right-dominant ACM (currently often described as ARVC, arrhythmogenic right ventricular cardiomyopathy), left-dominant ACM (also referred to as ALVC, arrhythmogenic left ventricular cardiomyopathy) for the cases in which the left ventricle appears to be most affected or biventricular ACM in individuals where there appears to be a more or less equal involvement of both the right and the left ventricle. ACM is often a genetic disease. In more than half of the cases a genetic variant associated with disease is identified. Those genetic variants mostly occur in the genes encoding for proteins which are part of the cardiac desmosome. This molecular structure plays a role in the electromechanical coupling of the cardiomyocytes. Disease causing variants have been shown to lead to a pleiotropy of effects, for example desmosomal disorganization and reduced cell adhesion between cardiomyocytes, abnormal calcium handling and aberrant nuclear signalling pathways. Increased understanding of these abnormal mechanisms has led to the discovery of a possible pharmacological treatment which interacts on specific pathways involved in ACM, like SB216763, a GSK3β inhibitor described by Asimaki et al.[2] New technologies, like modelling the disease with human cardiomyocytes derived from pluripotent stem cells from ACM patients and thereby capturing the patient's exact genetic makeup, enable unprecedented opportunities to study the disease in a more personalized manner and can even be used as a possible platform for drug testing on a patient-specific level.

Part 1 of this thesis is focused on the interpretation and characterization of genetic variants in ACM. In **Chapter 2** the challenges of variant interpretation are illustrated with respect to a specific genetic variant in the *LMNA* gene. New DNA-sequencing technologies allow for a multitude of genes being screened at the same time in patient care. Consequently, many genetic variants are identified. One of the current challenges is to correctly classify these variants. This is especially challenging for missense variants leading to a substitution of only a single amino acid (nonsynonymous substitutions), whereas proteins are often composed of hundreds to thousands of amino acids. Moreover, such variants are also frequently identified

in healthy subjects. One of the arguments for pathogenicity of variants is being absent or present at a very low frequency in a control population. In addition, several software tools are available that predict if such substitution influences the protein structure and function. Also, clinical data combined with genetic data can provide evidence of segregation of the variant with the disease and subsequently help in differentiating between harmless and harmful variants. Finally, functional assays can demonstrate a potential detrimental effect of a genetic variant. In general, a single argument by itself is insufficient to classify such variants as being pathogenic. Sometimes there are ambiguous results, which further complicates classification. We encountered this for the *LMNA* c.992G>A (p.(Arg331Gln)) variant, that was relatively frequently found in controls. By combining the results of various lines of evidence (including co-segregation and functional evidence) we were able to classify this genetic variant as pathogenic. The clinical phenotype is characterized by a relatively mild and late onset disease compared to that of carriers of other pathogenic *LMNA* variants in general. In more than half of the probands an additional variant of unknown significance was identified in genes implicated in the pathophysiology of dilated cardiomyopathy. It could therefore be that these additional variants ("genetic modifiers") play a role in the highly variable disease expression of this specific *LMNA* genetic variant. Indeed, in one family the combination of the *LMNA* variant with a likely pathogenic variant in the sodium voltage-gated channel alpha subunit 5 (*SCN5A*) gene was likely to contribute to the severity of the phenotype of the respective proband. Finally, our experimental results demonstrated that the decreased force development in cardiomyocytes is secondary to remodelling and not due to a direct effect on the contractile apparatus, which may hold true for pathogenic *LMNA* variants in general.

In **Chapter 3** we studied a *TTN* gene variant which was predicted to lead to a truncated form of the protein and evaluated its associated clinical phenotype. These kinds of variants are generally considered more radical with respect to their effect on the function of the protein than nonsynonymous variants, like the *LMNA* variant described in Chapter 2. However, titin is the human largest protein and truncating variants in *TTN* (*TTN*tv) occur at relatively high frequency in controls indicating that not all *TTN*tv are disease causing or leading to disease by itself. By haplotype and segregation analyses we collected additional evidence for the *TTN* c.59926+1G>A variant to be classified as pathogenic. We showed that atrial arrhythmias, especially (paroxysmal) atrial fibrillation, are an important feature of the phenotypic spectrum of *TTN*tv and occur even in the absence of gross cardiac structural abnormalities and risk factors for atrial fibrillation. A male predominance in the group of carriers with dilated cardiomyopathy was also observed and additional stressors were identified in six carriers, i.e., excess alcohol use, suspected myocarditis, anthracycline therapy, and inheritance of an additional pathogenic genetic variant in *SCN5A*. The male predominance and the identified additional stressors (including another pathogenic genetic variant) are

in support of a multiple-hit model in *TTN*tv cardiomyopathy, where an additional hit or possible more hits trigger the development of overt disease.

In **Chapter 4** we described the phenotype of the largest series of patients with truncating *DSP* variants (*DSP*tv). Like other gene-specific cardiomyopathies, there is incomplete penetrance and a wide spectrum of disease manifestations. The cardiac spectrum is characterized by a left-dominant or biventricular form of ACM. There was an enrichment of truncating variants in the nonsense mediated decay (NMD) competent regions of the gene compared to controls. Additionally, truncating variants in this region also appeared to be a lifetime risk factor for the development of malignant ventricular arrhythmias, which may be used in future risk stratification models predicting life threatening events in individual patients. The location of these type of genetic variants in *DSP* thus appears to be important and identifying variants in the NMD competent regions could be given a stronger level of supportive evidence when considering pathogenicity. In contrast to the situation in the *TTN*tv cohort described in chapter 3, we observed an overrepresentation of females in our *DSP*tv cohort. This observation was also reported in another large *DSP*tv cohort.[3] This could implicate that sex could be a disease modifier in *DSP*tv.

In **Chapter 5,** a database of subjects carrying two or more variants in one of the desmosomal genes was compiled and presented. The database consists of data of carriers identified after having performed a systematic literature search for carriers of multiple desmosomal genetic variants and that of unpublished carriers provided to us by our collaborators. Subsequently, all variants were reclassified according to the most recent guidelines resulting in three different groups consisting of subjects with more than one (likely) pathogenic (LP/P) desmosomal variant, subjects with a single LP/P desmosomal variant, or with no LP/P desmosomal variant. We were able to show that carrying more than one genetic desmosomal LP/P variant is associated with a worse survival and thereby provided strong evidence of a gene-dosage effect in arrhythmogenic cardiomyopathy (ACM). Since this is the largest cohort reported to date and since many of the LP/P variants previously described were now classified as (likely) benign or variant of unknown significance the survival curve depicted in Chapter 5 is currently the most accurate survival curve of carriers of more than one LP/P variant. This is highly relevant for clinical practise to determine treatment and follow up strategies.

Part 2 of this thesis is focused on identifying novel genes in the best-defined subform of ACM, arrhythmogenic right ventricular cardiomyopathy (ARVC). Based on the genetic overlap in the different subtypes of cardiomyopathy we screened the nine most common sarcomere genes in genotype negative ARVC patients in **Chapter 6**. The proportion of ARVC genotype negative patients carrying a rare and possibly pathogenic sarcomere variant was around 4%. However, without data from functional studies, segregation evidence, or both, these

variants should be regarded as non-significant and not be used in counselling ARVC patients and families. In **Chapter 7** we evaluated the occurrence of rare genetic variants in the *PLEC* gene in ARVC patients. Based on the understanding that plectin is responsible for linking the cytoskeleton of the cardiomyocyte to the desmosome, *PLEC* seemed to be a promising candidate gene in ARVC. We compared the proportion of ARVC patients carrying a rare *PLEC* variant to the estimated proportion of *PLEC* variants in the European cohort of the Exome Aggregation Database. In addition, the localization of plectin was analysed in heart tissue by immunofluorescence microscopy in ARVC patients with or without a rare *PLEC* variant. There was no enrichment of rare *PLEC* variants in the ARVC cohort and there were no notable phenotypic differences between ARVC patients, who carried an ARVC associated genetic variant, with and without a *PLEC* variant. Thus, it appears that rare variants in *PLEC* do not play a major role in ARVC. However, our immunofluorescence microscopy results do suggest that some *PLEC* variants cause abnormal plectin localization. This may suggest that some *PLEC* variants have an effect on the molecular level, but for which the relevance to the mechanism of ARVC development has yet to be determined. Together these results point to the direction that both sarcomere genes and *PLEC* do not play a major role in the pathophysiology of ACM, although we cannot exclude that some rare variants do.

FUTURE PERSPECTIVES

The paradigm of one single genetic variant leading to one disease in arrhythmogenic cardiomyopathy is shifting to a more complex model, in which the development of the phenotype not only depends on the mutated gene, but also on additional genetic variants, epigenetics (DNA modification by mechanisms other than changes in the DNA sequence, e.g., silencing of genes by methylation), other internal factors (e.g., hormonal influences), or external factors (e.g., exercise), or a combination of those. To incorporate the expanding knowledge on phenotype and aetiology and facilitate this increasing knowledge the more elaborate MOGE(S) Nomenclature described in the introduction can be used to summarize the phenotype and underlying disease mechanisms.[4] This classification system describes more aspects of the disease, including the aetiology. Knowledge of the aetiology can be used for aetiology-specific treatment. For example, in patients with dilated cardiomyopathy caused by pathogenic *LMNA* variants implantable cardioverter–defibrillator for primary prevention should be considered when having an additional clinical risk factor.[5] With development in new treatment modalities, like small molecule therapies, and gene replacement therapies, disease management will very likely be based on aetiology,[4] as is seen in the treatment of transthyretin amyloid cardiomyopathy patients with Tafamidis.[6] By describing different aspects, extrapolated from standard tests in clinical practice, one can make meaningful summaries of the phenotype, which can perhaps be used for more patient-specific treatments

or facilitate the development of personalized treatment regimes. In addition, the MOGE(S) classification system is flexible and expandible and is therefore very much suitable for future discoveries regarding aetiology and phenotypic manifestations.

Currently new DNA-sequencing techniques allow for vast amounts of genetic data to be collected, shifting the bottleneck in genetic analysis from the acquisition to the correct interpretation of the data. Despite these new technologies and great progress in understanding the genetics there are still ACM families and patients in whom no genetic cause has been identified. This could be due to the fact that the genetic variants lie within genes yet unknown to play a role in ACM, the variants reside outside exonic regions of the DNA which are not captured with targeted NGS panels or whole exome sequencing, our knowledge of identified variants is insufficient to conclude their disease involvement (see also next paragraph), or the disease within the family results from a more complex genotype in which multiple low penetrant genetic variants cause the disease.

Interpretation of variants

The correct interpretation of genetic variants is currently one of the main challenges we are facing. The American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) have provided a framework to systematically assess the likely effect of genetic variants.[7] There are software tools available which combine lines of evidence to classify variants. For example Gene-Aware Variant INterpretation (GAVIN) incorporates population data together with the predicted effect of the variant on gene and protein function.[8] The results of segregation and haplotype analysis provides insight in how to interpret genetic variants, as we have used for the interpretation of the *LMNA* c.992C>T and *TTN* c.59926+1G>A variants. Evidence regarding functional effects is an important part of the ACMG/AMP framework and is indispensable for the correct interpretation of variants. Standardized tools assessing these functional effects would be valuable. A good example is the recent quick and reliable method where nuclear morphology and percentage abnormal nuclei in dermal fibroblasts helped to confirm pathogenicity of *LMNA* variants.[9] Variants of unknown significance (VUS) found in *LMNA* could thus be analysed. Developing such (possibly high throughput) standardized assays also for other genes associated with ACM will greatly enhance interpretation of genetic variants found in these genes. Combining state of the art technologies in a patient-specific manner provides unique ways of supporting functional assessments. By generating cardiomyocytes derived from induced pluripotent stem cells (iPSC) from cardiomyopathy patients carrying a variant of unknown significance (VUS) it has become possible to recapitulate the cardiac phenotype in a patient and disease specific way. Subsequently, with the help of novel genome editing techniques, like clustered regularly interspaced short palindromic repeats/associated nuclease Cas9 (CRISPR/Cas9), it is now possible to examine the respective VUS. By combining the above techniques the opportunity

is now available to study the functional effects of a VUS in the genetic background of the specific patient.[10] Of note, these kind of analyses are costly and laborious. If we want to evaluate all variants of unknown significance found in patients and families high-throughput applications of these kind of analyses are needed.

Interpretation of variants has greatly benefited from the availability of large genetic datasets, which have recently become available. Currently there is the 'The Genome Aggregation Database', which contains more than 1000000 exome sequences and more than 15000 genome sequences. An enrichment of genetic variants in genes in patients provides strong evidence that the gene and such variants are implicated in the disease. Conversely, many genes previously thought to be involved in various cardiomyopathies are in fact not so convincingly associated as we previously believed, as the allele frequencies of variants in those genes are now shown to be comparable between patients and controls.[11] Other possibilities to explore in arrhythmogenic cardiomyopathy (ACM), when such large patients cohorts become available, is whether specific regions in genes could be identified in which disease associated variants cluster, as was shown to be the case in six hypertrophic cardiomyopathy (HCM) genes (*MYH7z, MYBPC3, TNNI3, TNNT2, MYL3* and *CSRP3*).[12] This type of clustering is also seen in the *TTN* gene in dilated cardiomyopathy (DCM)[13] and in Chapter 5 we demonstrated this for *DSP* in ACM. This kind of analysis can be helpful in the interpretation of variants by giving extra weight of evidence of pathogenicity if variants occur in such "hot spot" regions.

The electronic medical records of patients are of great value for the evaluation and analysis of segregation data and clinical phenotypes. Segregation data and clinical data regarding the phenotype is present in the electronic medical record, however it is not always readily accessible. From an organizational perspective much time and effort could be spared if systematically collecting and analysing these data would be easier and when this data could be interchanged between hospitals. A nationwide working group, consisting of medical specialists, patients group members, jurists, ethicists, bioinformaticians, researchers, statisticians, and government employees would be required to provide a feasible framework to achieve this. Take for instance the health care systems in the Nordic countries. These are mostly governmentally funded and are characterized by a combination of record-keeping and linking records on individual level. This has led to inter-connected longitudinal population-based registries with high quality data and subsequently powerful database systems, where especially Denmark is renowned for. In Denmark there are more than 200 databases in which almost every aspect of life is collected. The feature that makes these databases a unique and powerful research tool is the Central Personal Register (CPR) number, which is given to each Danish citizen at birth or upon immigration, and links all these databases together, thereby making population sized cohorts, which are readily accessible and contain high quality detailed

phenotypic data.[14] Easy accessibility of such databases would lead to the availability of high quality clinical and genetical data, which can be used for segregation analysis. One could even imagine that build-in (semi) automated pedigree analysis tools would facilitate the interpretation even more. Additionally, such databases would be informative regarding the frequency data of genetic variants in the general population and specific patient groups nationwide, which can aid interpretation.

Identification of modifiers

The above-described large population datasets enable researchers to identify factors playing a role in disease, including complex diseases. Finding such factors is challenging since their relative contribution to the disease expression is often small. The utilization of the enormous Danish datasets has however led to the identification of such factors. For example, it has thus been demonstrated that certain common genetic variants as well as specific infections are associated with certain complex psychiatric diseases.[15,16] The identification of modifiers in ACM, and many other diseases, would greatly benefit from such interwoven data infrastructure like in Denmark, since the identification of modifiers greatly depends on the detailed characterization of the phenotype and large patient data sets.

Arrhythmogenic cardiomyopathy (ACM) is a form of "cardiomyopathy" and is thus by definition not solely explained by ischemia or hypertension.[1] However ischemic heart disease and hypertension are major risk factors for heart muscle disease leading to heart failure and they have a high prevalence in the general population (4.5% and 31.4% in the Netherlands, respectively).[17-19] In addition, life-threatening arrhythmias occur most frequently in the setting of acute myocardial ischemia due to coronary artery disease.[20] One could imagine that these relatively common cardiovascular comorbidities, which are the leading risk factors for heart muscle disease, may also modify disease expression in ACM (**Figure 1A**). One could conceive that a patient carrying a genetic variant associated with ACM is also more susceptible to developing life threatening arrhythmia because of myocardial ischemia (**Figure 1B**) due to coronary artery disease or is more prone to developing cardiac dysfunction due to hypertension (**Figure 1C**). This concept is supported by the observation that rare genetic variants associated with primary electrical heart disease or familial cardiomyopathy were more prevalent in a sudden cardiac death cohort. In half of the older patients (50 years or older) carrying such a genetic variant sudden cardiac death occurred in the setting of acute myocardial infarction.[21] Vice versa some genetic variants in genes associated with cardiomyopathy are also associated with atherosclerosis.[22] Taken together, these observations implicate that one cause does not exclude the other and that relatively common cardiovascular comorbidity could explain part of the variability in disease expression in ACM. In addition, some genetic variants may also lead to cardiovascular disease other than primary heart muscle disease (e.g., atherosclerosis), which subsequently can lead to heart muscle

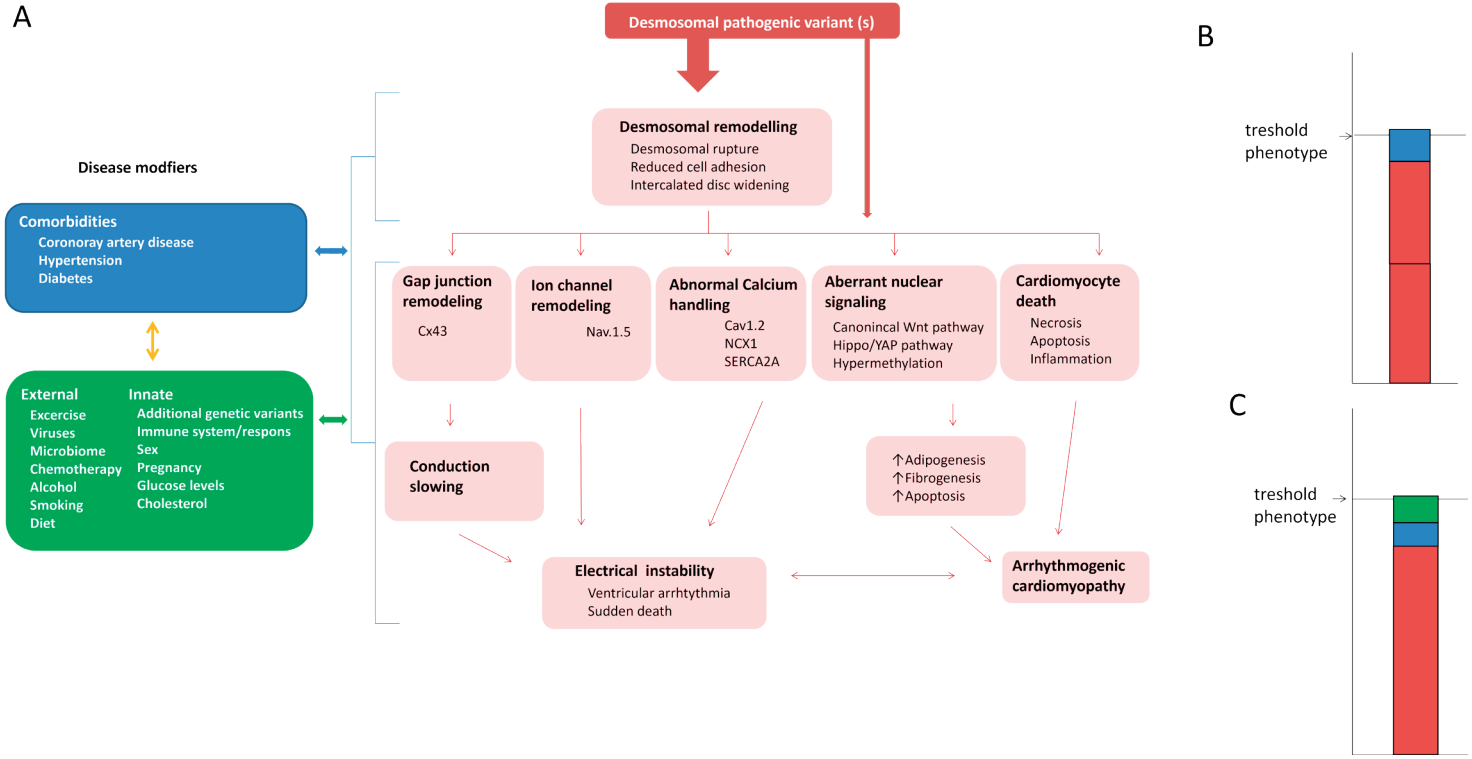

Figure 1. (A) Proposed cellular and molecular cascades underlying arrhythmogenic cardiomyopathy and the complex underlying aetiology. In autosomal dominant families with variable expression and age-related penetrance a genetic variant with a large effect contributes to much of the disease expression. One or more disease modifiers however are likely needed to reach the disease threshold. **(B)** schematic representation of a hypothetical carrier of two rare desmosomal variants with intermediate effect size (two red blocks) and coronary artery disease (blue). **(C)** schematic representation of a hypothetical carrier of a very rare desmosomal pathogenic variant (red block), which is expected to have a large effect, but also has mild form of hypertension (blue block) and carries a common genetic variant (green block) with a small effect on the disease expression. On the basis of figure 2 from Basso et al.[23]

disease. A detailed characterization of the blood pressure or presence of coronary artery disease, assuming that clinical work-up is complete, should be present in the electronic medical record. The above-mentioned data infrastructure ideally would facilitate access to such data, providing large cohorts and therefore statistical power to study such relatively common factors and their possible role in ACM. Blood pressure and data regarding coronary artery disease are just two examples and this could be extrapolated to other factors of interest, e.g., like data on cholesterol and glucose levels. These kinds of databases will be of enormous help in elucidating the complex interaction of genetic predisposition and environment, especially when it is also linked with biobanks.

A great example where the electronic health system is integrated with research activities with the goal to enable precision medicine is MyCode launched by Geisinger Health System from Pennsylvania (United States). It couples the electronic health system to a biobanking program and genealogic databases to investigate the genetic basis of disease and provides a means to evaluate the contribution of lifestyle and environment to the development of disease.[24] A recent example of a success of this design is the demonstration that there are ethnic differences in the association between truncating titin variants (*TTN*tv) in highly expressed exons and dilated cardiomyopathy (DCM). In patients from European descent a strong association with DCM was demonstrated, while their appeared to be no association with this disease in individuals from African descent.[25] Another great example of using large population-based cohorts, combined with biobank data, to better understand factors associated with disease expression is the Lifelines Cohort. The Lifelines Cohort is a large population-based cohort and biobank, in the northern part of the Netherlands consisting of more than 167000 participants from three generations.[26] In this cohort phospholamban *(PLN)* c.40_42delAGA carriers were identified. The *PLN* c.40_42delAGA variant is associated with ACM and is, like the other familial cardiomyopathies, characterized by incomplete penetrance. By evaluating phenotypic and genetic modifiers in *PLN* c.40_42delAGA carriers identified in the Lifelines Cohort, it was shown that a lower QRS duration is associated with the absence of symptoms in carriers.[27] The lifelines cohort is powerful tool, especially when combined with other population-based cohorts and biobanks. Illustrative of this is the participation of the Lifelines Cohort in the COVID-19 Host Genetics Initiative.[28] SARS-CoV-2 infection can lead to highly variable clinical manifestation, ranging from asymptomatic to a severe course of acute respiratory distress syndrome and death. Clinical risk factors associated with disease severity are male sex, age, and pre-existing comorbidities. These factors alone could not explain this highly clinical variability. The COVID-19 Host Genetics Initiative formed a network of international research groups with the aim to investigate the role of human genetics in SARS-CoV-2 infection and disease severity. By combining data of COVID-19 patients and controls from 19 countries they were able to show that some genetic factors were associated with SARS-CoV-2 disease susceptibility and disease severity.[28]

Another interesting new development which could be used for identifying factors implicated in arrhythmogenic cardiomyopathy (ACM) is the personal health record. The concept of the personal health record is to provide a complete and accurate summary of an individual's medical history and is controlled by the patient. New consumer wearables (e.g., smartwatches) can measure heart rate, blood pressure, oxygen saturation of blood, and even record an ECG. These data could all be collected in the personal health record in combination with the amount of exercise (e.g., number of steps, type of sport, time, and distances). These quantitative data could then be used to further elucidate the role of exercise in disease expression. It is important to expand our understanding of the effect of exercise, since the current advice has far-reaching consequences for patient choices regarding the future. The recommendations from the Sport Cardiology Section of the European Association of Preventive Cardiology advises patients with arrhythmogenic right ventricular cardiomyopathy (ARVC) or family members who are genotype positive but phenotype negative and carrier of a pathogenic desmosomal variant to not participate in competitive sports, since such individuals are deemed to more likely develop a phenotype and develop potential life-threatening arrhythmia and heart failure.[29]

In the Netherlands several founder mutations have been identified associated with familial cardiomyopathies, including arrhythmogenic cardiomyopathy (ACM).[30] There are several cohorts of hundreds to thousands of individuals all carrying an identical pathogenic genetic variant. Cohorts consisting of such carriers are relatively homogenous and represent ideal cohorts for the identification of genetic and environmental modifiers. Therefore the population of the Netherlands is very well suited to identify modifiers in ACM, or other genetic cardiac disease.[31] Registries, like the PLN-registry, which currently contains >1000 of individuals with the PLN p.(Arg14del) variant are ideal to investigate genetic and environmental factors modifying disease. Since exercise has been described as a modifier in ACM,[32] a registry like the PLN-registry, can be used to assess the influence of exercise in ACM caused by this specific genetic variant. Future findings in these kinds of studies may make it possible to give specific advice, in this case regarding exercise recommendations, based on the specific genetic variant or gene involved. In the Netherlands the cardiogenetic health care is provided by multidisciplinary teams of cardiologists, pediatric cardiologists, pathologists, clinical geneticists, radiologists, molecular geneticists, and genetic counsellors. Collaboration between centers led to nationwide registries with (gene-specific) cardiomyopathies. Due to the excellent level of patient care and intense collaboration the Netherlands is well suited to identify these modifiers and therefore play a prominent role in the development of a more personalized medicine in cardiogenetic health care.

Discovery of novel genetic regions

We have shown that there are gene dosage effects in arrhythmogenic right ventricular cardiomyopathy (ARVC) for rare (likely) pathogenic desmosomal variants. Recalling **Figure 6** in the introduction of this thesis, these variants are likely to have a large effect. However we know from genome wide association studies that there are common variants, which occur at a relatively high frequency in the general population, which increase the susceptibility for other inherited cardiac disease, like dilated cardiomyopathy.[33] These genetic variants, depicted on the right in **Figure 6** from the introduction of this thesis, are likely to have only a small effect on the disease expression. To detect these relatively small effects large numbers of patients are necessary. Since arrhythmogenic cardiomyopathy (ACM) is a rare disease studies like these were challenging to organize. However due to international collaboration[34] this type of study is becoming within reach and anticipated to yield valuable insight. In the near feature we could expect to see the first results of studies of this kind in ACM. Due to the decreasing cost of whole exome and genome sequencing and due to the availability of large control groups studies evaluating the possible contribution of rare genetic variants with specifically designed statistical methods[35] could be performed. Whole exome or genome sequencing of these cohorts will enable such analysis for all the human genes and allow for the possible detection of novel genes involved in ACM. Such approaches have proven to be successful in the identification of novel candidate genes in other fields.[36] Whole genome sequencing can provide information on most types of nucleotide variants, including variants outside the exons and mitochondrial DNA variants. Similar to hypertrophic cardiomyopathy (HCM), there could be intronic regions in ACM which harbour pathogenic genetic variants or variants in the mitochondrial DNA.[37] These data will also allow for the analysis of promotor and enhancer regions. It has been shown that genetic variants in such regions can control the dosage of mRNA and thus the protein amount of functional protein, which subsequently will influence disease expression. Whole genome sequencing in ACM patients provides an opportunity to evaluate a possible role for almost all type of nucleotide changes in ACM. Moreover, it will also provide the possibility for identifying copy number variants underlying ACM.

Only collaboration and standardized data collection, preferably interwoven with the electronic health record systems, will allow for large enough ACM patients cohorts with extensive characterization of the phenotype necessary for the elucidation of how these genetic and other factors interact to trigger disease, which will be the key to successfully manage ACM patients and their family members.

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Dutch summary | Nederlandse samenvatting

DUTCH SUMMARY | NEDERLANDSE SAMENVATTING

Aritmogene cardiomyopathie (ACM) is, net als de andere erfelijke cardiomyopathieën, een genetische hartspierziekte die zich in de loop van het leven ontwikkelt. De geschiedenis van de definitie cardiomyopathie en de verschillende classificaties worden beschreven in de Introductie. Verder wordt in de Introductie de ziekte ACM, het type cardiomyopathie waar dit proefschrift op gericht is, in meer detail besproken. **Hoofdstuk 1** geeft een overzicht van de pathologische kenmerken, genetische grondslag en onderliggende ziektemechanisme van ACM. Kenmerkend is de vervanging van het hartspierweefsel door vet- en bindweefsel. In eerste instantie werd de ziekte herkend in de rechterkamer van het hart en kreeg het de naam aritmogene rechter ventrikel (kamer) cardiomyopathie (ARVC). Later werd ontdekt dat de linkerkamer ook vaak betrokken is. Wanneer de linkerkamer het meest is aangedaan, wordt het ook wel aritmogene linker ventrikel (kamer) cardiomyopathie (ALVC) genoemd. Deze subtypes vallen onder de overkoepelende term ACM. Het subtype waarbij de rechterkamer het meest is aangedaan, wordt rechts dominante ACM genoemd. Het subtype waarbij de linkerkamer met name betrokken is links dominante ACM en de vorm waarbij beide kamers evenveel lijken aangedaan biventriculaire ACM.

In ongeveer de helft van de gevallen wordt een variant aangetoond in het DNA die de ziekte veroorzaakt. De overerving verloopt dan meestal via een autosomaal dominant patroon. In dat geval is er een genetische variant in een van de twee kopieën van een gen die leidt tot de ziekte. De genen die op de geslachtschromosomen liggen (de X en Y-chromosomen) worden hierbij niet meegerekend. Van elk van de ouders ontvangt iemand één kopie van een gen, wat betekent dat de aanleg voor de ziekte via één van de ouders wordt doorgegeven. Voor een schematisch overzicht van de overerving zie figuur 3 in de Introductie. De genetische varianten die leiden tot ACM worden meestal gevonden in de genen die coderen voor eiwitten die desmosomen vormen. Desmosomen spelen een rol in de verankering van hartspiercellen aan elkaar en in de elektrische geleiding. Varianten in genen die coderen voor desmosoomeiwitten kunnen uiteindelijk leiden tot een verminderde verankering tussen de hartspiercellen, celdood, abnormale geleiding van het elektrische signaal tussen de hartspiercellen, een abnormale calciumhuishouding en afwijkende signalering/regulatie binnen in de kern van hartspiercellen. Deze verscheidenheid aan effecten ligt aan de basis van ACM.

Nieuwe technieken in het aflezen van DNA, de afgelopen jaren ook toegepast in de patiëntenzorg, maken het mogelijk om veel genen tegelijkertijd te screenen. Het gevolg hiervan is dat er veel genetische varianten worden geïdentificeerd die lang niet altijd bijdragen aan het ziektebeeld. De gevonden varianten zijn vaak uniek en de families met ACM, waarin

zo'n genetische variant is aangetoond, zijn vaak klein; wat de interpretatie van een dergelijke variant bemoeilijkt. Onschuldige genetische varianten komen veel voor onder de bevolking. Een grote uitdaging op dit moment is het correct interpreteren van deze varianten. Deel 1 (hoofdstuk 2 t/m 5) van dit proefschrift is gericht op de interpretatie en beschrijving van dergelijke genetische varianten.

In **hoofdstuk 2** komen de uitdagingen van een correcte interpretatie aan het licht bij een specifieke missense variant in het *LMNA*-gen. Bij dergelijke varianten wordt een aminozuur binnen het eiwit vervangen door een ander aminozuur. Eiwitten zijn opgebouwd uit honderden tot duizenden aminozuren en het precieze effect op de functie van een eiwit in zo'n geval is moeilijk te voorspellen. Een argument dat voor schadelijkheid pleit, is afwezigheid of heel laagfrequente aanwezigheid in de algemene bevolking. Er zijn ook bio-informatica programma's die het effect op de functie van een dergelijke verandering voorspellen. Wanneer er klinische data beschikbaar zijn van de dragers, kan worden gekeken of de desbetreffende variant met de ziekte overerft. Tot slot kunnen functionele testen, waarbij het effect van een variant op het functioneren van een eiwit geanalyseerd wordt, meer duidelijkheid verschaffen of een variant schadelijk is of niet. Vaak is er bewijs nodig op meerdere vlakken, voordat een variant als schadelijk/betrokken bij de ziekte kan worden geclassificeerd. Bij de *LMNA* c.992G>A (p.(Arg331Gln)) variant was dit extra moeilijk. In een controlegroep werd deze variant frequent gevonden en door uitgebreid de verschillende aspecten van de variant in kaart te brengen, konden we deze variant classificeren als oorzakelijk (pathogeen). Opvallend hierbij is dat het klinische beeld milder is dan we gewend zijn bij dragers van varianten in dit *LMNA*-gen. Daarnaast vonden we ook in meer dan de helft van de indexpatiënten een additionele variant in een van de andere onderzochte genen betrokken bij hartspierziekten. Dit kan een aanwijzing zijn dat extra genetische varianten bijdragen aan het ontstaan van het ziektebeeld. Door functionele testen te combineren met elektronenmicroscopie konden we aantonen dat de verminderde krachtontwikkeling in de hartspiercellen door deze variant het gevolg is van een veranderde structuur binnen de hartspiercel.

In **hoofdstuk 3** geven we de interpretatie van een variant die leidt tot een voortijdig stopcodon ("truncerend") in het *TTN*-gen en beschrijven we de klinische karakteristieken. Dergelijke varianten kunnen leiden tot een verkort eiwit, maar zorgen over het algemeen tot nonsense gemedieerde degradatie (NMD), de afbraak van het coderende mRNA, met als gevolg haploinsufficiëntie (het verminderd aanwezig zijn van het betreffende eiwit). In het algemeen wordt aangenomen dat dergelijke varianten een groter effect hebben op het gen/eiwit dan bijvoorbeeld de missense varianten, zoals de hiervoor beschreven *LMNA*-variant. Titine (TTN) is het grootste eiwit in de mens en truncerende varianten in *TTN* komen relatief vaak voor in de bevolking. Dit impliceert dat ze niet allemaal schadelijk zijn. Met behulp van het analyseren van genetische markers konden we aantonen dat het ging om

een genetische variant die ontstaan is in een verre voorouder. Door de klinische kenmerken te combineren met het genetisch onderzoek konden we ook deze variant als pathogeen classificeren. Daarnaast bleek (paroxysmaal) atriumfibrilleren een belangrijk kenmerk van het fenotype, terwijl bekende structurele afwijkingen of risicofactoren voor het ontstaan hiervan afwezig waren. We zagen bovendien dat er meer mannen dan vrouwen waren aangedaan en dat er naast de genetische variant ook vaak andere factoren aanwezig waren die hebben bijgedragen aan de ontwikkeling van de met de *TTN*-variant geassocieerde hartspierziekte gedilateerde cardiomyopathie (DCM) zoals: chemotherapie, overmatig alcoholgebruik of een extra genetische ziekteveroorzakende variant. Dit tezamen laat zien dat er naast het dragerschap van de truncerende *TTN*-variant nog een additionele factor nodig kan zijn om de ziekte te krijgen.

In **hoofdstuk 4** beschrijven we een grote groep personen met truncerende varianten in het *DSP*-gen. Net als bij de andere genetische cardiomyopathieën is er een incomplete penetrantie en een grote variabiliteit in de uitingsvorm van de ziekte. Karakteristiek is dat de linkerkamer met name betrokken is. Bij het vergelijken van de locaties van de varianten in het gen met die in controle datasets, zagen we dat bij patiënten met ACM, deze varianten vaker voorkomen in gebieden van het gen die NMD ondergaan. Bovendien bleek dat het voorkomen van een truncerende variant in deze gebieden een voorspeller is voor het ontwikkelen van een levensbedreigende ritmestoornis gedurende het leven. De locatie van de variant lijkt dus belangrijk en het vinden van truncerende varianten in die gebieden zou als meer bewijs kunnen worden gezien voor het feit of de variant pathogeen is of niet. In tegenstelling tot de groep met de truncerende *TTN*-variant hierboven beschreven, vonden we in de patiëntengroep met truncerende *DSP*-varianten een oververtegenwoordiging van vrouwen. Mogelijk dat geslacht dus ook een rol speelt in de ziekteontwikkeling.

In **hoofdstuk 5** laten we zien dat het dragen van meer dan een pathogene variant in de desmosomale genen geassocieerd is met een slechtere uitkomst. Hiervoor werd, bij een groep patiënten met de verdenking op twee of meer ziekteveroorzakende varianten, eerst een gedegen herclassificatie van de gerapporteerde varianten uitgevoerd. Hierbij bleek een aanzienlijk deel van de varianten toch niet ziekte-veroorzakend. Dit pleit ervoor dat, na gedegen herbeoordeling van varianten, additionele genetische factoren bijdragen aan een ernstiger beeld en het op jongere leeftijd ontstaan van ACM.

Het tweede gedeelte van dit proefschrift (hoofdstuk 6 en 7) richt zich op het identificeren van nieuwe genen in ARVC, een subtype van ACM. De genetische oorzaak is in ongeveer de helft van de gevallen onbekend. Er is overlap in de genen die betrokken zijn bij de verschillende soorten cardiomyopathieën. Vanwege dit gegeven hebben we in **hoofdstuk 6** negen genen gescreend die betrokken zijn bij de opbouw en het functioneren van sar-

comeren in genotype negatieve ARVC-patiënten. In ongeveer 4% van deze patiënten werd een zeldzame, mogelijk ziekteveroorzakende variant gevonden. Echter, vanwege het missen van aanvullende informatie, zoals overerving van de varianten met de ziekte en resultaten van functionele testen, was het niet mogelijk uitspraken te doen over betrokkenheid bij de ziekteontwikkeling.

In **hoofdstuk 7** hebben we bekeken hoe vaak zeldzame varianten voorkomen in het plectine gen (*PLEC*) in patiënten met ARVC. Plectine speelt een rol in het verankeren van het cytoskelet aan de desmosoom en leek daarom een geschikte kandidaat. Echter, bij het vergelijken van de hoeveelheid zeldzame varianten in een grote genetische database met de hoeveelheid varianten in onze patiëntengroep, vonden we geen oververtegenwoordiging van *PLEC*-varianten bij ARVC-patiënten. Dit suggereert dat het *PLEC*-gen waarschijnlijk ook geen grote rol speelt in de ontwikkeling van ARVC. Op microscopisch niveau zagen we echter wel dat sommige *PLEC*-varianten leiden tot abnormale lokalisatie van het eiwit. Of dit een rol zou kunnen spelen in de mechanismen onderliggend aan ARVC, moet verder worden uitgezocht. Hoewel we niet kunnen uitsluiten dat sommige varianten in *PLEC* of de sarcomeer genen betrokken kunnen zijn bij de onderliggende pathofysiologie van ACM, lijkt er voor deze genen geen grote rol weggelegd.

Appendix

Acknowledgements | Dankwoord, About the author and Bibliography

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ABOUT THE AUTHOR

Edgar Theodoor Hoorntje was born in Groningen on the 28th of February 1988, as the second son of Jacobine and Theo Hoorntje-Holtrop. The family moved to Maastricht where he attended the primary school Openbare basischool de Binnenstad and finished primary school at the Utrechtse School Vereniging (Utrecht). In 2006 he graduated at the Utrecht Stedelijk Gymnasium and he was accepted to study medicine in Groningen (RUG). In 2010 he started his junior internships at the Martini Ziekenhuis (Groningen). He moved to Curacao in 2011 for his internships at the Sint-Elisabeth Hospitaal in Willemstad and in 2012 he moved back to Utrecht to work on his research project and do his final internship at the Department of Orthopedics. The focus of the research was bone regeneration and this led to his first scientific publication; CXCL12/Stromal-Cell-Derived Factor-1 effectively replaces endothelial progenitor cells to induce vascularized ectopic bone. During this period the foundation of his interests in science was laid and after one year working as a resident at the Department of Internal Medicine at the St. Antonius Hospital in Utrecht he started his PhD project in 2014 under supervision of prof. J. Peter van Tintelen, prof. Maarten P. van den Berg, and dr. Jan D.H. Jongbloed at the Genetics Department of the University Medical center Groningen. This resulted in his dissertation entitled 'Arrhythmogenic cardiomyopathy: beyond monogenetic disease', which he will defend on November 28th 2023 in Groningen at the RUG. During the final stretch of finishing his thesis, he worked for one year as a resident at the pulmonology and cardiology department (Martini Ziekenhuis, Groningen). In 2021 he started his specialization in general practice, which he finished in 2023. He is currently working as locum general practitioner. By serendipity, in the form of their shared supervisor, he met Mariam Kalule at the end of his first year of GP training. He is overjoyed she said 'yes'. They will marry on the 19th of May in 2024.

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