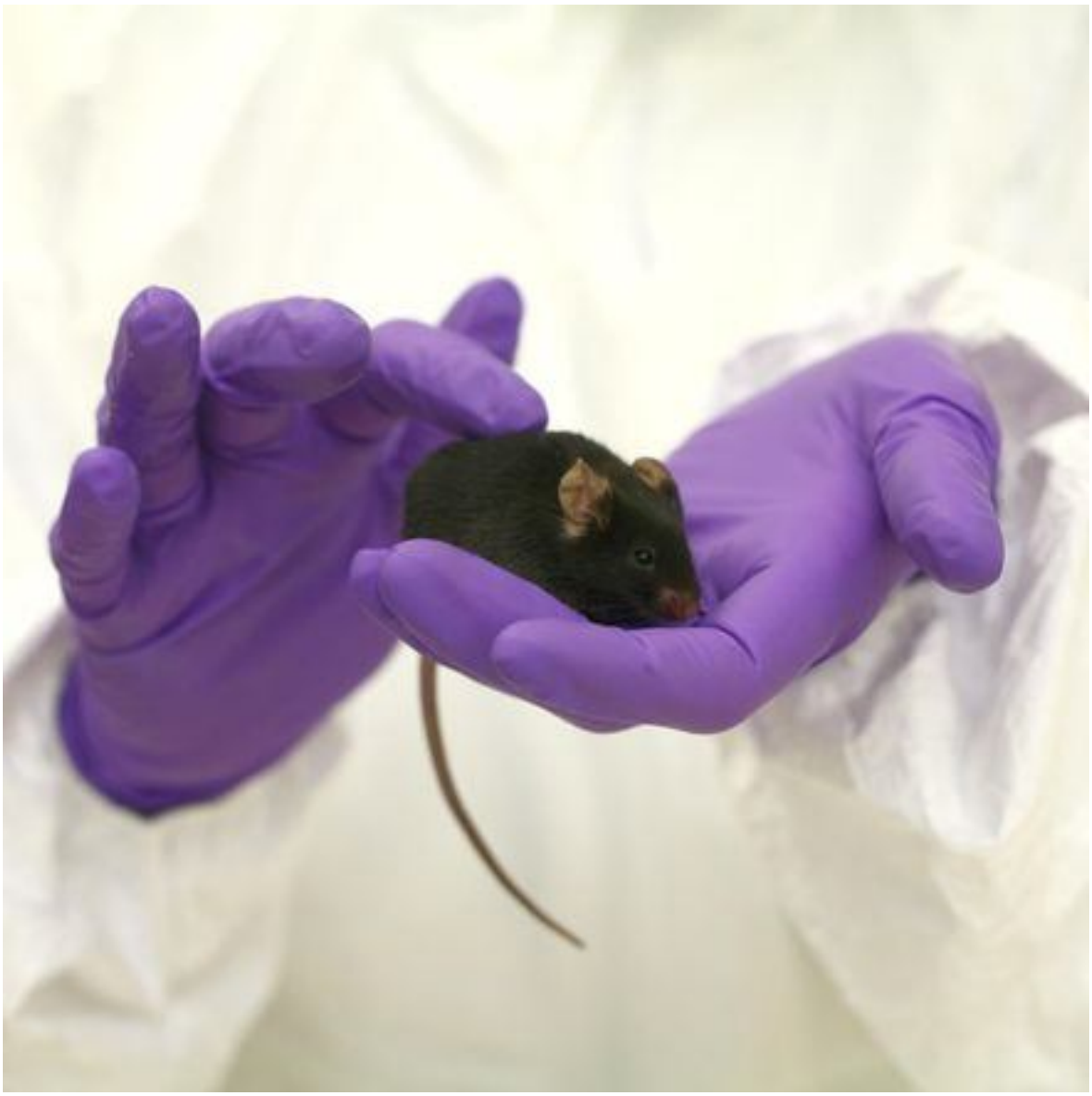
Animal models used for research in arrhythmogenic right ventricular cardiomyopathy placed in context



Biomedical Sciences | bachelor thesis | theoretical part

Sarah Vermij | April 2011

Contents

Abstract

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a progressive heritable disease, characterised by fibrofatty replacement of the right ventricular myocardium. In the last three decennia, much progress has been made in the research on this subject. The hypothesis has arisen that ARVC is a disease of the desmosome since mutations in the desmosomal proteins are associated more and more with the disease.

An important asset of ARVC research are mouse models that are deficient or haploinsufficient for a desmosomal protein, or express a mutated form of the protein. Therefore, the research question focuses on mouse models. These models are produced after the relation between a particular mutated protein and ARVC has been established using patient material and *in vitro* studies. Mouse models are available for the desmosomal proteins plakoglobin (PKG), plakophilin-2 (PKP2), desmoplakin (DSP) and desmoglein-2 (DSG2). Mutations in the fifth desmosomal protein desmocollin-2 (DSC2) have been associated with ARVC only recently, so no mouse model is available yet. Besides these models spontaneous ARVC has been described in cats and dogs.

Research on these models has revealed for instance that PKG and PKP2 deficiency causes heart rupture in cardiac development. PKG heterozygous mice were also shown to benefit from load-reducing therapy. Also, mutations in PKP2 are shown to cause gap junction (GJ) remodelling, which could be a substrate for arrhythmogeneity. Furthermore, DSP is needed for the association of the desmosome with IFs (IFs), and DSG2 heterozygous mice revealed that myocyte necrosis is the key initiator of myocardium dystrophy.

Mouse models in ARVC research have brought a cure and therapeutic measures for the patients closer. Also, it has greatly enhanced the knowledge on the desmosome.

Introduction

This research project focuses on the disease ARVC, or arrhythmogenic right ventricular cardiomyopathy. ARVC is also known as ARVD (ARV dysplasia) or ARVD/C (ARV dysplasia/cardiomyopathy). First, ARVC will be introduced including its history, symptoms, epidemiology, and causes. In order to create a complete picture of the disease, the diagnosis and treatment will be discussed as well.

ARVC is a disease of the desmosome, so this structure will be discussed closely, as components of the desmosome play a very important role throughout this project. Eventually, the research question is introduced, which concerns the animal models that are used in ARVC research. Then, in the chapter 'animal models' the research question will be answered extensively.

What is ARVC?

ARVC is a heritable form of cardiomyopathy, although a substantial part of the cases is not of known heritable origin. This is a progressive disease that primarily affects the right ventricle (RV), and is characterised by fibrofatty replacement of cardiomyocytes. These pathological phenomena are transmural, and occur most often in the 'triangle of dysplasia', that consists of the inflow tract, outflow tract and apex of the RV. In later stadia of the disease, this phenotype spreads to the left ventricle (LV) and septum [1].

Since its discovery, a lot of progression has been made in ARVC research. The pathology was described, diagnostic criteria were formulated, therapeutic measures were found and applied, and the genetic background was unravelled. This does however not at all mean that research in ARVC is 'done', since a lot of its features are still of unknown origin and there are other causes, genetic or other, that still have to be found.

Symptoms

In an early phase of the disease, patients with ARVC are mostly asymptomatic, although there is a risk of sudden cardiac death. This disease is one of the major causes of sudden death in the young and athletes [2]. When the disease presents itself, the most common symptoms are palpitations (in 27% of the patients), syncope (26%) and sudden cardiac death (23%). Cardiac arrest may also be a first presentation of the disease. In the clinic, patients present with abnormalities in the electrocardiogram and in the RV on a morphological level. The arrhythmias are of right ventricular origin and usually triggered by effort. They can occur even before histological evidence of right ventricular dysfunction can be found. As the disease progresses, the chances of biventricular heart failure increase. Also, congestive heart failure can occur when ARVC involves the ventricular septum and the LV [1].

History

Giovanni Maria Lancisi was the first to describe ARVC in his book *De Motu Cordis at Aneurysmatibus* in 1736. Although the name ARVC was of course not yet assigned, he described a family in which a certain disease occurred in members over four generations. The affected persons presented with palpitations, heart failure, dilation and aneurysms of the RV and sudden death [2]. Some centuries later, the first clinical and pathological series of patients with ARVC were described by Marcus in 1982 [3], Nava in 1988 [4] and Thiene also in 1988 [5].

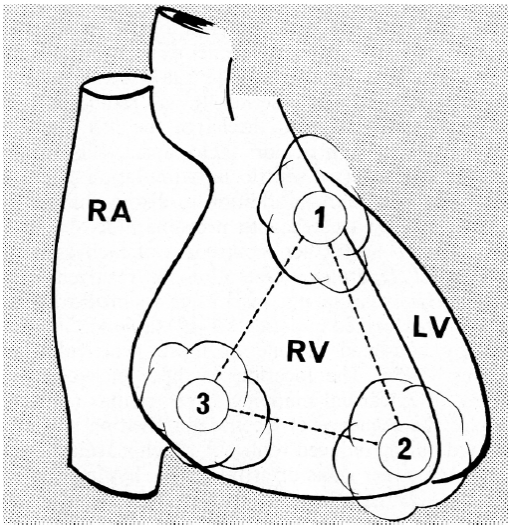


Figure 1 The triangle of dysplasia. 1, anterior infundibulum, 2, right ventricular apex, 3, inferior or diaphragmatic aspect of the right ventricle. RA, right atrium; RV, right ventricle; LV, left ventricle.[3]

Marcus *et al.* were the first to report the disease and emphasise the relation to the origin of arrhythmias from the RV. They observed many symptoms that still are regarded as standard characteristics of ARVC, such as cardiomegaly, ventricular tachycardia (VT), and left bundle branch block configuration. In this publication however, the disease is still called right ventricular dysplasia, but the term 'arrhythmogenic' is added when the VTs are the principle manifestation of the disease. Also, the triangle of dysplasia was already mentioned as the three areas in which dysplasia most often occurs: anterior infundibulum, right ventricular apex and the inferior or diaphragmatic aspect of the RV (triangle of dysplasia was already mentionedsee Figure 1) [3].

Nava *et al.* were the first to suggest a genetic origin as well as a variable penetrance of the disease. They also proposed that the disease is a cardiomyopathy instead of a dysplasia as progressive pathological processes are involved rather than a developmental abnormality [4]. After that, Thiene *et al.* showed that 20% of the sudden deaths in the young was attributable to ARVC [5]. Six years later in 1994, the first gene locus (ARVC) was identified by Rampazzo *et al.* at chromosome 14q23 [2], and the pathological profile was described in detail by Basso *et al.*[6] After that, research in ARVC has been progressing evermore, of which a relevant selection will be discussed in this thesis.

Epidemiology

The prevalence of ARVC has been estimated to be approximately 1 in 5000. However, the prevalence could be higher as there are many non-diagnosed or misdiagnosed cases [2]. Based on genetic modifications with an autosomal dominant inheritance, an estimated 50% of the ARVC cases is of familial origin.

ARVC has been studied in a relatively high extent in the Veneto region of Italy. This is attributable to the fact that ARVC was first described clinically and pathologically by Marcus, Nava and Thiene, who did their research in this Italian region [7]. In their review, Thiene *et al.* claim that the prevalence of ARVC is 1 in 2000 in this region [2]. They refer to the Nava study from 1988[4] as the source for this statement. Although Thiene *et al.* were themselves involved in many publications on ARVC in this region, neither the Nava study nor any other study found on the Veneto region contained support for this statement [4, 6, 8-11]. The only prevalence calculations were done on transplanted hearts, where the prevalence of ARVC was 1%, and in juvenile sudden cardiac death, where the

prevalence was 11.2% [6]. This means that it remains to be seen whether the claim that the overall prevalence of ARVC in the Veneto region is 1 in 2000 is right or not.

Causes

Between the discovery of ARVC as a disease and the genetic elucidation quite some time went by. The first link between ARVC and a mutated gene was made with mutations in the ryanodine receptor 2 (RYR2) gene in 2001 [12]. Then, in 2004 the genetic cause of Naxos disease, an autosomal recessive form of ARVC, was identified as a deletion in the plakoglobin (PKG) gene. The discovery of mutations in the plakophilin-2 (PKP2) gene encoding another desmosomal protein sometime later led to the idea that ARVC might be a desmosomal disease [13].
It is hypothesised that mutant desmosomes can still be functional in maintaining tissue integrity, but are very vulnerable in the areas of the heart with the most mechanical stress. These locations are the thinnest portions of the RV: the right ventricular outflow tract, inflow tract and the apex, together called 'the triangle of dysplasia' [13].

Twelve chromosomal loci for ARVC have been identified so far. The majority of these loci has already been associated with a mutated gene (see table 1). Five of these are desmosomal genes, and therefore ARVC is considered primarily a desmosomal disease. Mutations in these desmosomal proteins are associated more and more with the development of the ARVC phenotype. Mutations in TMEM43 (transmembrane protein 43), RYR2 and TGFβ3 (transforming growth factor beta 3) are also associated with the disease. The exact mechanisms of how the mutations lead to the disease phenotype remain however unknown. Also, in half of the ARVC patients the origin is not genetic, or the genetic origin is still unknown.

ARVC type	Locus [14]	Associated gene
ARVC1	14q23-q24	TGFβ3 [15]
ARVC2	1q42-q43	RYR2 [16]
ARVC3	14q12-q22	
ARVC4	2q32.1-q32.3	
ARVC5	3p23	TMEM43 [17]
ARVC6	10p14-p12	
ARVC7	10q22.3	
ARVC8	6p24	DSP [18]
ARVC9	12p11	PKP2 [19]
ARVC10	18q12.1-q12	DSG2 [20]
ARVC11	18q12.1	DSC2 [21]
ARVC12	17q21	PKG / JUP [22]

Table 1 ARVC loci and associated genes. TGFβ3: transforming growth factor beta 3, RYR2: ryanodine receptor 2, TMEM43: transmembrane protein 43, DSP: desmoplakin, PKP2: plakophilin-2, DSG2: desmoglein2, DSC2: desmocollin2, PKG: plakoglobin, JUP: junction plakoglobin.

Mutations in the desmosomal proteins may lead to a reduction in the amount of desmosomes and decreased structural integrity. Most importantly, mutations in the desmosomes may also lead to a change in distribution and amount of other proteins in the intercalated disc (ID), including gap junction (GJ) proteins; connexin43 (Cx43) in particular. This can affect intercellular conduction and thus enhance arrhythmogeneity [7]. These concepts will be

discussed extensively in later chapters. For now, it is sufficient to point at a study by Saffitz *et al.,* as they show that mice that are heterozygous for Cx43 +/- have a reduced amount of GJs [23]. The amount of Cx43 is thus correlated to the amount of GJs, so when in a particular study a smaller-than-normal Cx43 signal is observed, the number of GJs is probably decreased as well. This may lead to a reduced conduction velocity in the myocardium.

An interesting publication from 2011 by Lombardi and Marian points to the fact that the molecular pathogenesis of ARVC has at least two components, fibroadiposis and cardiac dysfunction. Adipogenesis has shown to be regulated by PKG. In ARVC, PKG translocalises partially from the desmosome to the nucleus and suppresses the canonical Wnt signalling pathway in the second heart field cardiac stem cells, which leads to adipogenesis [24]. Stem cells in the second heart field form the entire outflow part and some of the RV and atria [25]. A second heart field-specific transcription factor, Mef2C, regulates the development of these parts of the heart. The cells in the second heart-field are thus hypothesised to differentiate into adipocytes instead of cardiomyocytes because of mutations in PKG or other proteins. This causes fibroadiposis, while the cardiac dysfunction is caused by the impaired intercellular attachment [24].

In general, three theories have been proposed that explain the pathogenesis of ARVC. The first is the dysontogenetic theory, which states that ARVC might be a mild form of Uhl anomaly. Patients with Uhl anomaly have a very thin RV with or without tricuspid valve anomalies. Just as Uhl anomaly, ARVC might result from dysregulation of ventricular wall thickness, during development or postnatally. The second theory is the apoptotic theory, suggesting that ARVC is caused by increased apoptosis of cardiomyocytes. The third theory is called the transdifferentiation theory, which explains ARVC by the transdifferentiation of cardiomyocytes into adipocytes in response to stress [26]. The hypothesis of Lombardi and Marian can be seen as a fourth theory, and is opposite to the transdifferentiation theory. Both the apoptotic and the dysontogenic theory are supported in various articles, including by Yang *et al*.[26].

Diagnosis

The first time a guideline for the diagnosis of ARVC was proposed was in 1994, when the International Task Force Criteria were published by McKenna *et al*.[1] and showed in Table 1. These criteria were needed because a definitive diagnosis could only be made at necropsy or surgery, as the transmural fibrofatty replacement of right ventricular myocardium had to be demonstrated histologically. The diagnostic criteria as McKenna *et al.* proposed that the diagnosis of ARVC would be fulfilled by the presence of two major criteria from different groups, or one major plus two minor criteria, or four minor criteria [27].

The International Task Force Criteria were shown to be very useful in the diagnosis of ARVC, as they were specific. *In vitro* validation of several criteria has been done in 2008. This study showed among other things that myocardial atrophy is the most reliable and important morphological parameter of the *in vivo* diagnosis of ARVC.[9] However, problems with the original criteria are also observed, as they lack sensitivity and are often qualitative rather than quantitative. Modifications for these criteria have been suggested for example by Hamid *et al.,* to account for the fact that family members of a typical ARVC patient show a broader spectrum of the disease [1].

In 2010, the International Task Force Criteria were modified by Marcus *et al.,* in which new knowledge and technology were incorporated. This resulted in an increased diagnostic sensitivity, while the diagnostic specificity was not compromised. The lack of quantitative interpretation was addressed, as quantitative criteria for the imaging studies were proposed, and abnormalities were defined on the basis of comparison with control data.

Criteria category	Major	Minor
I. Global and/or regional dysfunction and structural alterations	Severe dilatation and reduction of right ventricular ejection fraction	Mild global right ventricular dilatation and/or ejection fraction reduction with normal LV
	Localised right ventricular aneurysms (akinetic or dyskinetic areas with diastolic bulging)	Mild segmental dilatation of the RV
	Severe segmental dilatation of the RV	Regional right ventricular hypokinesia
II. Tissue characterisation of walls	Fibrofatty replacement of myocardium on endomyocardial biopsy (EMB)	
III. Repolarisation abnormalities		Inverted T waves in right precordial leads (V2 and V3) (in absence of right bundle branch block)
IV. Depolarisation/conduction abnormalities	Epsilon waves or localised prolongation (>110 ms) of the QRS complex in right precordial leads (V1-V3)	Late potentials (signal averaged ECG)
V. Arrhythmias		Left bundle branch block type ventricular tachycardia (VT)
		Frequent ventricular extrasystoles (more than 1000/24 h)
VI. Family history	Familial disease confirmed at necropsy or surgery	Familial history of premature sudden death (<35 yr) due to suspected right ventricular dysplasia
		Familial history (clinical diagnosis based on present criteria)

Table 2 diagnostic criteria according McKenna *et al.*, 1994

An important consequence of these modifications is that the early and familial forms of ARVC could be diagnosed as well [28]. When the new Task Force Criteria were applied on suspected ARVC patients by Cox *et al.*, 64% of them were diagnosed with ARVC as well of 11% of their family members. This implied that the new criteria could increase the diagnostic yield of AVRC [29].

Diagnosis of ARVC relies on several techniques. Echocardiography is used as a first-line imaging approach to screen suspected ARVC patients and their family members. It is also very useful to keep track of the progression of the disease, allowing the detection of functional and structural abnormalities. RV angiography is seen as the gold standard for the diagnosis of ARVC. It can provide evidence of akinetic or diskinetic bulgings in the triangle of dysplasia. Magnetic resonance imaging (MRI) can be attractive as well, since it can distinguish fat from muscle and is non-invasive. However, a problem with MRI is the high degree of variability in the observed fatty infiltration, which even may be observed in healthy hearts. Electrocardiograms (ECG) are made very often, as a large range of abnormalities can be detected, such as epsilon waves, QRS complex prolongation, and depolarisation or conduction abnormalities. Therefore, ECG abnormalities are an important part of the International Task Force Criteria.

A new development in the diagnosis of ARVC is described by Asimaki *et al.* in 2009 [30]. In this publication, it was investigated whether the change in the distribution of desmosomal proteins in an endomyocardial biopsy (EMB) can be used for diagnostic testing for ARVC. This was done using immunohistochemical analysis on the cardiac tissue samples, with antibodies against PKG, PKP2, DSP and N-cadherin. Evidence for the specificity came from comparing the transmural sections of ARVC patients with those of patients with other heart diseases. Especially reduced immunoreactive signal levels of PKG at the IDs were a very specific feature in patients with ARVC, since aA reduced PKG signal is not observed in other forms of heart-muscle disease. Although this may sound very promising, more research is needed to confirm the diagnostic usefulness.

Another new development reviewed by Asimaki *et al*.[31] is the diagnosis of ARVC based on a conventional EMB. EMBs are hardly used in the diagnostic process, as the pathological processes are often absent in the endocardium and interventricular septum where the EMBs are taken. However, some biomarkers for ARVC have been identified in these EMBs that could make EMBs a diagnostic tool. Further validation has to been done before the analysis of biomarkers in EMBs can be used in the clinic.

Risk factors

Patients with ARVC at the highest risk for arrhythmic death are patients with syncope, patients that have been resuscitated from sudden cardiac death, very young patients, and those who have marked right ventricular involvement. Predictors of sudden cardiac death have also been identified, such as QRS dispersion [1]. For ARVC patients a risk stratification scheme was developed to predict the risk of cardiovascular mortality. Patients with right ventricular failure or left ventricular dysfunction were at high risk of cardiovascular mortality, which increased when one of these factors was combined with VT. Patients without VT had the best prognosis. Because ARVC is a relatively rare disease, information on the natural history is scarce, although this is needed to optimise therapeutic strategies. This risk stratification scheme can approach the natural history, and help with the development of therapeutic strategies [32].

Treatment

For the treatment of ARVC several strategies exist. In the first place, patients should avoid physical effort, as ventricular arrhythmias may be triggered by exercise and can result in sudden cardiac death. Clinical data suggesting this were confirmed by Kirchhof *et al.*. In heterozygous PKG-deficient mice they showed that manifestation of the ARVC phenotype is accelerated by endurance training.[33] Therefore, patients should avoid competitive athletics, and only practice physical activity with low intensity such as walking [1].

Another important strategy is to implant a ICD (implantable cardioverter-defibrillator). This device was already in 1993 proven to be life-saving, as Corrado *et al.* showed that 24% of the examined patients would have died in the course of the follow-up if they did not carry an ICD. However, 3% of the patients died anyway because of ARVC-related causes, so the ICD cannot prevent every death [10]. The effectiveness of the ICD was proven in many studies afterward, although some complications were seen. One of the most promising results was obtained by Hodgkinson *et al.* who found that ARVC5 patients have a very strong benefit from ICD. The 5-year mortality rate after ICD implantation in males was zero while in control subjects it was 28% [1].

Several antiarrhythmic drugs are applied regularly in ARVC patients, including sodium blockers, β-blockers, sotalol, amiodarone, and verapamil. Wichter *et al.* decided to examine the efficacy of the drugs, since little of that was known. The results indicated that sotalol was highly effective in patients with inducible or noninducible VT. Amiodarone was not more effective, but did show stronger side effects. When the patients did not respond to sotalol, verapamil and β-blockers could be used [34]. Marcus *et al.* however contradicted these results, as they found that neither β-blockers nor sotalol seemed effective in ARVC patients with an ICD. In contrast, amiodarone was very effective in the prevention of ventricular arrhythmias. Unfortunately, the publication did not mention side effects [35]. Therefore, more research is needed to further assess the efficacy of antiarrhythmic drugs.

As will be discussed later in greater detail, load-reducing therapy consisting of furosemide and nitrates prevented training-induced development of ARVC in heterogeneous PKG deficient mice. The load-reducing therapy might be a successful application in human ARVC patients as well, but because this study was published only in 2011, clinical application is still some time away [36].

Other promising drugs in the treatment of ARVC may be renin-angiotensin-aldosteron system (RAAS) inhibitors [37]. Fibrosis in the myocardium is shown to increase arrhythmia vulnerability in diseased hearts. In the process of fibrosis, RAAS regulates the synthesis of myocardial collagen. By inhibiting this system, less collagen will be synthesised, and thus the process of fibrosis will be slowed down. This also implies reduced arrhythmia vulnerability. This hypothesis was tested in aged mice, which were treated with the RAAS inhibitors eplerenone and/or losartan. The treated mice showed a clear increase in transverse conduction velocity and a reduction in anisotropic re-entrant arrhythmias. Also, interstitial fibrosis was significantly decreased in the treated group. On top of that, a strong correlation between the presence of fibrosis and arrhythmia inducibility was found. Chronic RAAS inhibition was thus found to limit aging-related interstitial fibrosis. Because arrhythmias and fibrosis both are important features of ARVC, RAAS inhibition may be relevant in ARVC treatment as well. However, in this study no specific ARVC models were used, and furthermore, the treatment did not alter the risk of sudden death. It is therefore possible that these findings may not have significant clinical implications, but further research on this subject may certainly be worthwhile.

Catheter ablation is sometimes applied in a specific group of ARVC patients. Indications for catheter ablation are monomorphic and well-tolerated VT with localised forms of the disease and drug-resistant or incessant VTs, or frequent ICD discharges. Successful outcomes of this technique have been reported several times [1], but only in the short term. Within three years, 60% of the patients show recurrence of VT, but in terms of survival the outcome is still quite good [2].

Concerning surgical interventions, right ventricular cardiomyoplasty can be applied to restore right ventricular function. This technique is also useful in right ventricular dysfunction caused by other diseases than ARVC. During the procedure, the RV free wall is wrapped with the left latissimus dorsi muscle flap. The distal part of the latissimus dorsi is then attached to the diaphragm and electrostimulated. A 10-year follow-up study revealed that the right ventricular cardiomyoplasty brought about hemodynamical and functional improvements, without perioperative deaths, long-term malignant arrhythmias or deaths related to RV dysfunction. RV cardiomyoplasty was thus shown to be an effective way to treat RV dysfunction, and this technique can be very useful to help patients in late stages of ARVC [38].

Heart transplantation is considered as the last resort in ARVC patients with progressive heart failure and unmanageable ventricular arrhythmias [2].

According to Hulot *et al.* who developed the risk stratification scheme discussed above, the only consensus in ARVC treatment exists by implanting a defibrillator in patients who survived cardiac arrest. The treatment for other ARVC cases remains however undefined. Hulot *et al.* suggest that high-risk patients risk according to the risk stratification scheme could be targets for aggressive therapeutic intervention. Patients at a low risk should accept treatment as well, because treatment with an antiarrhythmic drug in an early stage of ARVC can explain their good prognosis. Lifestyle recommendations are also advised, as the disease can progress more rapidly in cases of physical effort [32].

What is a desmosome?

Since ARVC is considered a disease of the desmosome, it is important to know what a desmosome is. Desmosomes are present in the ID, a very complex and essential part of cardiomyocytes that links the lateral edges of adjacent cells in the myocardium. IDs have at least two functions; one is to ensure mechanical coupling, and two is to enable fast conduction of electrical impulses. In the ID, three different protein complexes are present to facilitate these two functions; desmosomes, GJs and adherens junctions.

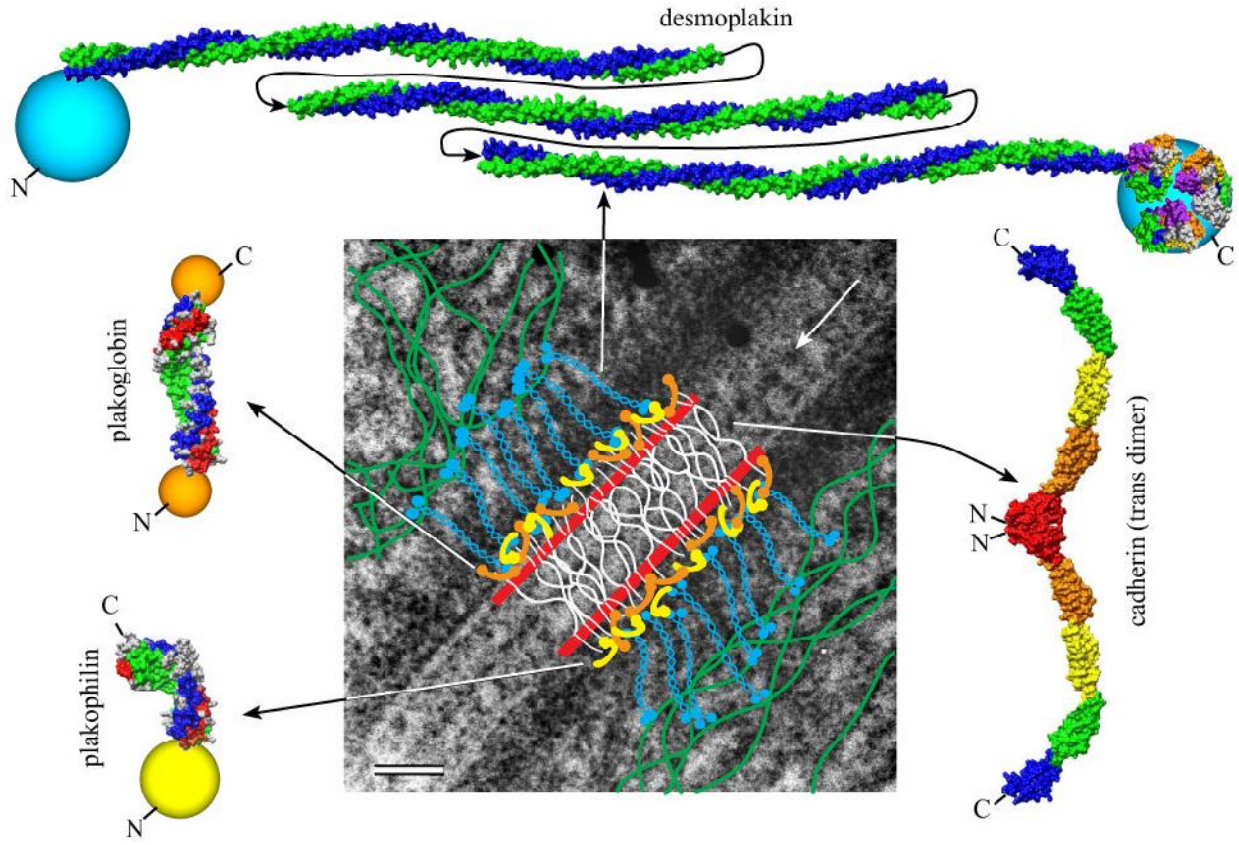


Figure 2 Protein components of the desmosome. Cell membranes of opposing cells are red. White strands are cadherin molecules [39]

Desmosomes are intercellular junctions that provide mechanical stability to tissues by joining lateral edges of adjacent cells. That makes them very important in tissues that undergo mechanical stress, such as skin and heart. Mutated desmosomes can probably maintain most of the mechanical stress, but may fail in the areas where the mechanical stress is the largest. That is why the triangle of dysplasia is the most vulnerable part in the heart, as the ventricle wall is the thinnest in that area [13].

The desmosome is built up of five proteins: plakoglobin (PKG), desmoplakin (DSP), plakophilin-2 (PKP2), desmoglein-2 (DSG2) and desmocollin-2 (DSC2). These proteins are arranged as seen in Figure 2. In this figure, the intracellular parts of the desmosome can be clearly separated in the outer dense plaque (ODP) and inner dense plaque (IDP). The ODP lies at the cell membrane and comprises the intracellular tails of cadherins (white), PKG (orange), PKP (yellow) and the N-terminal domain of DSP (blue). In the IDP, the rod and C-terminal domain of DSP are found. The latter is important to interact with IFs [39].

The intercellular part of the desmosome consists of cadherins, more specifically of DSG2 and DSC2, which interact in a heterophilic manner to connect the cells in which their transmembrane domains are located. Both proteins contain several calcium binding sites. A more schematic view of the desmosome, including DSG2 and DSC2, is shown in Figure 3.

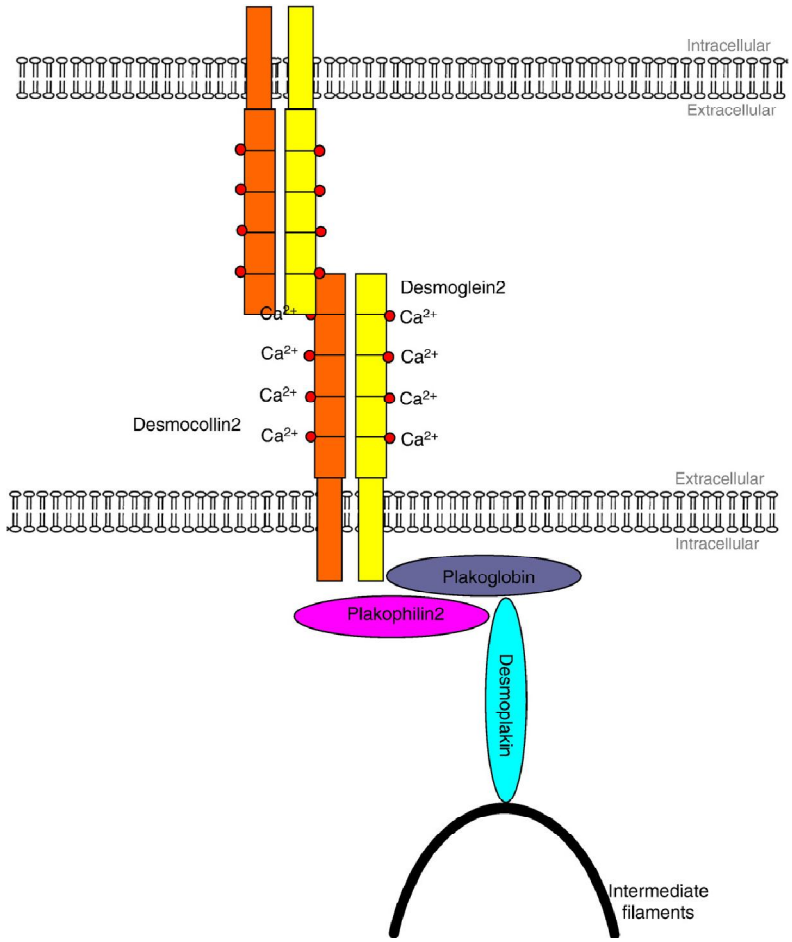


Figure 3 Desmosome[7]

Although desmosomes and adherens junctions are morphologically similar, desmosomes are specialised in resisting mechanical stress. Their state of adhesiveness can change, dependent on the situation. This feature is very important in wound healing and embryogenesis. Desmosomes are however not only important in providing mechanical stability, as they also play a role in signalling pathways in important processes such as morphogenesis and cell differentiation. Since desmosomes are important in many aspects, several diseases are associated with the failure of desmosomes. Acantholytic epidermolysis bullosa for example is associated with DSP deficiency, while PKP deficiency is linked to ectodermal dysplasia/skin fragility syndrome.

In the chapter animal models, each of the five desmosomal proteins will be discussed in detail.

Just like desmosomes, adherens junctions connect adjacent cells. But while desmosomes connect cells to IFs, adherens junctions connect to the actin cytoskeleton and the myofibrils. N-cadherin is one of its intercellular proteins [7].

GJs enable direct electrical and metabolic communication between adjacent cells by connecting the cytoplasm. Six connexin proteins form one connexon in the cell membrane, and can dock another connexon in the membrane of a neighbouring cell to form a GJ. In the ventricular myocardium, connexin43 (Cx43) is the most important connexin isoform, and will be mentioned often in this thesis.

Research question

A very strong hypothesis that arose during research in ARVC was that ARVC is a desmosomal disease. Over the course of the years, this hypothesis is ever strengthened. A very important tool in learning to understand how mutations in desmosomal proteins are responsible for the phenotype of ARVC is the production of transgenic animals, especially mice, that are haploinsufficient or completely deficient in one of the desmosomal proteins. It is therefore interesting to investigate which animal models are used, as well as how these models contributed to the understanding of the disease. Although mice take in the most prominent place in ARVC research, other animals may also be a promising asset, as spontaneous ARVC is described in cat and dog. Research on these models is however still in its infancy as the occurrence of ARVC is only recently described.

This leads to the following research question: **what animal models are used for research in the role of desmosomal proteins in ARVC?**

Since there are five desmosomal proteins, five mouse models can be expected. However, no transgenic mouse has been produced yet that is deficient in DSC2, since mutations in this protein have only relatively recently been related to ARVC. There are however publications available in which DSC2 deficiency is investigated *in vitro.* Transgenic mice are available for the other desmosomal proteins PKG, PKP2, DSP and DSG2. These will be discussed per desmosomal protein. Other publications on the proteins will be discussed also to understand the course the research has taken, considering the analysis of patient material or *in vitro* studies.

Animal models

Plakoglobin

General information

The protein plakoglobin (PKG), also known as junction plakoglobin (JUP) [22] and γ-catenin [7], is an important protein in the submembranous plaques of desmosomes and intermediate junctions where it binds cytoplasmic tails of cadherins [39]. It is found in both soluble and membrane-associated form [22], with a regulated equilibrium between the two forms. PKG is shown to be essential for cardiac development, mutations have been found to cause ARVC and Naxos disease [7]. The armadillo protein family, from which PKG is a member, is characterised by a series of *arm* repeats, which are each 42 amino acids long, and the structure of the protein is such that it looks like an armadillo.[40]. Interactions have been shown with 35 other proteins, including the desmosomal proteins DSG2 and PKP[41], and the tumour suppressor gene product APC [40]. PKG also has binding sites for microfilament anchoring junctions, such as zonulae adherents in epithelia [40]. The gene that encodes PKG has three splicing variants, one of which becomes the PKG protein [22]. Two single nucleotide polymorphisms (SNPs) are known [42].

Naxos disease

A mutation in PKG is associated with a subtype of ARVC that is called Naxos disease. The disease was first described by Protonotarios in 1986, who linked cardiac abnormalities to familial palmoplantar keratosis in patients originating from the Greek island of Naxos. Patients with palmoplantar keratosis and their living family members with cardiac abnormalities were examined by electrocardiography. Their symptoms included pale yellow keratosis in hand palms and foot soles, and scalp hair resembling steel wire (see Figure 4).

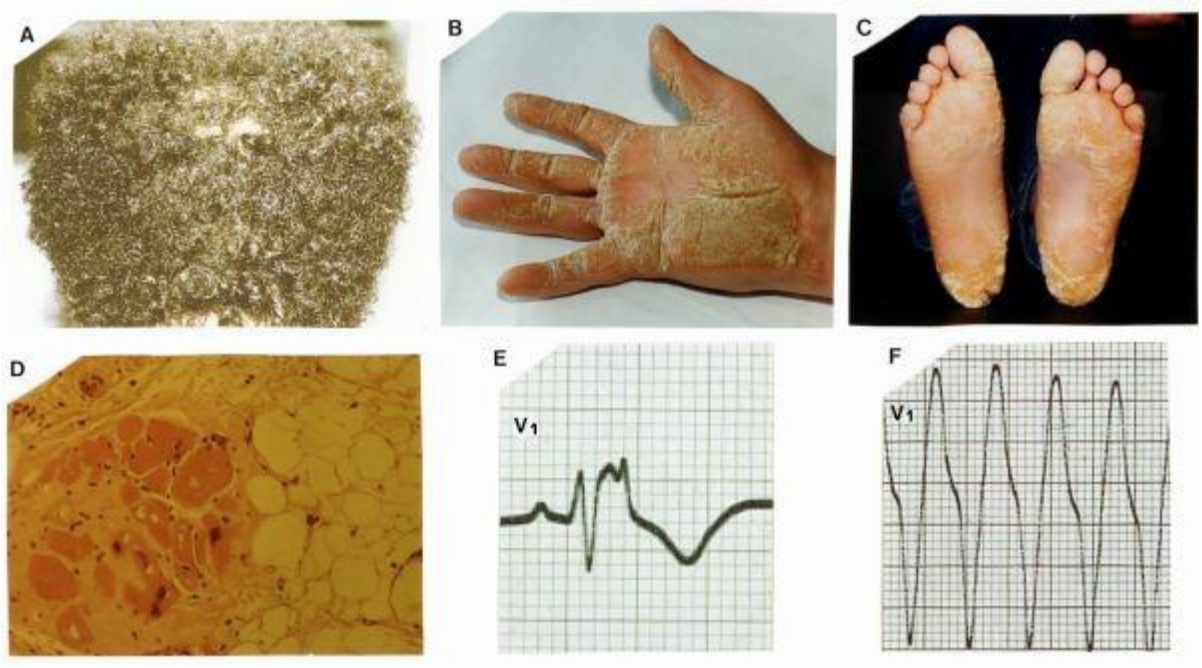


Figure 4 Phenotype of Naxos patients. A: woolly hair. B, C: keratosis. D: fibrofatty replacement of myocardium causes E: delayed activation. F: shows predisposition for re-entrant ventricular arrhythmias.[43]

Examination of the subjects revealed striking similarities in their cardiac abnormalities. The symptoms included cardiomegaly and echocardiographic evidence of predominantly right ventricular abnormalities. The RV was enlarged and dilated, and the wall showed motion abnormalities. The electrocardiographic disorders included T wave inversion, abnormal Q waves, low voltage, and arrhythmias of ventricular origin. There was no evidence of heart failure. The researchers pointed at a possible relation with ARVC, as the outcome of differential diagnoses for these patients was dilated cardiomyopathy or ARVC. Since the symptoms of the patients were so similar, the researchers concluded that this should be a hereditary disorder. Based on the pedigree of the patients an autosomal recessive trait was suggested.

It took 14 years to clear up the genetic cause. The genetic locus for Naxos disease was already mapped to chromosome 17q21 when in 2000 McKoy *et al.* showed that a homozygous two basepair deletion in the PKG gene on that chromosomal locus was specific for all the subjects with Naxos disease, while some family members were heterozygous for that deletion, and other family members as well as unrelated people showed homozygous normal alleles. ARVC patients without the ectodermal disease showed to be negative as well for this mutation [44]. Specifically, homozygous deletion of nucleotides 2157 and 2158 in patients with Naxos disease results in a frame shift mutation. This causes premature termination of translation, which truncates the C-terminal domain by 56 residues [45]. It is then impossible for cadherins to bind PKG, and the role of PKG in desmosomes is severely compromised. As shown by *in vitro* studies this mutation probably does not severely affect other functions of the protein. Further indications for this statement comes from PKG null mice, which die during embryogenesis due to heart defects [44]. The symptoms of the patients can be explained by the fact that the PKG mutation affects the primary role of maintaining cell-cell junction integrity. When this is compromised the tissues undergoing the greatest mechanical stress will be affected most, which correlates with keratosis on the hands and feet and cardiac abnormalities in Naxos patients. Concerning the woolly hair of the Naxos patients McKoy *et al.* hypothesised that the defect in PKG leads to fragility at desmosomal junctions in hair cells [44].

Kaplan *et al.* showed that myocyte GJs were remodelled because of this mutation. Myocardium from deceased Naxos patients was investigated on expression of various proteins, including Cx43, N-cadherin, DSP1, DSC2, PKG and PKP2. Results showed a reduced amount of PKG and Cx43 in the IDs. Other proteins localised normally. An interesting finding was that Cx43 was virtually absent in the highly phosphorylated P2-isoform, which is selectively located in GJs. Nonphosphorylated Cx43 was still present in nonjunctional pools. Electron microscopy showed a decrease in the number of GJs. All these data together indicate that GJs are remodelled in hearts of patients with Naxos disease. The GJ remodelling appeared most prominent in the affected regions of the RV, while Cx43 was also diminished at cell-cell junctions in the normal appearing LV. GJ remodelling also occurred in a Naxos patient who died before arrhythmias were present. PKG was shown to be expressed normally, but failed to localise to IDs.

Studies on plakoglobin knockout mice

Embryonic heart and skin defects in mice lacking plakoglobin [46]

In this study published by Bierkamp *et al.* in 1996 the role of PKG during embryonic development is examined. In order to achieve this, transgenic mice were bred with a null mutation of the PKG gene. First a targeting vector was created, then the PKG knock-out embryonic stem cell (ESC) clones were generated. The cells that had successfully underwent homologous recombination were microinjected into mouse blastocysts and transplanted into mice uteri. Offspring was checked for the homologously recombined PKG allele. The next step contained immunoblotting, using antibodies against PKG and β-catenin, followed by histological analysis.

The immunoblot analysis of PKG wild-type, heterozygous and homozygous-negative embryos clearly showed a loss-of-function mutation. Heterozygous animals appeared healthy, but their offspring did not contain homozygous-negative animals. This suggested a recessive embryonic lethality, which was confirmed using histological sections of these homozygous-negative embryos in different embryonic stages, compared to heterozygous and wild-type embryos. Especially the heart was less well developed. The thin heart wall enabled erythrocytes to pass through. This was contributed to a substantial loss in the number of desmosomes, and the desmosome plaques were also less dense. Furthermore, placenta and liver were smaller and supplied with less blood than the other embryos, which could be caused by the defect heart.

Other features of the PKG deficient mice included a detached superficial layer of the epidermis in snout, cheek, paws and tail region. This resulted in blister formation and a very thin epidermis, while the skin was generally very sensitive to mechanical stress. Ultrastructural analysis revealed major defects in skin architecture. There were for instance almost no contacts between cells in the granular layer of mutant skin. As well as in the heart, the number of desmosomes in the skin was reduced and the desmosomes itself were structurally altered.

In conclusion, the researchers interpreted their results as follows: the absence of PKG in the desmosomal plaque abolished the anchorage of cytokeratin filaments, which led to the observed phenotype, characterised by a defect heart and skin. Thus, PKG plays a crucial role in proper desmosomal function, particularly in cells where strong mechanic connections are needed.

Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart [40]

Ruiz *et al*. investigated in the same year the influence of PKG deficiency on the assembly of IDs in the embryonic heart. To achieve this a transgenic PKG deficient mouse was bred using similar techniques as described in the previous study. Subsequently, Northern blotting and immunoblot assays took place to determine the presence of PKG in the mice. Then, histological analysis was executed, using antibodies for β-catenin, DSP 1 and 2, and DSG 2. Electron and immunoelectron microscopy with anti-DSP antibodies was used as well. Functional analysis of the heart was done with an echo-tracking device.

When interbreeding was done, homozygous PKG -/- mice were not born, which was consistent with the previous study, indicating that -/- mice die in embryogenesis. To further investigate this, embryos in different stages were isolated and the conclusion was that -/- mice died between 12 and 16 days of gestation. The -/- mice did neither express PKG mRNA nor protein, while heterozygous and wt mice did.

After that, the cause of death in -/- mice was determined. The conclusion was that the embryos died of heart defects. That was based on the facts that the PKG -/- embryos were growth-retarded at day 12, blood supply of liver and placenta was reduced, pericardial cavities were filled with blood, and ventricles of the heart were ruptured. The amplitude of heart contraction was reduced, while the heart rate was increased. Part of these observations were also reported in the Bierkamp study. Furthermore, general heart morphology was the same in -/- mice, but the IDs were very different from control animals, as they did not show desmosomes or adherens junctions. Remarkably, epithelia did show desmosomes, in contrast to the hearts. On a more molecular level, control animals showed that DSP and DSG were segregated from β-catenin, while -/- mice showed colocalisation of DSP and β-catenin. Also, control animals showed the normal pattern of DSP-positive desmosomes and DSG-negative adherens junctions. -/- mice however showed no typical desmosomes and DSP was found in all plaque-bearing structures.

Heart rupture that occurred at day 12 of embryogenesis represents the primary defect in PKG -/- animals. It causes paleness, a reduction of size and blood supply of the placenta, and deterioration of liver parenchyme. The embryos that survive longest, 16-18 days of gestation, were swollen and covered with oedemas, while their kidneys and livers contained a lot of blood.

Thus, PKG was found to be essential for the segregation of desmosomes and adherens junctions during the development of IDs in the heart. In -/- mice desmosomes were not present, and adherens junctions developed that contained desmosomal proteins. The hearts showed architectural instability and dysfunction. Because the embryos die in the same stage of gestation, it is demonstrated that desmosomes and proper adherens junctions are required when the embryo becomes dependent of its own cardiovascular system. The fact that desmosomes are present in different epithelia of mutant mice is explained by molecules that are related to PKG that may contribute to desmosomal formation to compensate for the absence of PKG. Cardiomyocytes do not have such molecules available.

Age- and training-dependent development of arrhythmogenic right ventricular cardiomyopathy in heterozygous plakoglobin-deficient mice [33]

Now that the molecular consequences of PKG deficiency are clear, the scope of research with PKG deficient animals is broadened. Diminished mechanical adhesion between cardiomyocytes could result in right ventricular dysfunction and a thinner ventricular wall, which may cause a reduced mechanical stability. With that in mind Kirchhof *et al.* wanted to investigate whether desmosomal dysfunction can be the cause of ARVC. When endurance training was imposed on the mice and ARVC would aggravate, the hypothesis would be supported that susceptibility to mechanical cardiac strain contributes to ARVC in this model. Heterozygous PKG-deficient mice were used in this case instead of homozygous animals, because the latter all died in embryonic stage.

Heterozygous PKG-deficient mice were bred as previously described, with their wild-type siblings as controls. Techniques to analyse the animals included echocardiography, MRI and, *post mortem*, Langendorff apparati. Mice were subjected to defined stress tests and to endurance training, which consisted of swimming during 5 to 90 minutes per day for 8 weeks. Myocardial glucose uptake was measured using a fluor isotope and a positron-emission tomography (PET) camera, and *post mortem* the hearts were analysed with immunohistochemistry, using anti-PKG antibodies, and with electron microscopy. Right ventricular gene expression was analyzed. Amplification and labelling of RNA samples was followed by cDNA synthesis and labelling. This cDNA was hybridised to probe arrays and scanned confocally, after which the data were analysed. Statistical analysis was also performed to prove the significance of the outcomes.

The researchers found a correlation of training intensity with training-induced right ventricular enlargement in PKG heterozygous mice. The trained PKG +/- mice also showed increased spontaneous ventricular ectopy and VTs, which were mostly self-terminating, and prolonged activation times of the RV. Features of the trained and sitting rats that did not differ were absence of fibrosis, cardiomyocyte hypertrophy, and fibrofatty replacement. The structures of desmosomal and adherens junctions were not different either. This should not be confused with the results previous studies however: because these animals are heterozygous, there is functional PKG present, just not as much as in wildtypes.

Gene expression analysis revealed that training had induced expression of several hypertrophy-associated genes between trained and non-trained mice. There was no difference in gene expression between the different genotypes.

This study thus demonstrated for the first time that reduced PKG expression is sufficient for the development of an ARVC-like phenotype. Decreased expression or function of junctional proteins is a key element in the development of the disease. Training showed to reduce right ventricular function and provoke arrhythmias, so ARVC patients can be recommended to avoid endurance training.

Normally, PKG is upregulated in response to endurance training. The deficiency of one PKG allele is in this case sufficient to cause the phenotypic changes that are reported, so one intact allele is not sufficient to sustain normal cardiac PKG function. This clarifies the fact that myocardial wall stress elicits an abnormal response, and a reduced myocardial compliance could contribute to premature right ventricular dilatation in trained PKG +/- mice.

Surprisingly no fibrofatty replacement was found in the RV, while the right ventricular conduction velocity was decreased. This weakens the hypothesis that fibrofatty replacement slows down right ventricular conduction velocity. The researchers come up with some possible explanations. One is that the histological changes found in ARVC patients may develop later than the functional changes. The second explanation is that the structural abnormalities in ARVC patients are caused by genetic abnormalities that do not result from PKG deficiency. Thirdly, abnormal function of mutated junctional proteins may induce the structural changes in ARVC instead of the reduced levels of protein in the used murine model.

So, deletion of one PKG allele alters the RV in its contractile and electrophysiological characteristics. Myocardial structure and gene expression remain the same. Issues that still need to be solved are the mechanisms by which a PKG deficiency causes right ventricular dilatation and arrhythmias, and the cause of the structural abnormalities in ARVC hearts.

Load-reducing therapy prevents development of arrhythmogenic right ventricular cardiomyopathy in plakoglobin-deficient mice [36]

In 2011 Fabritz *et al.* published an article in which they introduced load-reducing therapy as treatment for ARVC patients. There is still no cure for this disease as considered in the introduction of this thesis, but avoiding endurance training could slow it down. Based on the theory that endurance training accelerates the development of ARVC in mice[33], Fabritz *et al.* hypothesised that chronically increased volume load may contribute to the development in ARVC in susceptible patients.

For this study adult PKG +/- mice and their wild-type littermates were used, which underwent a baseline echocardiography followed by 7 weeks of endurance training by daily swimming from 5 minutes per day increasing up to 90 minutes per day. The mice were randomly divided in two groups; one received load-reducing therapy with the loop diuretic furosemide and nitrates, administered via drinking water, and the other received only drinking water. Daily water intake was monitored. The therapy did not cause unwanted side effects, while the blood and RV pressure were reduced. After training, electrocardiography was executed, followed by the isolation of the hearts for electrophysiological examination in a Langendorff apparatus. The following step consisted of Western blotting of left ventricular tissue. Antibodies were used against PKG, N-cadherin and glyceraldehyde-3-phosphate (G3P) dehydrogenase. Also the membrane proteins were isolated and analysed by Western blotting. These samples were incubated with anti-calnexin, anti-Cx43, and anti-PKG or anti-β-catenin. After that, immunofluorescence was performed with antibodies specific for DSP, β-catenin, PKG and PKP2, and immunohistochemistry was performed with anti-Cx43 antibodies. To assess the relative gene expression of PKG +/- mice compared with wt tissue qPCR was executed on two housekeeping genes. Of course statistical analysis was done to reveal the significance of the results.

Results showed that load-reducing therapy actually prevents right ventricular enlargement and induction of VTs (VTs). In untreated trained +/- mice the RV was enlarged, and more VTs occurred, while in the treated trained +/- mice none of this was the case. The induction of VTs was associated with conduction block and macro-re-entry, and whether anatomic factors or functional effects caused prolongation of activation was further investigated by ventricular activation maps during pacing from the centre of the electrode. It appeared that longitudinal RV conduction velocity was reduced in untreated +/- mice, which was different from the treated hearts, which showed normal conduction velocities.

Furthermore, in +/- hearts compared to wt mice a decrease of PKG expression was observed, completely in concordance with the previous PKG studies. Also Cx43 concentration was lower in untreated +/- hearts than in the wt, and the wt and treated +/- hearts showed the same Cx43 levels.

From these results can be concluded that the applied load-reducing therapy does indeed prevent development of ARVC in a model with reduced PKG expression. The therapy prevented RV enlargement, conduction slowing and VTs. That made the treated PKG-deficient mice phenotypically indistinguishable from the wt animals. The hypothesis that reduced myocardial PKG concentration is a key component in the development of ARVC is supported by this study, as well as by previously described ones. Further characterisation of cell adhesion complexes in ARVC is however still needed. The overall message of this article is still that load-reducing therapy may be hope giving to ARVC patients, and the conclusions invite further validation of this therapy in other ARVC models as well as in patients.

PKG thus appears to be an extensively studied desmosomal protein. It was clear to see that the discussed studies reached a higher level in understanding and knowledge the later they were published. A large basis of knowledge is clearly built up on this subject in a relatively short time period. In the following sections the state of research concerning the other desmosomal proteins will be discussed, with an emphasis on the used animal models. Especially PKP2 is studied relatively intensively, however only one animal study is used up until now.

Plakophilin-2

General information

Plakophilin-2 (PKP2) is an armadillo protein that is part of the outer dense plaque of the desmosome, as is PKG. A difference with PGB is that PKP2 belongs to the $p120^{ctn}$ subfamily, which is distinct from the subfamily that PKG belongs to. The N-terminal domain is for instance 275-380 residues long, which is considerably longer than the N-terminal domain of PKG.[39] Its gene is located on chromosome 12p11. Homologues of this protein are verified in species like wolf, cow, mouse, chicken and zebra fish, indicating a strong evolutionary conservation [47].

PKP2 exhibits an important task in the desmosome by providing lateral association of the desmosomal cadherins DSC and DSG using its N-terminal domain. It is also found to interact with PKG and DSP, and more surprisingly with nucleoplasmic RNA polymerase III complexes [48]. The function of the latter is still unknown.

Just like the other desmosomal proteins PKP2 has an important role in modulation of GJs. This is shown in PKP2-deficient mice as well as *in vitro* where siRNA was used to silence expression of PKP2 [7]. More specifically, *in vitro*

loss of PKP2 expression causes Cx43 remodelling [49]. These studies also indicated the importance of PKP2 in normal heart function. The hearts of PKP2 knockout mice for instance showed lethal abnormalities, as will be discussed further [48]. In humans, autosomal dominant mutations in the PKP2 gene are the most common genetic cause of ARVC, contributing to approximately 70% of the familial ARVC cases [7]. There are 192 mutations identified, of which the majority is pathogenic (level on 15 April 2011) [50].

A study on plakophilin-2 knockout mice

Requirement of plakophilin-2 for heart morphogenesis and cardiac junction formation[48]

This study is the only publication to date using transgenic PKP2 deficient mice. The technique of generating the null mutation of PKP2 is comparable to the generation of PKG deficient mice discussed before. The produced heterozygous mice were mated, but no live offspring arised, suggesting that the PKP2 mutation was lethal in embryogenesis. This was indeed confirmed by genotyping embryos from different developmental stages using polymerase chain reaction (PCR) and inspecting them visually. Mutant embryos showed blood accumulation in the pericardial and peritoneal cavities around day 10.75 of embryogenesis (E10.75), and after that stage the expected Mendelian ratio of homozygous mutant embryos declined steeply. In E10.75 PKP2 -/- embryos was shown that PKP2 was absent in the heart, while in wt embryos the same treatment a strong PKP2 signal was observed in atria and ventricles.

The cause of lethality in -/- mice was determined by examination of embryos in stage E9.5 and later. E10.75 -/- embryos did not show homogeneously distributed blood throughout their bodies while the blood had accumulated in the pericardial and peritoneal cavity. In accordance with this finding, blood was present in neither the yolk sac nor the blood vessels, although the vasculature was intact. The -/- mutants also showed reduced trabeculation in the heart ventricles and thinner atria walls. The blood in the pericardial cavity was then explained by blood leakage through small perforation, because overt ruptures of the heart walls were not found. Later in embryonic development the -/- mice showed in addition blood leakage, and swollen pericardial and peritoneal cavities, after which they became necrotic.

Now the effects of the mutation were investigated on a more molecular level, using confocal laser-scanning and double-label immunofluorescence microscopy to assess the molecular localisations in the IDs of wt and -/- mice. An interesting finding was that in -/- mice DSP did not colocalise with any labelled junctional protein, among others PKG, PKP2 and DSG2, while in the wt mice DSP did colocalise with all labelled junctional proteins.

Electron microscopy showed furthermore that wt embryos had well-organised IDs in their cardiomyocytes with clear localisation of DSP in desmosome-like and fascia adhaerentes-type junctions, while in the hearts of -/- mice the location of DSP was severely altered. DSP was virtually absent from all junctions, but present in the cytoplasm, in granular aggregates between bundles of myofibrils or in association with IFs. The distribution of other plaque components however was not significantly different. The changes of cytoskeletal organisation caused by the PKP2 deficiency were also investigated. IFs were often observed in conspicuous arrays around the DSP aggregates. The PKP2 deficiency did not seem to have effect on the ultrastructure of adherens junctions and desmosomes in epithelial tissues, aorta and endothelium.

Biochemical analysis of cardiomyocytes showed that in wt mice junctional proteins such as PKP are not easily extracted by nondenaturating detergents such as Triton X-100, which is supported by previous demonstrations. In contrast, DSP, DSG2 and PKG were largely Triton X-100 soluble in PKP2 deficient mice, whereas cytoskeletal

assocociations of β-catenin and N-cadherin were unchanged. This suggests that PKP2 deficiency causes a reduction in the association of the cardiac junctional plaques with DSP, PKG and desmosomal cadherins. This is consistent with the reduced architectural stability of the IDs.

The conclusion based on these results is as follows. PKP2 deficiency causes defective cell-cell adhesion at the IDs and leakage of blood into the pericardial cavity. Plaque proteins are expressed in a lesser extent. These alterations all take place in the same developmental stage (E10.5-E12), and the pathological alterations are comparable with those caused by PKG mutation. [40, 46] Taking this together, it can be concluded that PKP2 is a key organiser of cardiac architecture during embryogenesis. This conclusion is also sustained by observations on PKP2-binding proteins in cultured cells.[48] PKP2 is the only PKP present in cardiac adhering junctions, and this may explain the sensitivity of the effects of the PKP-2 deficiency, for there is no other PKP that can compensate.

Furthermore, in PKG-deficient mice DSP is still firmly associated with the ID, while in PKP2 deficient mice this association is no longer present. PKP2 is thus essential for fixing DSP to the junctional plaques of cardiomyocytes. Nevertheless, several aspects of the ID stay intact, for instance several armadillo proteins such as β-catenin and PKG remain at their right places. This indicates that their binding to other plaque components is sufficient for their junctional integration.

The fact that several epithelia in PKP2 deficient mice appear to have normal desmosomes can be explained by the presence of PKP3, which might compensate in a structural and functional way.

Finally, the researchers point to the fact that other mutations than the obviously lethal null mutation in PKP2 can lead to human heart disease. The role of PKP2 in ARVC is established in a later stadium based on epidemiologic and *in vitro* studies.

Other studies concerning plakophilin-2 mutations

Research on the influence of PKP2 mutations has been done more extensively *in vitro* than in mice. Some of these articles will be discussed in the following sections. These studies have been found useful in unravelling the molecular pathology of ARVC. *In vitro* studies have been executed in two ways: either using cultured cells, mostly rat cardiomyocytes, or patient material. Cultured cells are used for a genotype-up approach while patient material is used for phenotype-down analysis.

Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy [51]

Gerull *et al.* were the first to show in 2004 an association between PKP2 and the occurrence of ARVC.[51] At that date, genomic loci and mutations in PKG, DSP and RYR2 had already been linked to ARVC. The association of PKP2 and ARVC was based on the study by Grossman *et al*.[48], using PKP2-deficient mice, as discussed above. To test the hypothesis that PKP2 mutations may lead to ARVC, DNA from patient material as well as material from family members was sequenced. Mutations in PKP2 of various kinds were detected in 27% of the ARVC patients. Also, in one right ventricular biopsy there was no truncated protein detected but only a decreased amount of wt PKP2, which indicated haploinsufficiency. Furthermore, certain mutations were identified in patients, while these mutations in others caused very mild symptoms. The cause for these differences in penetrance is unknown, but it may be related to a variety of factors, like gender, epigenetics or viral infections.

The investigators hypothesised that the lack of functional PKP2 challenged proper desmosome formation. Support for this idea came from the identification of recessive mutations in PKP1 which caused ectodermal dysplasia/skin fragility syndrome, characterised by small, poorly formed desmosomes and odd desmosome-keratin IF interactions. The murine study discussed above confirms this idea as well. It was however still unknown how the PKP2 mutation caused the disturbance of desmosome assembly.

Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy [52]

Now that the first association with ARVC and PKP2 was established, Van Tintelen *et al.* studied 96 ARVC patients and their family members.[52] They reasoned that if the PKP2 mutations occurred indeed as frequently as suggested by Gerull *et al.*, namely in 27% of ARVC patients, genetic screening could be an important tool in diagnosing persons at risk. This was evaluated by screening the ARVC patients and their relatives, with the goal to establish the prevalence and character of PKP2 mutations as well as phenotypic differences. More specifically, the material was analysed on PKP2 mutations as well as on haplotype, and after that the clinical data between mutation and nonmutation carriers were compared.

In this study an even higher prevalence of PKP2 mutations was found: 43% of the ARVC patients were carrying 14 different PKP2 mutations, that resulted mostly in a truncated or aberrant protein. There was however no specific correlation between genotype and phenotype, so the clinical course of ARVC cannot be predicted from DNA analysis alone. However, all five index patients with familial ARVC and sudden death in family members under 35 years of age were PKP2 mutation carriers.

Genetic analysis is thus shown to help identify persons at risk for ARVC, keeping in mind that part of the ARVC cases are of nongenetic origin. The high yield of PKP2 mutations demonstrates its predominance in genetic origin of the disease. This study has reinforced the position of PKP2 as a cause of ARVC.

Connexin 43 remodelling caused by inhibition of plakophilin-2 expression in cardiac cells [49]

As a natural next step the molecular pathogenesis of ARVC caused by PKP2 mutations can be investigated. Oxford *et al*.[49] investigated the influence of PKP2 on the function and distribution of the GJ protein Cx43. This was initiated by the findings of Saffitz *et al.* stating that disruption of mechanical coupling may lead to loss of electrical communication mediated by GJs. Oxford *et al.* wanted to confirm this hypothesis in a cellular model, where protein expression can be manipulated directly. In cardiac myocytes and epicardium-derived cells (EPDCs) PKP2 expression was silenced using siRNA. An important effect of this was the redistribution of Cx43 inside the cell, loss of GJ plaques and a reduction in Lucifer yellow-permeable GJs between cells. Cx43 and pkp2 are shown on beforehand to coexist in a macromolecular complex, which is affirmed in this study, and also this is the first study in which a PKP2 deficiency and Cx43-mediated cell-cell communication is demonstrated. Cx43 is clearly remodelled, as it seems that Cx43 is more concentrated in the perinuclear region instead at the cell membrane. Cx43 remodelling may be one of many factors that form a substrate for arrhythmias during ARVC.

A question that arises from this study is whether the ARVC-relevant mutations can alter the balance of epicardial-mesenchymal transformations, and whether that can cause fibrofatty infiltration. The cause of this fibrofatty

infiltration however is still a point of dispute; disturbance of the Wnt pathway has been proposed, as well as a differentiation of cardiac stem cells into adipocytes instead of cardiomyocytes [53].

To sum up, this is the first demonstration of an intermolecular crosstalk between PKP2 and Cx43. GJs appear to participate in the pathophysiological processes that lead to ARVC, at least in one group of patients. The exact mechanisms of the molecular crosstalk and its consequences to the function and communication of cardiomyocytes need still to be determined.

Abnormal connexin43 in arrhythmogenic right ventricular cardiomyopathy caused by plakophilin-2 mutations [54]

The connection between Cx43 and PKP2 was further investigated by Fidler *et al*. in 2008. They also point to the fact that abnormal gap junctional Cx43 expression has been reported as well in the autosomal dominant ARVC variants Naxos and Carvajal disease, caused by mutations in PKG and DSP respectively. Therefore, the hypothesis was that PKP2 mutations would lead to reduced expression and/or localisation of Cx43 at IDs.

The complete PKP2 gene was sequenced in ARVC patients, and endomyocardial biopsies were analysed for the presence and location of ID proteins. Also, mouse cardiomyocyte cell cultures were used in which PKP2 was silenced using siRNA. Cx43 levels were analysed using Western blotting after that, assessing Cx43 expression after PKP2 suppression.

It appeared that the suppression of PKP2 caused a decrease in Cx43 expression in all patients. This was confirmed by PKP2 suppression using siRNA in cultured cardiomyocytes. This could potentially explain the delayed conduction and propensity to develop arrhythmias in ARVC.

An interesting finding was that the level of the non-phosphorylated form of connexin43 was decreased more than the phosphorylated form. This could be explained by an increased degradation of the cytoplasmic form, which is non-phosphorylated, and the redistribution of Cx43 to perinuclear regions, where the protein is phosphorylated.

PKP2 silencing in cultured cells caused a dramatic reorganisation of Cx43, and the Cx43 expression pattern was similar to that seen in PKP2 mutated patients. Also, less GJ protein was noticed at cell borders. This, and the results from tissue of patients, suggest that PKP2 alterations have a deleterious effect on GJ localisation and expression.

The hypothesis that PKP2 mutations would lead to reduced expression and localisation of Cx43 at IDs was found correct, consistent with the fact that PKG and DSP mutations caused abnormal Cx43 expression. These findings point to a common mechanism for conduction delay and arrhythmias in ARVC.

Characterization of the molecular phenotype of two arrhythmogenic right ventricular cardiomyopathy (ARVC)-related plakophilin-2 mutations [55]

Only months after the Fidler study Joshi-Mukherjee *et al.* published another article concerning the changes in cellular function and structure that follow expression of mutated PKP2 in ARVC. The purpose was to investigate the changes in rat cardiomyocytes caused by expression of a mutated PKP2 protein where arginine at position 79 was replaced by a stop codon (R79x). Also cells expressing PKP2 with a frameshift at position 179 were used. Both of these mutations are in the N terminus of the protein, which is normally required for protein-protein interaction.

The mutated forms of PKP2 were created and a FLAG tag was placed at the C terminus. After that, adenoviruses were created which contained one of the two FLAG-tagged and mutated PKP2 genes, and the neonatal rat ventricular myocytes (NRVMs) were infected. To analyse the amount of PKP2 in each cell, antibodies against FLAG were obtained. Also, antibodies against DSP, β-actin, PKP2 and heat shock protein 90 (HSP90) were purchased. The anti-PKP2 targeted the C terminus of the protein and thus avoided detection of exogenous R79x and 179fs.

The expression and distribution of exogenous FLAG-tagged proteins R79X-FLAG, 179fs-FLAG and wtPKP2-FLAG were analysed by immunochemical techniques. To begin with, wt PKP2 was Triton insoluble, while R79x and 179fs were found Triton-soluble, suggesting a redistribution from the junctional complexes to the intracellular space. This was confirmed by immunolocalisation experiments where the wt PKP2 localised at sites of cell-cell apposition, while the truncated proteins were found in the intracellular space. This finding indicates that the N-terminal end of PKP2 lacks the necessary sequences for proper membrane localisation. Also, Cx43 showed to be reduced in R79X transfected cells.

Following this, dye transfer to assess intercellular communication was executed. Remarkably dye transfer was unaffected by R79X expression, despite the decrease in Cx43. This is different from the results from Oxford *et al.* that indicated that loss of PKP2 in rat ventricular myocytes caused an approximate 60% decrease in cell-cell dye coupling [49].

Co-immunoprecipitation experiments were executed after that to determine whether the mutations of PKP2 disrupted the association of PKP2 with DSP or Cx43. Wt PKP2 and 179fs retained the ability to associate with DSP and Cx43, but in the R79X cells DSP and Cx43 did not precipitate.

Then, the influence of the PKP2 mutations on apoptosis was investigated. This was done based on previous studies that suggested that ARVC relevant mutations induced apoptosis. The presence of apoptosis-related protein HSP90 was tested in the wt cells and in the cells expressing the mutant PKP2. A significant difference was found in R79X cells that showed a loss of HSP90. This suggests that this mutation causes active modification of the cellular and molecular phenotype.

In conclusion, R79X and 179fs failed to localise to the cell membrane, but the localisation of endogenous PKP2, DSP and Cx43 was not altered. R79X was showed to affect myocyte function, based on the following findings. Cx43 expression was reduced, the interaction of PKP2 with DSP was lost and HSP90 expression was almost lost. This lead to the hypothesis that the ARVC phenotype is not caused only by PKP2 haploinsufficiency but also by the presence of the R79X fragment. It must however be noted that dye transfer was normal in R79X cells. Thus, R79X may not be the cause of poor electrical coupling in ARVC. In patients however two factors come together, which are PKP2 haploinsufficiency and R79X expression. Both factors influence Cx43 and could together disrupt GJ-associated cellular functions, for instance electrical coupling. The fact that HSP90 is a cardioprotective protein implicates that the loss of it by R79X leads to facilitated activation of apoptotic pathways in the myocyte.

This study was the first to present the characterisation of the ARVC-relevant PKP2 mutation at the cellular and molecular level.

Loss of plakophilin-2 expression leads to decreased sodium current and slower conduction velocity in cultured cardiac myocytes [56]

The effect of PKP2 mutations on conduction in cardiomyocytes was further investigated by Sato *et al.* in 2009. Previously, PKP2 had been shown to localise preferentially to the voltage-gated sodium channel ($Na_V1.5$) of the ID, and Sato *et al.* aimed to investigate the association of PKP2 with sodium channels and its role in action potential propagation, more specifically the amplitude and kinetics of the sodium current and action potential propagation in a cardiomyocyte monolayer.

First, experiments were done to find out whether $Na_V1.5$ and PKP2 are present in the same molecular complex. This was done by concatenating glutathione S-transferase (GST) at the head domain of PKP2, and binding this recombinant protein to glutathione-sepharose beads. This was presented to a heart lysate. $Na_V1.5$ was indeed found to precipitate with PKP2, suggesting a physical interaction.

After that, the properties of I_{Na} were assessed as a function of PKP2 expression. To achieve this, short hairpin (sh)RNA was used to knock down PKP2 expression, and using a voltage clamp the sodium current was recorded. PKP2-silenced cells showed a decrease in peak current density, a shift in voltage dependence of steady-state inactivation and a prolongation of time-dependence of recovery from inactivation.

Referring to the findings of Oxford *et al.* where loss of PKP2 caused a strong decrease in cell-cell dye coupling[49], Sato *et al.* hypothesised that loss PKP2 would significantly affect propagation properties. It was indeed shown that loss of PKP2 caused slowing of action potential propagation, rate-dependent activation failure, and arrhythmic behaviour. Also, the conduction velocity was significantly decreased.

This was the first report showing a link between loss of PKP2 expression, impaired cardiac propagation, and loss of electric synchrony. Also the crosstalk between PKP2 and Cx43, and co-precipitation of Cx43 and $Na_V1.5$ was demonstrated. It has however to be seen whether these results, being obtained from an *in vitro* system, are relevant in the pathogenesis of ARVC.

Desmoplakin

General information

Desmoplakin (DSP) is part of the desmosome plaque together with PKG and PKP2. The transcript of the gene encoding this protein can be spliced in two ways, resulting in the variants DSPI and DSPII [57], weighing respectively 250 and 215 kDa [18]. DSPI is an obligate part of all desmosomes, while DSPII is predominantly or even exclusively present in stratified and pseudostratified epithelia [58], which implies that only DSPI is present in cardiomyocytes. Therefore, only DSPI will be discussed in the following sections, which will be abbreviated to DSP.

DSP is a relatively large protein, as the N- and C-terminal domains consist of almost 1000 amino acids, and its α-helix is of approximately the same size. It has been predicted that DSP forms dimers based on a coiled-coil interaction along the α-helix [39]. Its roles are both to provide lateral clustering of cadherins and to connect the desmosome to the IFs of the cell [18], by interacting with the IF subunit vimentin. Other interaction partners are PKP2, DSP and keratin [59]. The interaction with PKP occurs between the N-terminal domains of both proteins, and this is very important in the cadherin clustering properties. The C-terminal domain on the other hand is

responsible for the interaction with vimentin. Furthermore, the C-terminal domain contains three plakin-repeat domains (PRDs): PRD-A, -B and -C. These structures form beads on a string, where the linker residues between PRD-B and PRB-C are conserved in other plakin family members and are therefore hypothesised to play an important role in binding of intermediate filaments. This is confirmed in co-sedimentation studies, where the presence of this linker greatly enhanced the binding of constructs to vimentin [39].

DSP mutations are the cause of ARVC in 16% of the Italian patients, and in 6% in patients from the USA. Two autosomal recessive inherited syndromes have been associated with a mutated form of DSP: Carvajal syndrome and a Naxos-like disorder. The symptoms differ slightly; Carvajal patients present with dilated cardiomyopathy, woolly hair and palmoplantar keratoderma, while the Naxos-like patients have ARVC, woolly hair and an epidermolytic skin disorder. DSP mutations are also associated with typical autosomal dominant ARVC, considering the electrocardiographic and structural features [13]. Left ventricular involvement occurs in half of the patients, and the involvement of DSP mutation in that symptom was identified in 2005 [60]. The early left-ventricular involvement is not seen in ARVC that is caused by other desmosomal mutations, and it is therefore hypothesised that the DSP mutation results in disruption of the cytoskeletal integrity, which should explain this high prevalence of early left ventricular involvement [13].

Carvajal disease

In 1998 Luis Carvajal-Huerta from Ecuador published an article in which an association of woolly hair and palmoplantar keratoderma with epidermolytic hyperkeratosis was shown for the first time [61]. These symptoms were very often associated with dilated cardiomyopathy, which defined a cardiocutaneous syndrome.

Carvajal had studied 18 patients over the course of 27 years. They all showed palmoplantar keratoderma, woolly hair (see

Figure 5) and cutaneous lesions, associated with cardiac abnormalities. Based on the pedigree of several patients, an autosomal recessive inheritance pattern was likely.

Figure 5 A 15-year-old girl with woolly hair and palmar keratoderma [61]

Cardiac abnormalities began at an early age, except for two brothers, whose first symptoms presented in their thirties. The first abnormalities were only electrocardiographic and asymptomatic. Later the patients showed echocardiographic and hemodynamic features similar to dilated cardiomyopathy. Also either low voltage or intraventricular conduction disturbances or ventricular arrhythmias was observed in every patient.

Naxos disease was already discovered before this publication. Naxos patients also show palmoplantar keratoderma, curly hair and cardiac abnormalities. Carvajal concluded however that his findings were similar but not identical to this disease, because the Naxos patients do not show epidermolysis and the analysis of the skin biopsies showed different results.

Two years later the cause of Carvajal disease was cleared up by Norgett *et al.*, showing that all the included Carvajal patients were homozygous for the 7901delG mutation in the DSP gene [62]. This mutation led to a premature stop codon 18 amino acids downstream the mutation. The C-terminal domain is truncated because of this, and the interactions with IFs are impaired.

The structural and molecular pathology of Carvajal disease was cleared up further in 2004 [63]. An 11-year-old Ecuadorian patient was studied extensively. At initial cardiologic examination she was still asymptomatic but showed premature ventricular contractions, left ventricular dilatation and apical hypokinesis. Later she was hospitalised and died, and she and other affected family members were proven to be homozygous for the 7901delG mutation in the DSP gene. *Post mortem* the heart of the girl was studied more extensively. The right atrium and LV were dilated. The apoptotic index was high in both sides of the heart. Furthermore, the distribution of DSP, PKG, Cx43, N-cadherin and DSC was assessed. The immunoreactive signal for DSP was virtually absent, PKG and Cx43 signals were diminished, and N-cadherin and DSC were normal in patients compared to controls. Furthermore, the IF protein desmin failed to localise at IDs in the cardiomyocytes.

Although there was only one heart from a Carvajal patient available, it seems likely that the features reported here are representative for other patients, because the clinical characteristics were similar to those of other patients. The fact that desmin does not localise at the ID supports the hypothesis that a DSP mutation challenges binding to IFs. The conduction abnormalities and arrhythmogenesis that was observed could be explained by the DSP mutation as well, because this probably caused a Cx43 depletion, that affects formation or maintenance of electrical junctions.

At last, the researchers point to another mutation in DSP that had been recently discovered, namely the Gly2375Arg mutation. The clinical data resulting from that mutation seem to be more closely related to ARVC than to the more diffuse, dilated and aneurysmal cardiomyopathy associated with the 7901delG mutation. This however does underline the hypothesis that mutations in DSP can lead to cardiac and cutaneous diseases.

Studies concerning desmoplakin mutations

Novel mutation in desmoplakin causes arrhytmogenic left ventricular cardiomyopathy [60]

At the time this study was published, only disease-causing mutations in DSP and PKP had been identified as genetic causes of ARVC. It was already established that this disease inherited most often in an autosomal-dominant fashion. The two autosomal-recessive variants of ARVC, Naxos and Carvajal syndrome, had already been identified as well.

In this study a novel DSP mutation was shown to have a role in left ventricular involvement of ARVC. 27 members of a large family were analysed. 10 members of this family were diagnosed with ARVC according to the diagnostic criteria, with the help of ECG and exercise testing. Also, certain affected patients and controls were analysed with cardiac magnetic resonance (CMR), to assess ventricular volumes and function. All 10 ARVC patients showed predominant LV involvement. Normally this is more common in advanced disease, while in these cases the patients were still in an early stage of ARVC. More common symptoms such as inferior and/or lateral T-wave inversion were observed as well.

Whole blood samples from all subjects were then used to perform a genome-wide scan to map the causative gene, which was possible because the family had a sufficient number of affected individuals. This identified a single disease locus on chromosome 6. The DSP gene was a plausible candidate gene, which was confirmed using an intragenic microsatellite repeat. This marker correlated completely with the disease phenotype. After that, the site of mutation in the DSP was determined. This appeared to be a single nucleotide insertion, 2034insA, which caused a frameshift and premature stop codon 8 residues downstream of the mutation. Another confirmation came from the fact that this mutation introduced also a *Mse*I restriction enzyme site. Indeed, restriction degradation occurred in all affected subjects but not in controls. Finally, Western blot was used to confirm the transcription of both the wt and the truncated protein in patients. Because the mutation is located in the amino terminus, the rod and the carboxy terminus are lost, which disrupts binding to desmin. The amino terminus is shortened as well, which impairs cell adhesion.

The cardiac phenotype observed in these patients resembles that of Carvajal syndrome. This can be explained by the fact that the 7901delG mutation in DSP that is found in Carvajal patients also truncates the carboxy terminus. The patients included in this study however did not show cutaneous abnormalities. This can be explained by the fact that the 7901delG is inherited dominantly, and the patients are heterozygous for this mutation. The 2034insA mutation in Carvajal disease however is inherited recessively, and the patients are homozygous for this mutation. Thus, it can be concluded that in 7901delG patients expression of DSP, albeit partly in the mutated form, is sufficient for protection from palmoplantar keratoderma. It is however insufficient to sustain normal cardiac function, as can be concluded from the cardiac abnormalities.

Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy [64]

This study, published in 2002, was the first to identify DSP as a causative gene in ARVC. At the time, only for ARVC2 the causative gene had been elucidated. The index case for this study was an 18-year old male who experienced cardiac arrest while performing physical exercise, after which he was diagnosed with ARVC. His family, including 26 members in four generations, gave informed consent to be evaluated and to give blood samples for a DNA study. The 1994 McKenna criteria[27] were used for diagnosis, which turned out to be positive for ARVC in 11 family members. These individuals manifested severe or moderate or mild forms of the disease. Follow-up was done during 2 to 17 years, depending on the patient.

Genetic investigation was done, which revealed that none of the patients had a mutation that was already known to cause ARVC. That is why a genome-wide scan was executed, using 700 microsattelite markers. Significant positive lineage was found on 6p24. At the time, this was a novel locus for ARVC, and therefore it was named ARVC8, since numbering ARVC types had ended at ARVC7. A critical region was defined, which contained the gene DSP. This was a good candidate gene, because the relation between DSP and Carvajal disease had already been established. DSP was shown to have the missense mutation C1176G in every clinically affected individual, and in

some of the relatives whose clinical status is unknown. The mutation could not be detected in any of 240 control subjects, which suggests that this nucleotide substitution is pathogenic. On a protein level, the mutation caused a replacement of the serine residue (S299R). This altered the coiled region of the protein, which is probably involved in protein-protein interactions. Furthermore, the first 584 amino acids of the N-terminal domain, wherein the mutation is located, are already known to be involved in PKG binding. This firmed the base for the hypothesis that this mutation could be pathogenic.

DNA sequencing revealed also another variant in DSP, I305F. This was however a common polymorphism in the control population, and probably not pathogenic.

The researchers concluded that this S299R mutation should be rare, as this is the only family in which this specific mutation occurs among 16 families in which the linkage with ARVC loci had been established. The fact that the patients did not show keratoderma, in contrast with Carvajal patients, can be explained by the fact that DSP binding to IFs is reduced but not lost, or that the loss of the binding of DSP with IF could be compensated for by another desmosomal protein. It is also possible that the absence of skin defects in heterozygous carriers of the S299R mutation can be explained by considering that this mutation does not affect DSP-IF binding. A keratoderma phenotype will then be caused by another mutation that does target DSP-IF binding.

The most important conclusion drawn from this and previous publications is that ARVC might result from defects in intercellular connections. Besides RYR2 mutations, the elucidated ARVC causes are all mutations in desmosomal proteins, DSP and PKG. The mechanism that is proposed is as follows: mechanical forces apply to adherens junctions and activate stretch-sensitive calcium-permeable channels. The stretching of cardiomyocytes modulates the elementary calcium release from RYR release channels. Therefore, since the mechanical stress response is genetically impaired, this might affect intracellular calcium concentration and excitation-contraction coupling, leading to apoptosis and necrosis. This could promote fibrosis and adipose substitution. The selective involvement of the RV can be explained by its extensibility, in comparison with that of the LV. The hypothesis of the key pathogenic role played by altered intracellular calcium levels is supported by the RYR2 mutations that cause ARVC.

Studies on desmoplakin transgenic mice

Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal lineage[65]

The role of DSP in desmosome formation had already been investigated in mice before the relationship between ARVC and DSP had been established. In this study, several issues were to be solved. One was the possibility that desmosomes that are assembled from different proteins may have different properties and functions. Another was the potential redundancy of desmosomal proteins, which would make the use of knockout mice useless. At last, the role of cytoskeletal linkages to desmosomes had to be clarified.

To address these issues, DSP -/- mouse embryos were generated. The embryos were analysed by either whole mount immunofluorescence after dissection from their deciduas or by sectioning and conventional indirect immunofluorescence after partial dissection from their deciduas. This happened at various stages of development. Antibodies were used against various domains of DSP, E-cadherin, PKG, PKG, the keratin K8, DSCII, DSGII and PKP2 for confocal and electron microscopy.

Heterozygous (+/-) embryos appeared to develop normally. -/- embryos however did not survive until gastrulation. Targeting of DSP thus gave rise to early embryonic lethality. The cause of that lethality was examined by analyzing

DSP expression in the embryos at stage E3.5. This stage was chosen because DSP was suggested to be essential for the trophectoderm to withstand the mechanical stress imposed on the cells at blastocoel cavity formation. The embryos without a DSP signal did however form a trophectoderm and a blastocoel cavity, indicating that DSP expression is not required for this process.

Next, the embryos were examined at the early postimplantation stage, at E5.0. 25% of those embryos from heterozygous matings failed to stain with DSP. As controls, DSP staining was observed in the maternal vessels, and E-Cadherin was stained normally. Morphologically, these embryos were also similar to embryos with DSP staining. Thus, these data thus indicate that DSP mutant embryos can proceed through implantation.

Another stage later in development, at E6.0, DSP -/- mice were shown to be significantly smaller than normal. In wt embryos, DSP staining was restricted to surface endoderm, while this signal was absent in the small embryos. As a control, E-Cadherin-staining was however still normal in -/- embryos. This indicated that adherens junctions were still functional, despite the absence of cellular expansion. Since reduction in adherens junctions and cell degradation was detected from stage E6.5, further analysis was restricted at E6.0 and earlier.

The fact that -/- embryos were smaller at stage E6.0 is an interesting finding because wt embryos show a strong increase in cell proliferation at that stage. -/- embryos were apparently struggling with the massive wave of proliferation and cellular reorganisation, but this was not caused by an increase in apoptosis. The embryos were still alive, and it seemed that the defect was due to fundamental failure in extraembryonic tissues, because expression of DSP and desmosome formation only begin in wt embryonic ectoderm from E7.0.

Furthermore, -/- embryos showed abnormalities in the organisation of keratin IF networks, which showed after anti-K8 staining. These defects provide evidence that biochemical perturbations in the DSP null cells precede overt morphological aberrations visible in E6.0.

The structural consequences of DSP ablation were further analysed by localizing the desmosomal cadherins. -/- embryos clearly showed reduced amounts of K8, DSG2, DSC2 and PKP2. E-cadherin localisation was however not affected.

After that, E6.0 mutant embryos underwent detailed morphological investigation. Mutants were extremely fragile to mechanical dissection. They did however partially establish epithelial polarity and progressed through proamniotic cavity formation. The cells showed clear defects in cell-cell adhesion, especially in tissues normally expressing DSP, leading to gaps in the endoderm.

The number and size of desmosomes was furthermore decreased in the ectoplacental cone and endoderm compared to the postnatal epidermis. Also, the numbers of IFs and association of IF with desmosomes was smaller than in the epidermis. There were no desmosomes found in the embryonic ectoderm. Overall, mutants showed a 10 times reduction in desmosome number and a twice reduction in their size. This was consistent with the immunofluorescence data, which suggested that the absence of DSP results in a defect in desmosome assembly. The largest desmosomes found in the mutants did not show attached keratin filaments. Taking this all together, the researchers concluded that keratin filaments were disorganised because the number of desmosomes was reduced drastically and because the remaining desmosomes cannot attach IFs efficiently.

GJs, adherens junctions and tight junctions appeared to be largely normal in mutant E6.0 animals. This is different from E12.5 PKG null muscle cells, where adherens junctions were larger and more prominent than normal, and desmosomes were absent. Possible causes for this are for instance the functional differences in PKG and DSP, and tissue-specific differences between muscle cells and embryonic endoderm. To assess the influence of desmosome

shortage on PKG distribution and/or partitioning, immunoelectron microscopy was done using an anti-PKG antibody. This showed a weaker PKG signal in mutant tissue, which is probably explained by the smaller overall size of the desmosomes in the DSP deficient cells.

The conclusions of the paper were the following. First, DSP is required for linkage between IFs and desmosomes, which can be drawn from the disruption of keratin filament network and lack of attached IFs to the remaining desmosomes. This confirms previous findings, in which the DSP's tail segment binds IFs and its head domain associates with desmosomes. Second, DSP is required for assembly and/or stabilisation of desmosomes. This comes from the finding that lack of DSP results not only in IF disorganisation, but also in dramatic reduction in the number of desmosomes. The precise role DSP plays in assembly and stabilisation of the desmosomes has however still to be cleared up.

Desmosomes play an important role in embryogenesis. While adherens junctions are essential already in blastocoel cavity formation and epithelial polarisation, desmosomes are shown here not to be essential until the elongation of the egg cylinder, which clearly fails in mutants. The first problems appear however already between E5 and E6, when the proamniotic cavity forms and is considerably smaller than in control E6 embryos. It appears that the big increase in cell proliferation accompanied by required major changes in cellular organisation cannot occur without functional desmosomes to withstand the mechanical and organisational stress. It is also possible that cell signalling pathways may be perturbed because of the lack of desmosomes. An interesting finding hereby is that the DSP null embryonic ectoderm also became disorganised, because desmosomes are not present there at that developmental stage. This could be explained by a possible relation between desmosome assembly and signalling pathways between endoderm and ectoderm. The researchers point to the fact that this should however still be elucidated in the future. To sum up, these findings are a reflection of the functional importance of desmosomes during dynamic processes of tissue remodelling.

Compared to the PKP2 deficient mice, it is remarkable that the PKP2 deficient mice survive until stage E10.5-E12, which is 5 to 6 days longer than DSP knockout mice. DSP is shown to be essential to bind IFs to the desmosome, while PKP2 provides lateral association of desmosomal cadherins. Both proteins are however very important in the formation of the desmosome, and therefore a deficiency of either one causes great errors in embryonic development.

Finally, these findings are compared to the Bierkamp[46] and Ruiz[40] studies in which PKG knockout embryos were analysed. The most obvious difference was the timing when disturbances in development were detected. PKG -/- mice were normal until E12.5, which is more than 6 days longer than DSP -/- mice. A hypothesis for this difference is redundancy, as presumably PKP can compensate for the PKG loss until a certain stage. Another difference is that the PKG knockout showed a mixed type adhering junction with DSG2 spread diffusely over the cell surface. In DSP -/- embryos, DSG2, DSC2 and PKP2 were still clustered and colocalised. A final difference is that PKG null muscle cells showed larger adherens junctions, while DSP mutants showed unaffected adherens junctions. These differences suggest that PKG and DSP play different roles in desmosome formation and stabilisation. Future research should be performed, especially to compare the functions of these two proteins in the same cell type. This has been executed almost two decades later, in the study described below.

Desmosomal dysfunction due to mutations in desmoplakin causes arrhythmogenic right ventricular dysplasia/cardiomyopathy [26]

Four years after the publication discussed previously[65], another study was published concerning DSP transgenic mice in 2002.[26] In contrast with the former publication, this one does concern the relationship between a DSP mutation and ARVC. The DSP mutation had already been related to ARVC using patient material. The aim of this study was to establish a cause and effect relationship between the DSP mutations and ARVC.

This study used patient material, a desmosome-forming cell line and transgenic mice. In patient material, the frequency of DSP mutations was investigated by DNA sequencing. The cell line that was used was the human tongue squamous cell carcinoma SCC-9, and protein expression was assessed by immunohistochemistry. Cardiac-restricted DSP transgenic mice were generated, and at last, statistical analysis was done to investigate the significance of the findings.

DNA analysis of the North American patient population showed that 6% of the patients carried mutations in the DSP gene, including 1 nonsense and 3 missense substitutions. None of these mutations was identified in 200 control individuals. Two of these mutations, V30M and Q90R, occurred in the head region of the DSP protein, influencing the binding to the linker proteins PKG and PKP. One nucleotide mutation, 699G>A, was not detected in the cDNA, indicating that this substitution leads to haploinsufficiency. R2834H affects the DSP C terminus, which normally associates with IFs. The effects of these missense substitutions were assessed *in vitro* and *in vivo.*

To investigate the mutations *in vitro,* the SSC-9 cell line was transfected with WT DSP NTP (wt DSP, but with a truncated N-terminal domain), V30M DSP NTP, Q90R DSP NTP and W233X DSP (first 233 amino acids). WT DSP NTP was located at the cell membrane, but V30M and Q90R DSP NTP were found in the cytoplasm. This indicated that the V30M and Q90R mutations affect the localisation of DSP *in vitro.* W233X DSP was, as expected, very unstable and formed perinuclear aggregates. In addition, the cells were also transfected with FL DSP (full length). The N-terminal mutants, V30M and Q90R DSP FL, were still not located at the cell membrane. WT and R2834H DSP FL were present at the cell membranes. This suggests that the C-terminal mutation R2834H does not affect the function of the DSP N terminus.

Furthermore, the effects of these mutations on the interactions between DSP and PKG, PKP1 and PKP2 were investigated. This was done using coimmunoprecipitation. PKG coimmunoprecipitated with WT DSP NTP, but not with V30M or Q90R DSP NTP, suggesting that V30M and Q90R disrupt the binding ability of DSP N terminus. The coimmunoprecipitation with PKP1 and PKP2 was similar.

Then, transgenic mice were generated. This was successful for R2834H and WT DSP mice, but V30M and Q90R-Tg mice were not born. It was demonstrated that after E13.5 no more embryos were present. At E13.5, V30M-Tg and Q90R-Tg mice showed severely slimmed ventricular walls and ventricular dilatation, compared to R2834H-Tg, WT-Tg and nontransgenic (NTG) littermates. These mutations thus cause cardiac development abnormalities with cardiac noncompaction and early embryonic lethality, although death occurred in a later stadium than in DSP-/- mice [65].

After that, the phenotypes of WT-Tg and R2834H-Tg were compared, showing similar expression levels of DSP. At the age of 6 months, the R2834H-Tg mice showed many differences with NTG and WT-Tg mice. Their heart weights were relatively high, the ventricular cardiomyocyte cross-sectional area was 40% larger, the LV function was significantly decreased, and left ventricular end systolic dimension was increased. The structure and cardiac function of the R2834H-Tg was also severely compromised at the age of 9 months, showing a significant increase in

end diastolic volume and decrease in ejection fraction in both ventricles, as well as reduced RV wall thickness and RV thickening. This suggested RV dysfunction. There were no differences between NTG and WT-Tg mice.

Histologically, the R2834H-Tg mice showed also major differences with the NTG and WT-Tg mice. Histological studies in R2834H-Tg mice revealed focal myocyte loss and replacement by fibrous tissue in both ventricles. WT-Tg mice showed normal cardiac histology. Furthermore, R2834H-Tg mice showed neutral lipid accumulation in their cardiomyocytes, and increased cardiomyocyte apoptosis.

The R2834H-Tg mutation was furthermore shown to affect the interaction between DSP and desmin filaments. The colocalisation efficiency of DSP and desmin at IDs was significantly reduced. Other junctional proteins such as PKG, PKP2 and Cx43 localised normal. The IDs itself were altered as well, being irregularly shaped.

To examine the relative expression level and solubility of junctional proteins, Western blot experiments were done. In these experiments, proteins were isolated from the total cell lysate, the Triton-X100 insoluble fraction (proteins associated with the cytoskeleton) and the Triton-X100 soluble fraction (proteins unassociated with the cytoskeleton) were assessed. DSP and desmin were overexpressed in WT-Tg and R2834H-Tg mice. A remarkable finding was that a large amount of PKG was present in the soluble fraction of R2834H-Tg mice compared to NTG and WT-Tg mice. Also, the expression of PKP2 and β-catenin was upregulated in R2834H-Tg mice, especially in the soluble fraction. Finally, Cx43 appeared to be redistributed in to the soluble fraction. Taken together, these data suggest a disassociation of several junctional proteins from cell-cell junctions in the R2834H-Tg mice.

In short, this study identified 4 novel mutations in DSP. The effects of these mutations were identified *in vitro* and *in vivo*. A nonsense mutation in the N terminus of DSP, W233X, can lead to haploinsufficiency, whereas the V30M and Q90R mutations being missense mutations in the N terminus of DSP disturb the normal localisation of DSP. Overexpression of V30M and Q90R in embryonic mouse hearts leads also to embryonic lethality. This leads to the speculation that these N-terminal mutants disrupt the normal function of desmosomes and cause desmosomal instability. The desmosomes cannot cope with the mechanical stress, leading to cardiac dysfunction and lethality.

The overexpression of the C-terminal DSP mutation R2834H leads to extensive cardiac defects in adult mice. The desmosomes can be formed sufficiently to allow cardiac development, but after 6 and 9 months, severe defects like myocyte apoptosis, cardiac fibrosis and dysfunction are seen. Most of the defects were observed in both ventricles, which is consistent with previous findings in which ARVC patients with DSP mutations show LV involvement, in contrast with other ARVC-causing mutations. The mice did however not show myocyte replacement by adipose tissue, as observed in human patients. This can be explained by differences between the species, for instance in epigenetics. The R2834H mutation led also to failure of colocalisation of DSP and desmin at the IDs. This might lead to instability of desmosomes. Furthermore, it is believed that secondary changes to the disturbed DSP-desmin interactions are redistribution of desmosomal and GJ proteins, which may further contribute to the cardiac phenotypes. These data also suggest that desmosomes perform important roles besides the maintenance of tissue integrity, including apoptosis and lipid metabolism.

The researchers conclude that disruption of desmosomal integrity leads to ARVC, leading to defective mechanical linkage and abnormal localisation of proteins of cell-cell junctions and changes in GJs. These defects in the desmosomal so-called 'final common pathway' might lead to the ARVC phenotype, which includes fibrosis, adipocyte infiltration and arrhythmias. The hypothesis that ARVC is a disease of the desmosome is thus supported by this study. However, mechanisms of pathogenesis must still be elucidated further.

The research in the role of DSP in ARVC has thus already formed a firm basis. The amount of publications on this

subject is however rather low, with significant time gaps in between. The last two of the desmosomal proteins discussed in this thesis, DSG2 and DSC2, have however still less publications to discuss.

Desmoglein-2

General information

Desmogleins (DSG) are calcium-dependent glycoproteins and cell-adhesion molecules. Together with DSCs they are called cadherins, by virtue of their homology to the cadherin class of proteins [20]. The DSG family consists of four members, DSG1-4 [2]. The desmocollin family of cadherins contains three members, DSC1-3 [2]. The genes encoding the desmosomal cadherins are clustered in a small multigene family on chromosome 18q12.1. The domain architecture and characteristic sequence motifs are conserved between all members of the cadherin class [39]. Cadherins are single pass transmembrane proteins, with one transmembrane, one cytoplasmic and five extracellular domains. The intracellular portions interact with PKG and PKP2. Between cadherin molecules, recognition sites are composed of a motive consisting of the three amino acids HAV. This motive is localised to the adhesion dimer interface. The cadherins have a rod-shaped morphology, formed by linking the five subdomains with Ca^{2+}. Cadherins from opposing cells bind each other to form heterodimers, making them very important for physical interactions between two cells [7].

DSG2 consists of 1117 amino acids [66], whose homologues have been found in various mammals, like chimpanzee, mouse and wolf, as well as in the chicken [67]. DSG2 is expressed in many tissues, including the myocardium, in which it is the only DSG isoform [68]. The connection between ARVC and DSG2 mutations has been established in 2006, as will be discussed below.

Studies concerning desmoglein-2 mutations

Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy[69]

At the time this article was published, causative mutations in DSP, PKG, and PKP2 had already been identified. The hypothesis that ARVC is a disease of the desmosome was therefore evermore strengthened, what motivated Pilichou *et al.* to screen ARVC patients for DSG2 mutations.

An Italian ARVC population was clinically evaluated using a detailed personal and family history, physical examination, ECG, and echocardiogram. More invasive techniques including angiography and right ventricular EMB were performed only if necessary for diagnosis. Mutation screening was done in the coding regions of DSP, PKP2 and TGFβ3 genes. Based on the phenotype, mutations in RYR2 and PKG could already be exluded, because the patients did not show effort-induced arrhythmias or gross skin and hair impediments. More than half of the patients were negative for mutations in DSP, PKP2 and TGFβ3, and thus screened for DSG2 mutations by denaturing high-performance liquid chromatography and direct sequencing. A control group was also screened for the DSG2 sequences, to exclude the possibility that detected abnormalities were in fact SNPs. The splice site mutation that was found later was analysed further by mRNA isolation and PCR to amplify the suspected fragments. The identified mutations that altered the restriction sites were confirmed by restriction digest.

In five subjects, a right ventricular EMB was obtained, fixed and processed for histology. The diagnosis of ARVC was based on the presence of myocardial atrophy and fibrofatty tissue replacement. Three of these samples were also ultrastructurally investigated, using electron microscopy.

Nine mutations have been identified in DSG2 in 10% of the ARVC index cases, while PKP2, DSP and TGFβ3 mutations are responsible for 42.5% of this patients population. Of the 9 DSG2 mutations, 5 were missense, 2 insertion-deletions, 1 a nonsense, and 1 a spice site mutation. None of these mutations were found in controls. The five missense mutations, as well as Y87C, G100R and N266S, occurred in evolutionary highly conserved residues, indicating that these mutations could be pathogenic. K294E and E331K were located in the extracellular domains. These amino acid changes may destabilise the rod structure of the protein. Furthermore, the 4 basepair insertion in exon 9 introduces a premature stop signal, resulting in a truncated DSG2 molecule without transmembrane and cytoplasmic domains. These effects would also hold true for the nonsense mutation. The single base pair deletion in exon 14 would also cause a truncated protein, but with the transmembrane domain and without the intracellular segments, that would compromise normal interaction with PKG. The splice site mutation was shown to activate an alternative cryptic splice site in exon 13, as a result of which the mRNA contains a 38 basepair deletion and codes for a truncated protein of 646 amino acids that misses the cytoplasmic domain.

Clinical findings showed typical ARVC symptoms in mutation carriers, while family members not carrying DSG2 mutations were negative at clinical examination.

Light microscopy findings included a significant amount of fibrofatty tissue, as well as dysmetric and dysmorphic nuclei and prominent cytoplasmic vacuoles in all samples. Electron microscopy revealed furthermore a decrease in desmosome number and increase in desmosome gap in patient material. Also, abnormal small junctions and abnormally located desmosomes with pale internal plaques were observed.

In conclusion, DSG2 was identified as a novel disease gene involved in ARVC. 7 of the 9 identified mutations are located in the extracellular N-terminal domain, which impairs intercellular adhesion. This has however still to be determined in further studies.

The cellular consequences of the mutations are already shown in various studies, including DSG2 depleted transgenic mice and *in vitro* antisense experiments against DSC2. Those last experiments showed that lack of DSG2 caused decreased number of desmosomes, associated with increased asymmetry and detachment. This correlates with the findings of this paper. Further speculations are that these mutations lead to myocyte detachment, cell death and fibrofatty repair. The penetrance of the DSG2 mutations however has not yet been established. The fact that epidermis and hair were normal in all patients can be explained by compensation by other DSG isoforms. As DSG2 is the only form in the heart, mutations in DSG2 will lead to a cardiac-specific phenotype.

Ultimately, the researchers point to the fact that clinical diagnosis of ARVC is hard, and that mutation analysis could be a good way to make the diagnosis more easy.

DSG2 mutations contribute to arrhythmogenic right ventricular dysplasia/cardiomyopathy [68]

A rather surprising publication was done almost a year after the Pilichou *et al.* study discussed previously, also claiming to be the first to associate DSG2 mutations with ARVC. The methods were quite similar to those of Pilichou *et al.*, however, the identified mutations were different. All five identified mutations changed a highly

conserved amino acid in a functional domain of DSG2. As well as in the previous study, none of the subjects have woolly hair or keratoderma.

Two of the identified mutations, R45Q and R48H, for instance led to a disruption in the furin-cleavage site of the DSG2 proprotein. This proprotein has an N-terminal propeptide sequence that keeps the DSG2 inactive until it is posttranslationally cleaved to form the mature, active protein. The proprotein convertases cleave C-terminal to the recognition motif RXKR, and mutations in this recognition site prevent endoproteolytic processing and abolish the function of DSG2. This is what probably happens because of the R45Q and R48H mutations. Also, the C506Y mutation was identified. This meant a loss of a sulphur residue, which normally participates in disulfide bonding in the extracellular anchor domain. The fourth mutation, G811C, occurred in the intracellular cadherin-typical sequence of DSG2. This region normally binds DSG2 to PKG, which is probably challenged by this mutation.

One individual carried the R48H mutation as well the W305X mutation. His unaffected mother shares the W305X but not the R48H mutation, and with no information from the father or siblings, the mutation may also be *de novo.* Three of the four probands carried a single heterozygous missense mutation. The fourth proband however had both a missense on one allele and a nonsense mutation on the other. This means that the DSG2 mutations can cause ARVC in a recessive or dominant way of inheritance. If the disease inherits recessive, the three probands could carry a second mutation that has not yet been identified. The disease can also inherit dominantly, with low penetrance, as has been reported for other genetic causes of ARVC.

The low penetrance was evident, as described in other forms of ARVC. This is however not automatically the case for the DSG2 mutations. One of the cases carrying a W305X mutation had a mother and sister with the same mutation, but no phenotypic manifestations. This can be explained by incomplete penetrance, but this mutation may also be insufficient to result in ARVC, implicating that these patients may have another disease-causing mutation. Whether haploinsufficiency is indeed the mechanism causing ARVC has yet to be determined, and for this, individuals with mutations that prevent transmembrane anchoring of DSG2 are required.

In conclusion, the researchers report that DSG2 mutations contribute to the development of ARVC, although the mechanism of the development of the disease is not yet known. It does however provide further evidence that errors in desmosomal proteins are important of the pathogenesis of ARVC.

A study on desmoglein-2 transgenic mice

Myocyte necrosis underlies progressive myocardial dystrophy in mouse DSG2-related arrhythmogenic right ventricular cardiomyopathy [70]

This 2009 study by Pilichou *et al.* is not the first study concerning DSG2 mutations in transgenic mice. A publication on DSG2 null mice had already been done in 2002, in which the function of this protein was examined [71]. All DSG2 -/- embryos and a large part of DSG2 +/- embryos died shortly after implantation. The blastocysts were normally formed, but immunofluorescence analysis showed abnormal DSP distribution. DSG2 seemed to be especially essential in the inner cell mass, and evidence was showed that DSG2 is needed for normal ES cell proliferation, when DSP is not yet present. DSG2 has thus an important role in the survival of ES cells and embryos, especially in the stadium in which desmosomes are not yet formed.

Pilichou *et al.* examined an explanted heart of an individual carrying a N266S mutation. This mutation was already shown by Pilichou *et al.* in 2006 to occur in ARVC patients [69], located between the second and third extracellular

cadherin domains, being critical for Ca^{2+} binding. Then, in a mouse model the N271S mutation was introduced, which is equivalent to the N266S mutation in humans. This way, the phenotype of the transgenic mice could be compared to the human heart.

Three groups of transgenic mice were created: one group with cardiac-specific overexpression of FLAG-tagged wt DSG2 (Tg-WT), and the other two with N271S DSG2 (Tg-NS) in a high (Tg-NS/H) or low level (Tg-NS/L). The Tg-NS/H mice died significantly sooner than the Tg-NS/L mice. ECGs were recorded, showing typical ARVC symptoms, such as increased QRS duration and VT. The activation patterns of the hearts were analysed in Langendorff perfusion. Among a lot of other aberrations, the Tg-NS mice showed abnormal activation patters, indicating the presence of a conduction block, excessive conduction slowing and ventricular arrhythmias.

Gross cardiac morphology and main cardiac features were also different between the Tg-NS and Tg-WT and WT hearts. The Tg-NS hearts were increased in weight, with white streaks in the myocardium. The walls of RV and LV were increasingly thinning with age. Chamber dilatation and aneurysms were observed as well. Cardiomyocyte diameter was increased, while the thickness of the septum was also decreased.

Histological examination showed necrosis, inflammatory infiltrates and calcification in Tg-NS mice. These abnormalities progressed in time. In a later stage, nuclear abnormalities were also evident, as was lipid accumulation within the areas of necrosis. There were however no adipocytes present. These abnormalities were more severe in mice with a high level of the mutated DSG2 than in mice with a low level of that protein.

The first manifestation of the disease in the hearts of NT-Tg mice was however always necrosis. Macrophages would then be recruited to phagocyte the cardiomyocytes that became calcified. Also, the integrity of the sarcolemma was shown to be disrupted.

TUNEL-analysis was then executed to assess apoptosis in the myocardium. Apoptosis did not precede necrosis, as the level of cleaved caspase-3 levels did not increase much before three weeks of age, while necrosis was increased in an earlier stage. Electron microscopy was also done to reveal the ultrastructure of the desmosomes. The Tg-NS mice showed structurally normal desmosomes, with the desmosomal proteins and desmin and Cx43 distributed normally at the IDs.

In conclusion, it was interesting to see that the mice that expressed a mutated form of the protein showed the same pathognomonic feature of ARVC in humans. It is demonstrated that the disease is initiated by necrotic cell death, which triggers an inflammatory response and calcification, followed by fibrous tissue replacement. Apoptosis was only observed in a small extent. These represent hitherto underexposed aspects of ARVC, even though these mutations only account for a small subset of the patients.

It has already been shown that a mutation in a residue that is involved in Ca^{2+} coordination can lead to impairment of intercellular interactions of dimers. This N271S mutation affects one of these critical residues. The desmosomes however are structurally normal, in human as well as in mice. This implicates that the mutant DSG2 influences desmosome function rather than structure. A point mutation can thus be enough for a disease phenotype, while in other cases haploinsufficiency alone is not sufficient for that[40, 48].

On an electrophysiological level, the Tg-NS mice show a highly arrhythmogenic phenotype, and a high prevalence of ventricular arrhythmias. Sudden death was reported in 30% of the cases, in advanced as well in early stages of the disease. Conduction in the ventricles was affected as well. These findings are all parallel to symptoms in the human disease.

Adipocytes were not observed, but there was lipid accumulation where necrosis occurred. This was consistent with findings in other experimental ARVC animal models[26, 72]. As this does occur in human ARVC myocardium, this can be explained by differences between the species. Also, mice do not show epicardial fat, in contrast to humans.

Thus, transgenic mice overexpressing the DSG2 mutation N271S show pathognomonic features of human ARVC. Myocyte necrosis is the key initiator of the dystrophy of the myocardium. Further research should be done to unravel the molecular mechanism of ARVC pathogenesis.

Desmocollin-2

General information

Desmocollin (DSC) belongs to the cadherin family of proteins as well as DSG, and therefore contains five extracellular domains at the N terminus, one transmembrane domain, and a cytoplasmic tail at the C terminus. DSC is also known as desmosomal glycoprotein, DG [21]. In humans, there are three DSC isoforms, DSC1-3. DSC2 is ubiquitously expressed, and appears to be the only DSC in cardiac tissue [73]. The gene maps to chromosome 18q12.1 [21], and interaction partners are DSG2, PKG, DSG1, PKP2 and PKP2 [74].

DSG2 and DSC2 have overlapping functions in binding PKG and PKP2, which is probably mediated by the intra-cellular cadherin segment (ICS), a conserved region in the cytoplasmic domain. Alternative splicing of the DSC2 gene yields two subforms, DSC2a and DSC2b. The DSC2a protein contains the full ICS, while the ICS is truncated in DSC2b. The ratio between the a and b isoforms is tissue specific [75].

Of all the desmosomal proteins, DSC2 was the last to be shown to have a role in ARVC. More specifically, mutations in DSC2 attribute to ARVC11. Unfortunately, no mouse model is available yet, so two studies will be discussed shortly, one in which the relation between ARVC and DSC2 mutation is established, and one in which the consequences of a DSC2 mutation are addressed.

Studies concerning desmocollin-2 mutations

Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in the desmosomal gene desmocollin-2 [73]

Sometime before this publication, DSG2 mutations had been shown to cause ARVC. Because DSC2 is also a desmosomal cadherin, and it is the only desmosomal protein in cardiac tissue that has not yet been related to the disease, DSC2 was a plausible candidate gene for ARVC. Therefore, 77 patients that were diagnosed with ARVC but did not carry a mutation PKG, DSP, PKP2 or DSG2 were screened for mutations in DSC2. DNA from whole blood and paraffin-embedded tissue was extracted. Primers were used for each DSC2 exon, PCR amplification was performed, and these products were sequenced. Four subjects carried a heterozygous mutations; a deletion or an insertion. These mutations were also found in three family members. More specifically, in family A the 1430delC was detected in exon 10. This leads to a frameshift and a premature termination codon at position 480. In family B, C and D an insertion of two bases was found in exon 17 (2687_2688insGA). Four residues downstream a termination codon was introduced because of this.

These two mutations are both regarded as pathogenic, as they introduce premature stop codons. Especially the 1430C mutation can be harmful, as one-half of the DSC2 protein is lost, including the transmembrane and cytoplasmic components. This can lead to a non-functional mutant protein, but also to haploinsufficiency, as nonsense-mediated mRNA decay can occur.

Although DSC2 is expressed in many tissues, still no hair or skin abnormalities were detected. This is consistent with previous findings in which PKP2 and DSG2 mutations also caused only a cardiac phenotype. Besides that, disease penetrance is incomplete, for some subjects did carry a DSC2 mutation but did not meet the ARVC diagnostic criteria.

An interesting finding is that although ARVC classically presents with right ventricular involvement, the majority of probands had a more prominent left ventricular involvement. This is also reported in ARVC patients with mutations in PKG, DSP and DSG2, but the diagnostic criteria do not allow for left ventricular disease. This has to be investigated further.

In conclusion, in this publication DSC2 mutations are identified for the first time in ARVC. This provides even more evidence that ARVC is a disease of cell adhesion.

A novel desmocollin-2 mutation reveals insights into the molecular link between desmosomes and gap junctions[75]

Since DSC2 mutations are now shown to be related with ARVC, the molecular mechanisms can be elucidated. In this study, a patient is analysed that carried a truncation mutation of DSC2 and a missense variant in DSG2.

The patient was clinically evaluated, including history, physical examination, ECG, and echocardiography. The ARVC diagnosis was done according the latest diagnostic criteria, however, the patient did not fulfil these criteria. She did show clear evidence of a sub-clinical ARVC phenotype. RV biopsy samples were analysed histologically and immunohistochemically. At last, *in vitro* experiments were performed on HL-1 cells and neonatal rat cardiomyocytes (NRC) to localise certain proteins, and to assess the consequences of the DSG2 and DSC2 mutations.

Mutation screening revealed a heterozygous deletion in exon 16 of DSC2 (2554delA). This introduced probably a premature stop codon at position 855, truncating DSC2 by 47 amino acids of the DSC2a isoform. The patient also carried a heterozygous nucleotide change in exon 11 of DSG2 (1550C>T), which changed an alanine into a valine at position 517. The patient's daughter also carried both mutations, and had a borderline diagnosis of ARVC.

EMB showed interstitial and replacement fibrosis. N-cadherin was clearly present, but the immunoreactive signal for PKG was severely depressed at the ID. These findings are consistent with data from other ARVC cases.

Western blotting revealed that DSC2 and DSG2 expression was not significantly reduced. Other desmosomal proteins and desmin were at control levels, but Cx43 was mildly reduced. Also, a shift in electrophoretic mobility of Cx43 implicated a lower proportion of the highly phosphorylated protein. Immunofluorescence showed that Cx43 localised normally.

In vitro, mutant DSC2 and DSG2 localised normally, so the pathogenic effect should arise from the altered protein function in the desmosomes. Functional studies on the DSG2 mutation however did not uncover a pathological mechanism.

The mutation in DSC2 will cause a truncation of the DSC2a isoform, while DSC2b is probably not affected by the mutation. Pulldown experiments revealed that DSC2a wt bound to PKG and PKP2, while DSC2b bound to PKP2. Mutant DSC2a however did not bind PKG and DSP, while binding to PKP2 was unaffected. This suggests that the mutation interferes with intra-desmosomal protein interactions.

Patient myocardium showed changes in Cx43 total protein level and phosphorylation. This suggests a link between the DSC2 mutation and this GJ protein. This was confirmed by experiments that showed that only DSC2a WT could bind Cx43, and not the mutated form of DSC2a or DSC2b wt.

These results show a direct interaction between DSC2a and Cx43, and indicate that the mutation in DSC2a could contribute to changes in GJ proteins because of the inability to interact with Cx43. Furthermore, it is likely that the presence of mutant DSC2a reduces the capacity of desmosomes to retain PKG. This could contribute to the observed redistribution of PKG from the IDs. This may modulate Wnt-signalling pathways, and stimulate adipogenesis and fibrogenesis. Also, the patient did not have arrhythmias. This may be caused by the absence of a specific physiological trigger, for instance increased adrenergic tone.

In conclusion, this publication provided insight into the disease mechanisms of ARVC. Cx43 changes occur before any clinical manifestation that is significant enough to fulfil the diagnostic criteria. Also, the newly defined interaction between DSC2a and Cx43 may contribute to the interdependence between desmosomes and GJs, and this can open new doors on the way to new therapeutic approaches in ARVC.

Other animal models

The mouse is without doubt the most widely used animal model in ARVC research. Research in mice has many advantages, such as the possibility to produce transgenic mice relatively easily. This makes studying the influence of a mutation or insufficiency possible. Also, mice also are small, so housing is relatively easy, and reproduce fast. Spontaneous ARVC does however not occur in mice. Therefore, it is possible that the ARVC-like phenotype that occurs in a transgenic mouse lacking a desmosomal protein does not resemble the human ARVC phenotype to a sufficient extent. That is why research is also needed in animals that are evolutionary and physiologically more close to humans. It is also desired that these animals are spontaneous ARVC models. Up to this date, spontaneous ARVC has been described in two species, dog and cat.

ARVC in domestic cats

Fox *et al.* were the first to publish on the occurrence of spontaneous ARVC in another species than man [76]. Twelve domestic cats with ARVC were investigated. The cats were analysed using similar techniques as in humans, for instance ECG and echocardiography. They showed combinations of the following symptoms: right-sided congestive heart failure, supraventricular tachyarrhythmias, VTs, polymorphic ventricular arrhythmias and right bundle-branch block. Furthermore, all cats had enlarged hearts. Morphological abnormalities were also consistent with those in humans. These were RV dilatation, RV wall thinning, aneurysms in the RV and cavity dilation in RA and LA. Histopathology showed myocardial atrophy, RV myocarditis, inflammatory infiltrates, and fibrofatty replacement. The latter is especially interesting since mouse models of ARVC did not show this phenomenon. Apoptotic myocytes were also present to a significant extent.

In conclusion, ARVC in cats is very similar to that in humans. One important difference is however that none of the cats died of sudden cardiac death, which is common in human ARVC cases. Besides that, ARVC cats can be regarded as the clinical equivalent of human ARVC patients, and could be an important investigative tool to further investigate the pathophysiological mechanisms.

ARVC in boxer dogs

In 2004, Basso *et al.* published on the spontaneous occurrence of ARVC in boxer dogs [8]. The 23 dogs that were diagnosed with ARVC showed similar symptoms as seen in humans, such as sudden death, ventricular arrhythmias of suspected right ventricular origin, and syncope. The histopathological findings also correlated strongly with those found in human ARVC-afflicted tissue, showing myocyte loss and fibrofatty replacement, predominantly in the RV. Apoptosis was also identified, with a higher rate in the RV than in the LV. Each dog that died of sudden death showed myocarditis. 10 of the 23 investigated dogs showed familial occurrence. It is particularly interesting that the dogs showed fibrofatty replacement in the myocardium, because that is not detected in mouse myocardium.

These findings were all confirmed by Oxford *et al.* in 2007 [77]. This study also examined the subcellular localisation of N-cadherin, PKP2, PKG, desmin and Cx43. This showed that N-cadherin was not distributed differently in ARVC-afflicted dogs. The signals from DSP and PKP2 were however weaker than in controls. The localisation of the proteins seemed normal. PKG was not observed at all, and Cx43 was not present in at the end-end sites of the cells, indicating a loss of Cx43 at the ID. Desmin was detected throughout the cell, but not at IDs, as in control dogs. These results indicate a loss of GJ plaques. Also, genetic screening was executed for DSP, PKP2, PKG and Cx43, but none of these genes were mutated in the subjects. The genetic cause of ARVC in these animals could unfortunately not be revealed.

The hypothesis that ARVC is associated with disruption of the mechanical and electrical coupling between cardiomyocyte was supported by the data. The mechanism by which loss in mechanical coupling affects electrical communication is however not yet clarified. This mechanism could include either intermolecular communication between the proteins of desmosomes and GJs or the physical strain imposed on the GJs that is caused by the absence of functional mechanical coupling.

In summary, dogs with ARVC can be an appropriate model for ARVC. They are preferred above cats, because dogs with ARVC show sudden cardiac death, while domestic cats do not. However, still no publication can be found in which cats or dogs with AVRC are used to test therapeutic measures.

Conclusion

In this thesis, the research question as stated in the introduction has been answered extensively. The research question was: **what animal models are used for research in the role of desmosomal proteins in ARVC?**

This question has been answered for each desmosomal protein: PKG, DSP, PKP2, DSG2 and DSC2. Except for DSC2, for each of these proteins a transgenic mouse model was available. In some cases, the protein was knocked out completely, while other animals were heterozygous for the wt allele, or a mutated protein was expressed as it was identified in human ARVC patients. It is important to note that the production of these transgenic mice was only done after previous research that made the connection between a certain type of ARVC and a mutation in the desmosomal protein, based on patient material and family trees. Furthermore, in many cases the effects of the absence of the desmosomal protein was investigated *in vitro* before the transgenic mice were bred.

For PKG, null and heterozygous mice were bred. Null mice were not born, since the PKG deficiency caused heart rupture at E12. This was shown to be caused by an absence of functional desmosomes. Since PKG was the first to be discovered as a desmosomal protein involved in ARVC, more specifically in an ARVC subtype called Naxos disease, the research on therapeutic measures is relatively far ahead. Load-reducing therapy and avoidance of physical effort was tested on PKG heterozygous mice, with positive outcome. ARVC patients are therefore advised to limit their physical efforts to activities with low intensity. Load reducing therapy however still awaits testing in the clinical phase.

PKP2 has been studied relatively intensively, however only one publication concerning knockout mice can be found. This means that the effects of a PKP2 deficiency are cleared up *in vitro* more than in mouse models. In mice, the effects of the absence of PKP2 were studied. The deficiency caused defective cell-cell adhesion, and PKP2 was concluded to be a key organiser of cardiac architecture during embryogenesis. *In vitro,* the intermolecular crosstalk between PKP2 and Cx43 was established, and it was shown that PKP2 mutations lead to reduced expression and localisation of Cx43 at the ID. This means that a PKP2 mutation may influence GJs as well. Also, loss of PKP2 was shown to lead to decreased sodium current and a slower conduction velocity. These findings have however not been validated in mouse models yet.

Mutations in DSP were in the first place associated with Carvajal disease, which is just like Naxos disease an autosomal recessive subtype of ARVC. Later, the mutations causing a dominant form of ARVC were identified. These mutations were introduced in mice to examine its effects. DSP was clearly needed to establish the linkage between IFs and desmosomes. Also, DSP is needed for the assembly and stabilisation of desmosomes. Another study concerning transgenic mice established the relationship between DSP mutations and ARVC, as the DSP deficiency led to defective mechanical connections between cells, and changes in GJs.

The cadherin proteins DSG2 and DSC2 provide the actual intercellular connections in a heterophilic manner. After mutations in DSG2 were associated with ARVC twice, both publications claiming to be the fist, the DSG2 mutations were investigated in transgenic mice. This showed that myocyte necrosis is the key initiator of the dystrophy of the myocardium, and that mice overexpressing the DSG2 mutations showed pathognomonic features of human ARVC, which may indicate that the found mechanisms may be the same in human patients.

The mutations in DSC2 were relatively recently discovered and associated with ARVC, which may explain why no mouse model has been developed yet. One *in vitro* publication can be found in which insight is provided into the disease mechanisms of ARVC. In this publication, the interaction between DSC2 and Cx43 is indicated, and it is shown that Cx43 changes occur before any clinical manifestation that can fulfil the diagnostic criteria.

Ultimately, spontaneous models of ARVC are addressed in this thesis. In both domestic cats as boxer dogs ARVC can occur spontaneously. In cats, on a clinical and cellular level the presentation of ARVC seems very similar to that in humans. The similarities between the ARVC phenotypes of dogs and humans are even better, since sudden cardiac death has been described in dogs rather than in cats. These models have to be examined more closely on the similarities with humans. Then they could be an important asset in ARVC research, as their physiology is more closely to humans than mice.

Although the transgenic mice have helped to greatly enhance our knowledge on the effects of mutations of desmosomal proteins, the production of transgenic mice never stands alone. It is not just preceded by research on patient material and *in vitro*, it will also be followed by further research steps. The mechanisms of the disease will be evermore investigated in transgenic mice, and confirmation of the unravelled mechanisms will probably come from other animal models such as dogs and cats with ARVC. However, this does not mean that the mechanisms are the same in human disease. Therefore, therapeutic methods that are developed based on the animal research, such as RAAS inhibition, will be tested in a clinical phase. This will confirm whether the mechanisms as elucidated in the animals are valid in human disease as well.

On the whole, mouse models have greatly enhanced our understanding of ARVC and of the desmosome and the ID in general. Although the molecular pathogenesis is unravelled more and more, ARVC research is however not yet far advanced, as hardly any clinical study has been published. The fact that the disease is relatively rare means that international research will probably not pay much attention to it. However, since the disease is a major cause of sudden death in the young, this attention is indeed needed.

References

Image on title page:
http://www.understandinganimalresearch.org.uk/resources/images_library/details/195/black_mouse_in_gloved_hands/

1. Azaouagh, A., et al., *Arrhythmogenic right ventricular cardiomyopathy/dysplasia: a review and update.* Clin Res Cardiol, 2011.
2. Thiene, G., D. Corrado, and C. Basso, *Arrhythmogenic right ventricular cardiomyopathy/dysplasia.* Orphanet J Rare Dis, 2007. **2**: p. 45.
3. Marcus, F.I., et al., *Right ventricular dysplasia: a report of 24 adult cases.* Circulation, 1982. **65**(2): p. 384-98.
4. Nava, A., et al., *Familial occurrence of right ventricular dysplasia: a study involving nine families.* J Am Coll Cardiol, 1988. **12**(5): p. 1222-8.
5. Thiene, G., et al., *Right ventricular cardiomyopathy and sudden death in young people.* N Engl J Med, 1988. **318**(3): p. 129-33.
6. Basso, C., et al., *Arrhythmogenic right ventricular cardiomyopathy. Dysplasia, dystrophy, or myocarditis?* Circulation, 1996. **94**(5): p. 983-91.
7. Noorman, M., et al., *Cardiac cell-cell junctions in health and disease: Electrical versus mechanical coupling.* J Mol Cell Cardiol, 2009. **47**(1): p. 23-31.
8. Basso, C., et al., *Arrhythmogenic right ventricular cardiomyopathy causing sudden cardiac death in boxer dogs: a new animal model of human disease.* Circulation, 2004. **109**(9): p. 1180-5.
9. Basso, C., et al., *Quantitative assessment of endomyocardial biopsy in arrhythmogenic right ventricular cardiomyopathy/dysplasia: an in vitro validation of diagnostic criteria.* Eur Heart J, 2008. **29**(22): p. 2760-71.
10. Corrado, D., et al., *Implantable cardioverter-defibrillator therapy for prevention of sudden death in patients with arrhythmogenic right ventricular cardiomyopathy/dysplasia.* Circulation, 2003. **108**(25): p. 3084-91.
11. Corrado, D., et al., *Sudden death in young competitive athletes: clinicopathologic correlations in 22 cases.* Am J Med, 1990. **89**(5): p. 588-96.
12. Tiso, N., et al., *Identification of mutations in the cardiac ryanodine receptor gene in families affected with arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2).* Hum Mol Genet, 2001. **10**(3): p. 189-94.
13. van Tintelen, J.P., et al., *Molecular genetics of arrhythmogenic right ventricular cardiomyopathy: emerging horizon?* Curr Opin Cardiol, 2007. **22**(3): p. 185-92.
14. *ARRHYTHMOGENIC RIGHT VENRICULAR DYSPLASIA, FAMILIAL, 1: ARVD1* 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/107970.
15. *TRANSFORMING GROWTH FACTOR, BETA-3; TGFB3*. Online Mendelian Inheritance in Man 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/190230.
16. *RYANODINE RECEPTOR 2; RYR2*. 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/180902.
17. *TRANSMEMBRANE PROTEIN 43; TMEM43*. 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/612048.
18. *DESMOPLAKIN; DSP*. Online Mendelian Inheritance in Man 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/125647.
19. *PLAKOPHILIN 2; PKP2*. Online Mendelian Inheritance in Man 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/602861.
20. *DESMOGLEIN 2; DSG2*. Online Mendelian Inheritance in Man 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/125671.
21. *DESMOCOLLIN 2; DSC2*. Online Mendelian Inheritance in Man 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/125645.
22. *JUNCTION PLAKOGLOBIN; JUP*. Online Mendelian Inheritance in Man 2011; Available from: http://www.ncbi.nlm.nih.gov/omim/173325.
23. Saffitz, J.E., et al., *Effects of diminished expression of connexin43 on gap junction number and size in ventricular myocardium.* Am J Physiol Heart Circ Physiol, 2000. **278**(5): p. H1662-70.
24. Lombardi, R. and A.J. Marian, *Molecular Genetics and Pathogenesis of Arrhythmogenic Right Ventricular Cardiomyopathy: A Disease of Cardiac Stem Cells.* Pediatr Cardiol, 2011.
25. Abu-Issa, R. and M.L. Kirby, *Heart field: from mesoderm to heart tube.* Annu Rev Cell Dev Biol, 2007. **23**: p. 45-68.
26. Yang, Z., et al., *Desmosomal dysfunction due to mutations in desmoplakin causes arrhythmogenic right ventricular dysplasia/cardiomyopathy.* Circ Res, 2006. **99**(6): p. 646-55.
27. McKenna, W.J., et al., *Diagnosis of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Task Force of the Working Group Myocardial and Pericardial Disease of the European Society of Cardiology and of the Scientific Council on Cardiomyopathies of the International Society and Federation of Cardiology.* Br Heart J, 1994. **71**(3): p. 215-8.

28. Marcus, F.I., et al., *Diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia: proposed modification of the Task Force Criteria.* Eur Heart J, 2010. **31**(7): p. 806-14.
29. Cox, M.G., et al., *Arrhythmogenic right ventricular dysplasia/cardiomyopathy diagnostic task force criteria: impact of new task force criteria.* Circ Arrhythm Electrophysiol, 2010. **3**(2): p. 126-33.
30. Asimaki, A., et al., *A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy.* N Engl J Med, 2009. **360**(11): p. 1075-84.
31. Asimaki, A. and J.E. Saffitz, *The role of endomyocardial biopsy in ARVC: looking beyond histology in search of new diagnostic markers.* J Cardiovasc Electrophysiol, 2011. **22**(1): p. 111-7.
32. Hulot, J.S., et al., *Natural history and risk stratification of arrhythmogenic right ventricular dysplasia/cardiomyopathy.* Circulation, 2004. **110**(14): p. 1879-84.
33. Kirchhof, P., et al., *Age- and training-dependent development of arrhythmogenic right ventricular cardiomyopathy in heterozygous plakoglobin-deficient mice.* Circulation, 2006. **114**(17): p. 1799-806.
34. Wichter, T., et al., *Efficacy of antiarrhythmic drugs in patients with arrhythmogenic right ventricular disease. Results in patients with inducible and noninducible ventricular tachycardia.* Circulation, 1992. **86**(1): p. 29-37.
35. Marcus, G.M., et al., *Efficacy of antiarrhythmic drugs in arrhythmogenic right ventricular cardiomyopathy: a report from the North American ARVC Registry.* J Am Coll Cardiol, 2009. **54**(7): p. 609-15.
36. Fabritz, L., et al., *Load-reducing therapy prevents development of arrhythmogenic right ventricular cardiomyopathy in plakoglobin-deficient mice.* J Am Coll Cardiol, 2011. **57**(6): p. 740-50.
37. Stein, M., et al., *Reduction of fibrosis-related arrhythmias by chronic renin-angiotensin-aldosterone system inhibitors in an aged mouse model.* Am J Physiol Heart Circ Physiol, 2010. **299**(2): p. H310-21.
38. Chachques, J.C., et al., *Right ventricular cardiomyoplasty: 10-year follow-up.* Ann Thorac Surg, 2003. **75**(5): p. 1464-8.
39. Stokes, D.L., *Desmosomes from a structural perspective.* Curr Opin Cell Biol, 2007. **19**(5): p. 565-71.
40. Ruiz, P., et al., *Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart.* J Cell Biol, 1996. **135**(1): p. 215-25.
41. TyersLab.com. *BioGRID 3.1 Interaction Summary JUP* 2011; Available from: http://thebiogrid.org/109931/summary/homo-sapiens/jup.html.
42. *Plakoglobin - SNP results.* NCBI - Entrez SNP; Available from: http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp&cmd=search&term=plakoglobin
43. Protonotarios, N. and A. Tsatsopoulou, *Naxos disease.* Indian Pacing Electrophysiol J, 2005. **5**(2): p. 76-80.
44. McKoy, G., et al., *Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease).* Lancet, 2000. **355**(9221): p. 2119-24.
45. Kaplan, S.R., et al., *Remodeling of myocyte gap junctions in arrhythmogenic right ventricular cardiomyopathy due to a deletion in plakoglobin (Naxos disease).* Heart Rhythm, 2004. **1**(1): p. 3-11.
46. Bierkamp, C., et al., *Embryonic heart and skin defects in mice lacking plakoglobin.* Dev Biol, 1996. **180**(2): p. 780-5.
47. HomoloGene. *HomoloGene:3364.* 2011; Available from: http://www.ncbi.nlm.nih.gov/homologene?term=plakophilin-2.
48. Grossmann, K.S., et al., *Requirement of plakophilin 2 for heart morphogenesis and cardiac junction formation.* J Cell Biol, 2004. **167**(1): p. 149-60.
49. Oxford, E.M., et al., *Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells.* Circ Res, 2007. **101**(7): p. 703-11.
50. Van Der Zwaag, P.A., J.D. Jongbloed, and P. van tintelen. *ARVD/C Genetic Variants Database* 2011; Available from: http://arvcdatabase.info/Default.aspx.
51. Gerull, B., et al., *Mutations in the desmosomal protein plakophilin-2 are common in arrhythmogenic right ventricular cardiomyopathy.* Nat Genet, 2004. **36**(11): p. 1162-4.
52. van Tintelen, J.P., et al., *Plakophilin-2 mutations are the major determinant of familial arrhythmogenic right ventricular dysplasia/cardiomyopathy.* Circulation, 2006. **113**(13): p. 1650-8.
53. Merner, N.D., et al., *Arrhythmogenic right ventricular cardiomyopathy type 5 is a fully penetrant, lethal arrhythmic disorder caused by a missense mutation in the TMEM43 gene.* Am J Hum Genet, 2008. **82**(4): p. 809-21.
54. Fidler, L.M., et al., *Abnormal connexin43 in arrhythmogenic right ventricular cardiomyopathy caused by plakophilin-2 mutations.* J Cell Mol Med, 2009. **13**(10): p. 4219-28.
55. Joshi-Mukherjee, R., et al., *Characterization of the molecular phenotype of two arrhythmogenic right ventricular cardiomyopathy (ARVC)-related plakophilin-2 (PKP2) mutations.* Heart Rhythm, 2008. **5**(12): p. 1715-23.
56. Sato, P.Y., et al., *Loss of plakophilin-2 expression leads to decreased sodium current and slower conduction velocity in cultured cardiac myocytes.* Circ Res, 2009. **105**(6): p. 523-6.
57. Green, K.J., et al., *Structure of the human desmoplakins. Implications for function in the desmosomal plaque.* J Biol Chem, 1990. **265**(19): p. 11406-7.
58. Cowin, P., H.P. Kapprell, and W.W. Franke, *The complement of desmosomal plaque proteins in different cell types.* J Cell Biol, 1985. **101**(4): p. 1442-54.

59. TyersLab.com. *BioGRID 3.1 Interaction Summary DSP*. 2011; Available from: http://thebiogrid.org/108166/summary/homo-sapiens/dsp.html.
60. Norman, M., et al., *Novel mutation in desmoplakin causes arrhythmogenic left ventricular cardiomyopathy.* Circulation, 2005. **112**(5): p. 636-42.
61. Carvajal-Huerta, L., *Epidermolytic palmoplantar keratoderma with woolly hair and dilated cardiomyopathy.* J Am Acad Dermatol, 1998. **39**(3): p. 418-21.
62. Norgett, E.E., et al., *Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma.* Hum Mol Genet, 2000. **9**(18): p. 2761-6.
63. Kaplan, S.R., et al., *Structural and molecular pathology of the heart in Carvajal syndrome.* Cardiovasc Pathol, 2004. **13**(1): p. 26-32.
64. Rampazzo, A., et al., *Mutation in human desmoplakin domain binding to plakoglobin causes a dominant form of arrhythmogenic right ventricular cardiomyopathy.* Am J Hum Genet, 2002. **71**(5): p. 1200-6.
65. Gallicano, G.I., et al., *Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage.* J Cell Biol, 1998. **143**(7): p. 2009-22.
66. *Desmoglein 2 [Homo sapiens]* Available from: http://www.ncbi.nlm.nih.gov/protein/CAA81226.1.
67. *HomoloGene:1464. Gene conserved in Amniota* Available from: http://www.ncbi.nlm.nih.gov/homologene/1464
68. Awad, M.M., et al., *DSG2 mutations contribute to arrhythmogenic right ventricular dysplasia/cardiomyopathy.* Am J Hum Genet, 2006. **79**(1): p. 136-42.
69. Pilichou, K., et al., *Mutations in desmoglein-2 gene are associated with arrhythmogenic right ventricular cardiomyopathy.* Circulation, 2006. **113**(9): p. 1171-9.
70. Pilichou, K., et al., *Myocyte necrosis underlies progressive myocardial dystrophy in mouse dsg2-related arrhythmogenic right ventricular cardiomyopathy.* J Exp Med, 2009. **206**(8): p. 1787-802.
71. Eshkind, L., et al., *Loss of desmoglein 2 suggests essential functions for early embryonic development and proliferation of embryonal stem cells.* Eur J Cell Biol, 2002. **81**(11): p. 592-8.
72. Garcia-Gras, E., et al., *Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy.* J Clin Invest, 2006. **116**(7): p. 2012-21.
73. Syrris, P., et al., *Arrhythmogenic right ventricular dysplasia/cardiomyopathy associated with mutations in the desmosomal gene desmocollin-2.* Am J Hum Genet, 2006. **79**(5): p. 978-84.
74. TyersLab.com. *BioGRID 3.1 Interaction Summary DSC2*. 2011 [cited 2011; Available from: http://thebiogrid.org/108158/summary/homo-sapiens/dsc2.html.
75. Gehmlich, K., et al., *A novel desmocollin-2 mutation reveals insights into the molecular link between desmosomes and gap junctions.* Heart Rhythm, 2011.
76. Fox, P.R., et al., *Spontaneously occurring arrhythmogenic right ventricular cardiomyopathy in the domestic cat: A new animal model similar to the human disease.* Circulation, 2000. **102**(15): p. 1863-70.
77. Oxford, E.M., et al., *Molecular composition of the intercalated disc in a spontaneous canine animal model of arrhythmogenic right ventricular dysplasia/cardiomyopathy.* Heart Rhythm, 2007. **4**(9): p. 1196-205.