Chapter 11

Summary Discussion

Summary
Heart failure is a complex condition, in which the heart is unable to generate sufficient blood flow to meet the metabolic requirements of tissues and organs. Cardiomyopathies (CM), in particular dilated cardiomyopathy (DCM), are a common cause of heart failure. The mortality rate is high and the costs of treating heart failure is 1-2% of total health care expenditure in developed countries.[1]

In the last two decades, there have been important breakthroughs in unraveling some of the mechanisms of cardiomyopathies. Moreover, the familial character of cardiomyopathies is increasingly been recognized, with 30-50% of familial disease in dilated cardiomyopathy (DCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC).[2,3] This last finding was an indicator for the involvement of genetic factors of cardiomyopathies and has led to the discovery of many genes underlying diverse types of cardiomyopathies. Most of the inherited cardiomyopathies are genetically highly heterogeneous. Moreover, some of the genes proved to be mutated in different forms of cardiomyopathies and even arrhythmia syndromes (Figure 1). From the points of view of cost and time, it may seem that making a genetic diagnosis in these CM types would be impractical, particularly if there are no clear phenotypic clues for performing a targeted DNA analysis. Moreover, the boundaries between different clinical entities are disappearing as overlapping clinical phenotypes are being recognized more frequently. All of these developments have led to intensive cooperation between cardiologists, geneticists and clinical geneticists in cardiogenetics outpatient clinics. The studies presented in this thesis fit into this fast-moving field and add to our knowledge on these developments.

The first part of this thesis focuses on cardiomyopathies mainly involving the left ventricle (LV). In **Chapter 2** the results of screening of the lamin AC gene (*LMNA*) in patients that were referred to our joint cardiology-clinical genetics outpatient clinic are described. In this study *LMNA* mutations were identified in 6% of patients referred with a primary cardiomyopathic problem. These mutations were all found in the subgroup of DCM patients with familial disease that presented with concomitant cardiac conduction disease.

Moreover, four patients with familial cardiac conduction disease were studied and we identified one *LMNA* mutation that was described earlier in a family in which several members only demonstrated cardiac conduction disease. This particular mutation is therefore believed to give more predominant cardiac conduction disease, with DCM appearing only in a

subset of patients. This underscores the importance of screening for *LMNA* mutations in patients with conduction disease who have a family history of cardiac conduction disease, DCM or sudden cardiac death. We also studied eight patients with a primary neurological disease (neuromuscular disease) and associated DCM and found as many as seven LMNA mutations.

Since de novo LMNA mutations do occur and signs of associated muscular disease may only develop over time, *LMNA* screening should be considered not only in familial DCM with cardiac conduction disease, but also in 'pure' DCM cases.

In **Chapter 3** a large multi-generation family with an autosomal dominantly inherited form of myocardial fibrosis is described. The clinical picture was dominated by extensive myocardial fibrosis with a high rate of sudden death at relatively young ages. Genome-wide linkage analysis mapped the disease phenotype to a region on chromosome 1 containing the *LMNA* gene. Regular PCR-based screening techniques failed to show an *LMNA* mutation, but Southern blotting, cDNA sequencing, and multiplex ligation-dependent probe amplification (MLPA) revealed a deletion encompassing the start codon containing exon of the *LMNA* gene. These observations underscored the role of deletions underlying laminopathies and added a distinct cardiomyopathic entity, characterized by fibrosis and rather limited cardiac dilatation, in the spectrum of idiopathic DCM.

A deletion underlying the cardiac phenotype is also described in **Chapter 4**. It describes two families with exercise-related ventricular arrhythmias and also sinoatrial and atrioventricular nodal conduction abnormalities, atrial fibrillation and atrial standstill. Moreover, LV dysfunction was present in several individuals. Linkage analysis pointed to the *RyR2*- and *ACTN 2*-containing region on chromosome 1 (1q42-q43). Although PCR-based screening did not reveal any abnormalities in these genes in the two families, an exon-3 *RyR2* deletion was identified using MLPA. The findings of this study extended the clinical phenotype of *RyR2*-related disease with reduced LV function and DCM.

Reduced LV function can also be observed in approximately 16% of patients with Marfan syndrome, an inherited generalized connective tissue disorder, even in the absence of valvular abnormalities or previous aortic surgery. **Chapter 5** describes the results of genotype-phenotype studies in a large series of patients fulfilling the Ghent criteria for Marfan syndrome and LV dilatation (as defined by left ventricular end diastolic diameter corrected for age and body surface >112%). *FBN1* is the major gene underlying Marfan

syndrome. *FBN1* mutation-positive Marfan syndrome patients who carry a mutation most likely to lead to haploinsufficiency (in particular large deletions/ null-alleles or frameshift mutations) more frequently had LV dilatation than missense mutation carriers. In addition, *FBN1* mutation-negative Marfan syndrome patients demonstrated LV dilatation significantly more often than *FBN1* mutation-positive patients. Although this observational study does not provide an explanation for LV dilatation, it might point toward a role for the extracellular matrix, via FBN1/TGFBR, in the evolvement of LV dilatation.

In **Chapter 6**, two families with a desmin-related myopathy due to a novel head domain mutation (p.S13F) in the desmin gene (*DES*) are described. They demonstrate a highly heterogeneous clinical picture, varying from isolated DCM to a generalized skeletal myopathy. The muscle biopsies revealed intracytoplasmic desmin aggregates. In **Chapter 7** we describe 27 carriers of this p.S13F *DES* founder mutation (including additional family members from the families described in chapter 6 and three other families). Four of these patients demonstrated right-sided heart failure. Besides, two patients fulfilled the ARVC task force criteria and one of them had histology findings compatible with this diagnosis. This led to the hypothesis that desmin-related myopathy caused by the p.S13F *DES* mutation overlaps desmosome cardiomyopathies. This prompted us to study the desmosomal proteins in myocardial samples of p.S13F mutation carriers. We demonstrated normal amounts of desmosomal proteins, yet the intercalated disks were highly convoluted and elongated with a zigzag appearance suggesting a localized effect of the mutant protein on cellular connections.

In the second part of the thesis, the focus is on right-sided heart failure. In **Chapter 8** the results of a national collaborative study on *PKP2* mutations are presented. Of the 56 ARVC patients fulfilling the generally accepted task force criteria, 24 (43%) turned out to carry a *PKP2* mutation. Four mutations were identified more than once, and haplotype analyses suggested these mutations to be founder mutations. We also related the results of earlier family investigations to these findings, which led to the observation that a *PKP2* mutation could be identified in 70% of proven familial ARVC cases, whereas *PKP2* mutations were absent in proven sporadic ARVC patients. However, no genotype-phenotype relationships could be identified.

In ***Chapter 9***, four small, possibly distantly related, families with ARVC were studied using high-density, genome-wide single nucleotide polymorphisms (SNP) arrays. This study was based on the concept that in low-penetrance Mendelian disease, the chromosomal regions that contain the disease-

causing mutation are identical-by-descent and that those mutation-containing regions are larger than the haplotypes found coincidentally and are identical-by-state. Using this haplotype sharing test, a single, large haplotype run of 91 SNP markers on chromosome 12 was identified. The validity of the haplotype sharing test was confirmed by the fact that this longest haplotype did indeed contain the disease-causing gene, because we identified a novel splice-site mutation in the *PKP2* gene. In ***chapter 10***, the characteristics of the mutation and details of the clinical spectrum in the families are given. The c.2489+4A>C mutation identified led to an aberrant mRNA. The families demonstrated great clinical variability and female members also showed non-penetrance.

DISCUSSION AND FUTURE PERSPECTIVES

The unraveling of the genetic factors underlying cardiomyopathies began in the early 1990s. It has resulted in the identification of many disease-associated genes and contributed enormously to our understanding of the pathophysiology underlying the two most frequent types of cardiomyopathies: hypertrophic cardiomyopathy (HCM) and DCM.[5] Initially, HCM seemed to be due to mutations in genes encoding sarcomeric proteins whereas DCM seemed to be a disease mainly caused by mutations in the cytoskeleton. This basic concept lasted until 2000, when the first mutations in the sarcomeric genes in DCM were identified, which has led to the discovery of a series of genes involved in both disorders (Figure 1).[4,6]

Nowadays it is recognized that genes involved in cardiomyopathies encode proteins involved in several structures within the cardiomyocyte (Figure 2). First of all, this applies to the sarcomeres, the basic contractile force-generating units that form the myofibrils. These sarcomeres consist of thin and thick filaments that slide. The thick filaments are composed of myosin and myosin-binding proteins, whereas thin filaments contain actin, α-tropomyosin and troponins (C, I and T). The giant molecule titin can be considered as a template for the sarcomere as it extends from the Z-disk to the M-line of the myocytes. The Z-disks, located at the end of the sarcomeres, are a collection of inter-digitating proteins (e.g. α-actinin, Cypher/Zasp, filamin, MLP, myopalladin, nebulette, telethonin/T-cap) that maintain myofilament organization by cross-linking to titin and the thin filaments.[8,9]

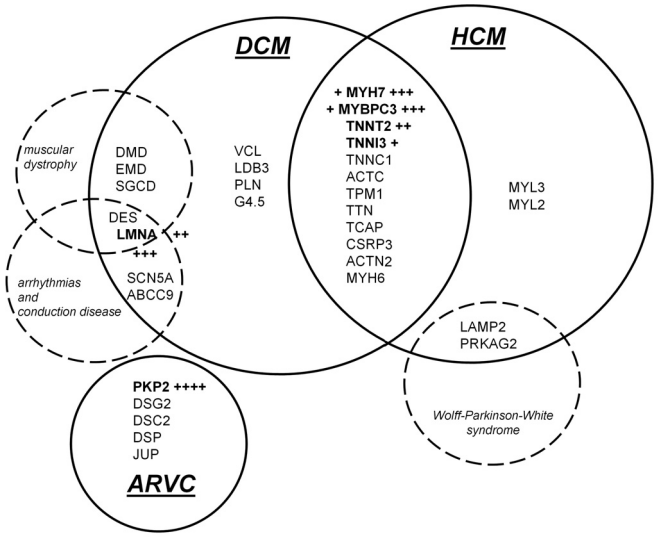

Figure 1: Genetic heterogeneity and overlap in genes causing cardiomyopathies.[4V] The genes causing cardiomyopathy are: *ABCC9* (ATP-sensitive potassium channel), *ACTC* (cardiac α-actin), *ACTN2* (α-actinin-2), *CSRP-3* (muscle LIM protein), *DES* (desmin), *DMD* (dystrophin), *DSG2* (desmoglein-2), *DSC2* (desmocollin-2), *DSP* (desmoplakin), *EMD* (emerin), *JUP* (junctional plakoglobin), *LAMP-2* (lysosome-associated membrane protein-2), *LDB3* (cypher/ZASP), *LMNA* (lamin A/C), *MYBPC3* (myosin-binding protein C), *MYH6* (α-myosin heavy chain), *MYH7* (β-myosin heavy chain), *MYL2* (regulatory myosin light chain), *MYL3* (essential myosin light chain), *PKP2* (plakophilin-2), *PLN* (phospholamban), *PRKAG-2* (AMPK-γ2 subunit), *RYR-2* (ryanodine receptor type-2), *SGCD* (δ-sarcoglycan), *SCN5A* (cardiac sodium channel), *G4.5* (Tafazzin), *TCAP* (titin-cap/telethonin), *TGF-β3* (transforming growth factor β-3), *TNNC1* (cardiac troponin C), *TNNI3* (cardiac troponin I), *TNNT2* (cardiac tropinin T), *TPM1* (α-tropomyosin), *TTN* (titin), *VCL* (metavinculin).

+, ++, +++ give an indication of the prevalence of mutations in a certain gene involved in a specific cardiomyopathy: + ~5%, ++ ~10%, +++ ~20-40%, ++++ >40% (this is not shown for the genes involved in <5% of cases).

The cardiac muscle fibers consist of a series of cardiomyocytes that are linked together at the intercalated disk (Figure 2). This structure contains gap junctions (formed by connexins), important for electrical coupling, and adherens junctions (containing N-cadherin, catenins, and vinculin) and desmosomes (containing desmin, desmoplakin, desmocollin and desmoglein). The latter two structures are important for the structural connection between the cardiomyocytes, which are surrounded by a membrane (sarcolemma) linked to the sarcomere and the extracellular matrix by the extrasarcomeric cytoskeleton. Elements involved in this skeleton are, for example, intermyofibrillar and subsarcolemmmal components, intermediate filaments (such as desmin), microfilaments (γ-actinin) and

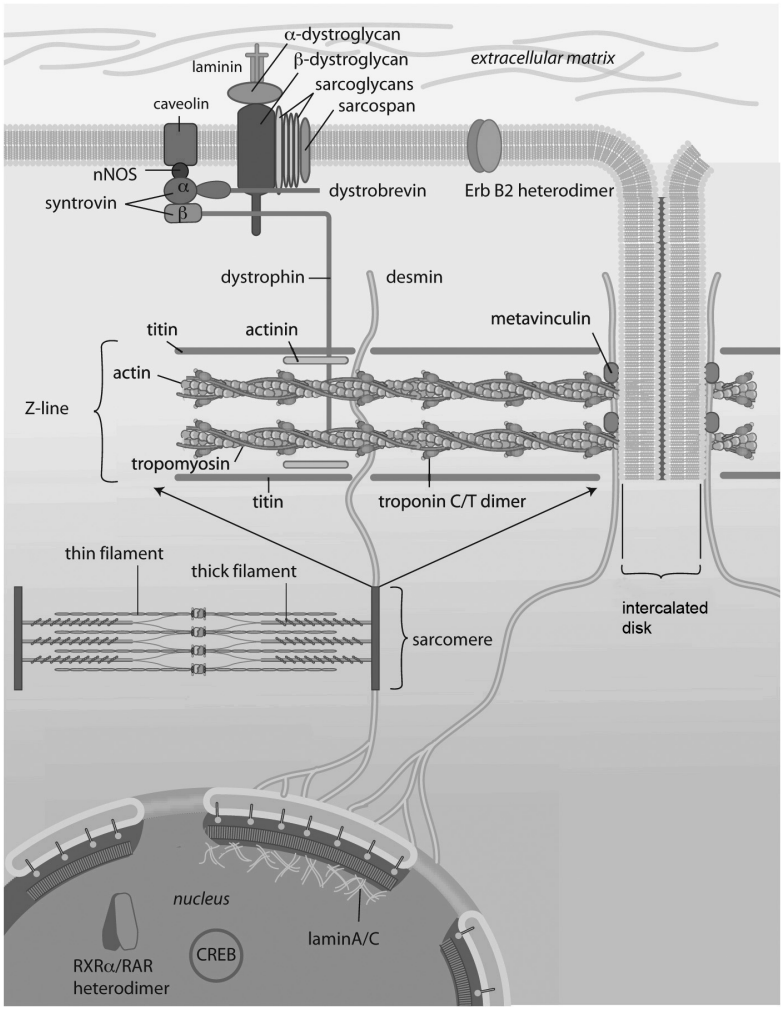

Figure 2. Cardiomyocyte showing some proteins involved in the development of dilated cardiomyopathy (DCM).
A simplified sarcomere is shown to highlight positions of thick and thin filaments relative to the Z-line, which is shown more in detail. The intercalated disk is shown without desmosomes and gap junctions for clarity.[7] (color image: page 271)

microtubules.[10] These structures support subcellular structures and transmit mechanical and chemical signals within and between cells. Desmin forms a scaffold throughout the extrasarcomeric cytoskeleton, surrounding the Z-disks and connects to the subsarcolemmal costameres.[10,11] The microfilaments link the sarcomere (via α-actinin) to the costamere, which are subsarcolemmal domains flanking the Z-disks and overlying the I-bands, along the cytoplasmic side of the sarcolemma. These costameres connect various cytoskeletal

networks and link the sarcomere and sarcolemma, and are believed to be important for force transduction. The main elements of these costameres are the DAG (dystrophin-associated glycoprotein complex)[12], spectrin-based complex and the focal adhesion-type complex. The latter connects the cytosplasmic proteins (like vinculin, talin, tensin, paxillin and zyxin) with cytoskeletal actin filaments and transmembrane proteins like α- and β-dystroglycan, α-β-γ-δ- sarcoglycans, dystrobrevin and syntrophin.[12-15] At the sites where actin filaments attach to the costameric complexes, there are several actin-associated proteins present such as α-actinin, and muscle LIM protein.

Dystrophin links actin (at the N-terminal part) to β-dystroglycan. This forms the link through α-dystroglycan to the extracellular matrix via α-2-actinin. Interestingly, ion channels co-localize with some of these proteins.[16-19]

All these proteins that link extracellular matrix to the nucleus could be involved in the pathophysiology of DCM or ARVC. Disruptions in these proteins are believed to produce diverse effects, such as an impaired generation of force (e.g. due to mutations in the sarcomeric proteins), impaired transmission of force (e.g. owing to mutations in cytoskeletal elements), impaired cell-cell interaction, Ca^{2+} homeostasis, reduced energy supply, altered signaling, gene expression or transcriptional activation, etc.

Although the proteins are involved in many pathophysiological mechanisms, these all result in a common pathway of ventricular dilatation and systolic dysfunction.

Shifting clinical concepts

Apart from the "HCM-sarcomere, DCM-cytoskeleton concept", there was another paradigm that had to be set aside the last couple of years, namely the strict separation of cardiomyopathies from channelopathies. Channelopathies were initially considered to cause only primary arrhythmia syndromes, but not cardiomyopathies as a primary manifestation. However, recent observations have also elucidated a role for ion-channel mutations and related proteins in the pathogenesis of DCM.[20-24] This movement in time which is regularly observed in genetic disorders, is commonly referred to as "splitting and lumping". After the initial "splitting" state, in which ion-channel mutations were related to arrhythmias (sarcomere gene mutations to HCM, and cytoskeleton gene mutations to DCM), disorders are now being lumped together as they have been shown to share identical genes, probably leading to some different pathophysiological effects not yet identified.

This lumping together also applies to the clinical arena. For example, initially LV involvement was an exclusion criterion for a disorder like ARVC, but recent developments highlight the recognition of LV involvement in ARVC, or even "sole LV involvement ARVC".[25, 26]

This thesis also presents observations that add to the lumping together, like the occurrence of presumed isolated conduction disease in families with an R225X mutation in the *LMNA* gene, underscoring a role for non-ion-channel mutations in familial arrhythmia/conduction disease (chapter 2), the occurrence of DCM in *RyR2* deletion carriers (chapter 4), and the recognition that in allegedly left-sided disease due to *DES* mutations, there is right ventricular involvement, including ARVC phenocopies (chapter 7). Finally, LV involvement in connective tissue disorders is another example of lumping (chapter 5).

Classification of cardiomyopathies

The changing clinical and pathophysiological spectrum and the problems in classifying the cardiomyopathies are also reflected in the new classifications of cardiomyopathies recently published by working groups of both the American Heart Association (AHA)[27] and the European Society of Cardiology (ESC).[28] The 2006 AHA statement considers cardiomyopathies to be a group of genetically determined diseases of the myocyte, including those showing a primarily arrhythmic phenotype, in the absence of overt structural changes (Figure 3). The ESC classification, however, considers cardiomyopathies primarily as diseases of the heart, leading to structural and functional abnormalities (Figure 4). Notwithstanding this difference, both classifications reinforce the idea of dividing cardiomyopathies into familial/genetic and non-familial/non-genetic forms.

DNA diagnostics in DCM and ARVC

There are now nearly 40 known genes underlying DCM and it can therefore be considered a highly heterogeneous disorder. The overwhelming majority of genes in DCM are responsible for many fewer than 1.5% of cases per gene. One important exception is DCM with cardiac conduction disease, in which an *LMNA* mutation is found in more than 20% of patients.[29] Other potentially relevant genes in DCM associated with conduction disease or atrial fibrillation (AF) are *SCN5A* and *TTN*. In isolated forms of DCM it is important to consider screening for *MYH7*, *ZASP*, *TTN* and *DYS* in X-linked forms/males while, to a lesser extent, mutations in *TNNT2* could be considered.

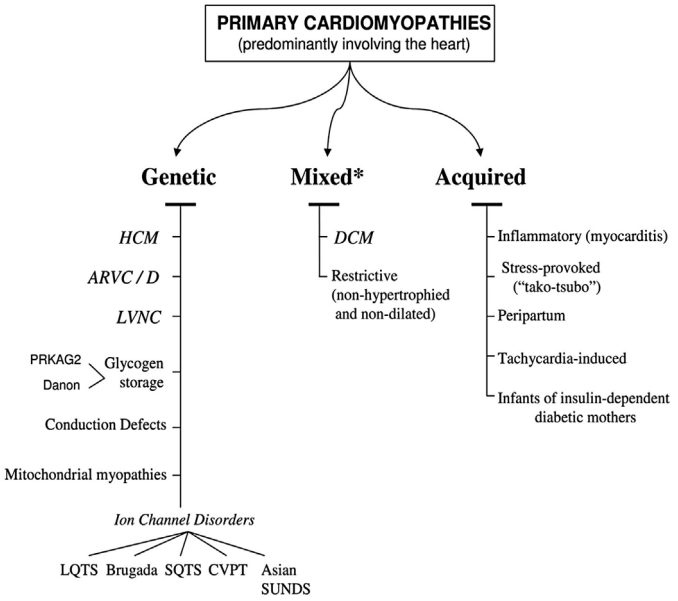

Figure 3: Primary cardiomyopathies in which the clinically relevant disease processes solely or predominantly involve the myocardium. The conditions have been segregated according to their genetic or non-genetic etiologies. *Mixed CM are mainly non-genetic in origin, although familial disease with a genetic origin has been reported in a minority of cases. [27]

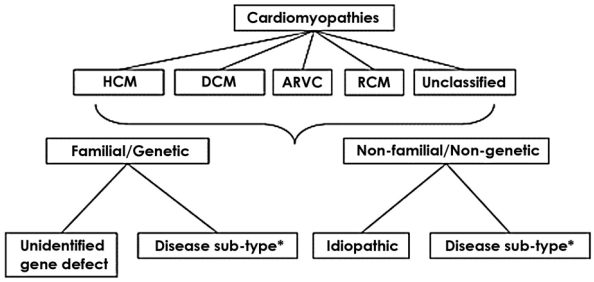

Figure 4: Classification system proposed by the European Society of Cardiology (ESC).[28]

In ARVC, eight genes have been identified, including recently the *TMEM43* gene. Mutations are mainly identified in genes encoding desmosomal proteins, in particular *PKP2*, in which they have been identified in some 50% of Dutch patients, or in an even higher percentage if familial disease is taken into account.[30]

Future aspects

Why is there no major gene in DCM?

Several founder mutations have been described in HCM, in particular the c.2373insG mutation in the myosin-binding protein C gene (*MYBPC3*) is highly prevalent in the Netherlands.[31] In the northern part of the Netherlands, we have identified several other founder mutations, such as the c.3776delA mutation in the myosin-binding protein C gene (*MYBPC3*), found in 11% of our HCM index patients (unpublished data), and others in ARVC patients (chapters 8 and 10). As nearly all DCM families show a clinical picture with late onset disease, often at well over age 40 years, no reproductive selection against these mutations is anticipated. We therefore expect that there will be founder mutations in DCM as well. However, with the exception of the p.S13F mutation in the *DES* gene, we have not identified any such mutations in the *DES*, *SCGD*, *TNNT2*, *MYH7*, *MYBPC3*, *LMNA*, and *TNNI3* genes in the more than 100 DCM index patients we have screened so far (unpublished data). Several causes can be suggested to explain this absence of founder mutations in DCM.

First of all, as families are often too small for linkage analysis and non-penetrance is likely to occur, we have to focus on candidate gene screening, and given the enormous genetic heterogeneity, it may be a matter of luck to pick the right genes to screen. We note though that our research in *LMNA* (chapter 2) showed that the yield of mutations in that gene did not differ from studies performed by others, arguing against a founder mutation in this particular gene.

Another cause might be underestimation of the role of the *TTN* gene in the pathogenesis of DCM. Apart from three families, in which linkage analysis pointed towards the locus containing *TTN, (*with subsequent identification of the mutation)[32,33], a more population-based screening of a part of *TTN,* demonstrated mutations in 3% of patients.[34,35] The huge size of the gene, encompassing more than 300 exons, might explain the reluctance of research groups to screen for *TTN* mutations.

A third possibility is that we are not seeing or underestimating the role of certain mechanisms possibly involved in the pathogenesis of DCM, including exogenous factors, such as myocarditis or alcohol abuse. Myocarditis, including viral infections that influence the myocardium directly or in a later stage while the virus persists, is believed to play an important role in the pathogenesis of DCM. Persistent viral genome expression, as demonstrated

by biopsy-proven RT-PCR of enterovirus, adenovirus, parvovirus B19, and HHV-6 is associated with progressive impairment of the LV function, while subsequent improvement of the LV was associated with spontaneous viral elimination.[36,37] Interestingly, it has been demonstrated in experimental models that enteroviral protease 2A in vitro cleaves the cytoskeletal protein dystrophin and disrupts the sarcolemmal membrane.[38,39]

Excessive alcohol intake is another example of an exogenous factor known to contribute to the pathogenesis of diastolic dysfunction and DCM.[40,41] It is important because alcohol use is often downplayed or even denied by the patient, so that this factor is often overlooked.[42] Other exogenous factors are also likely to be discovered in DCM, analogous to the role of strenuous exercise in ARVC, because experiments in heterozygous plakoglobin-deficient mice that were trained for endurance, were shown to have accelerated development of right ventricular dysfunction and arrhythmias.[43]

A fourth reason why major genes have not yet been identified in DCM might be that recognition of deletions is not possible with the PCR-based genetic screening techniques regularly used at the moment. This thesis gives examples of deletions underlying the cardiac phenotype in both *LMNA* and *RyR2* (chapters 3 and 4), and some have also been described in other cardiac disorders, such as the congenital long QT syndrome.[44] As haploinsufficiency is also the main underlying mechanism in the pathogenesis of *PKP2*-related ARVC (because of the high number of nonsense/frameshift mutations identified), this could well be a mechanism relevant to other cardiomyopathies (Chapters 8 and 10). However, *LMNA* (Chapter 3) and *PKP2* screening of large series of DCM and ARVC patients by MLPA only revealed deletions in a relatively small subset of patients.[45]

Mitochondrial protein complexes are important for the synthesis of ATP, providing energy. Although many of these protein components are encoded by nuclear DNA, a subset is encoded by the mitochondrial genome itself. This mitochondrial genome is exclusively matri-lineally inherited and in the case of mutated mitochondrial DNA, the number of affected copies per cell can vary highly, a phenomenon referred to as heteroplasmy. Because many tissues depend upon the energy supplied by mitochondria, we often see a diverse yet more generalized clinical picture. However, there are some indications that certain mitochondrial mutations exclusively or at least predominantly produce cardiac disease, including DCM.[46-48] Because of the heteroplasmy, lack of cardiac tissue samples, and aging effects, it is not always feasible to assess the role of mitochondrial mutations in DCM, and their exact role in the

pathogenesis of familial isolated DCM remains to be established.

DCM as a complex polygenic disease

Apart from the above reasons, we might be overestimating the role of monogenic inheritance, even in familial DCM. Although familial forms of DCM and ARVC are common, the majority of cases are sporadic, because these individuals are the only ones in their families known to be affected. We feel we should consider DCM as a "complex genetic disease" because of the genetic heterogeneity of familial DCM, the wide spectrum of non-cardiac disorders or exogenous factors involved in its pathogenesis, and because an estimated 30% of DCM is familial. In a complex genetic disease, a decline in heritability as age-of-onset rises can be anticipated. The genetic influence is an interplay of several genetic variations (polygenic) of low penetrance and higher prevalence, while there is an inverse relationship between magnitude of the genetic effect and allele frequency as shown in Figure 5. As outlined in chapter 1, familial DCM is defined as a family with two or more members affected according to the proposed criteria, but a large proportion of the families we and others identify have only two affected members. It is possible that these familial cases are not related to a single, high penetrance gene but are due to a set of mutated genes, as shown in the middle part of Figure 5.

DCM: genotype-phenotype correlations

The relatively low numbers of patients identified so far with cardiomyopathies such as DCM or ARVC with specific mutations or genes involved, hamper

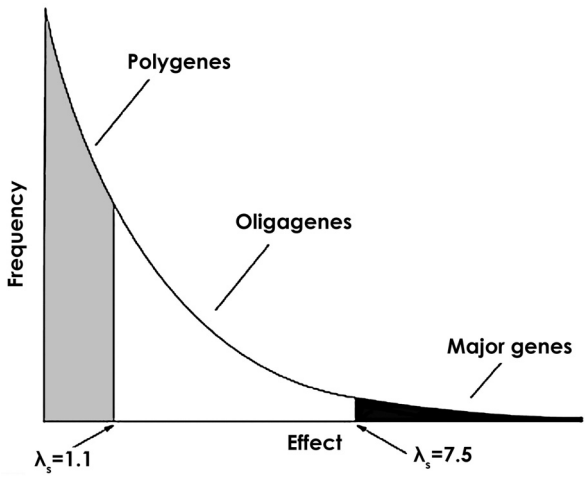

Figure 5. Inverse relationship between allele frequency and phenotype effect as postulated by Wright.[49,50]

evaluation of the genotype-phenotype relationships and this, in turn, unfortunately stops the return of knowledge from the bench to the bedside and does not allow us to add to the cardiologist's clinical tools.

However, breakthroughs can be expected as the numbers of patients with identified mutations increase. One important development is the upsizing of screening capacities using solid phase sequencing techniques; these will enable comprehensive screening of many patients for a great number of genes, which should lead to the discovery of more genes underlying the cardiomyopathies. For instance, we will search for new genes with the approach described in chapter 9, using a SNP array-based technology combined with haplotype sharing techniques. We will focus our work on large series of patients from the northern parts of the Netherlands and we should be able to identify regions identical-by-descent, as described in chapter 9. This will help us identify genomic regions underlying DCM and familial DCM.

Apart from screening for known genes and searching for new genes, a major challenge lies in exploring factors that underlie the clinical heterogeneity and non-penetrance (which can be as high as 70%, e.g. in *PKP2* mutation carriers). Age of disease onset ranges from early childhood to late in senescence and the natural history varies greatly with regards to survival and NYHA functional class, even within members of the same family. Additional features may help to phenotype patients or families correctly because additional cardiac or extracardiac manifestations have been described in relation with DCM, such as conduction disease, mitral valve prolapse, hearing loss, or skeletal muscle disease.

These steps can only be achieved if there is good cooperation between the different cardiogenetic centers, providing enough power for studies to obtain meaningful results. The initiative to set up the Dutch GENCOR database (see www.gencor.nl) and the recent establishment of the national Durrer Cardiogenetic Research Centre in Amsterdam are major steps in facilitating large-scale research in these relatively rare disorders. Although concentrating knowledge in university centers is important from the point of view of research and improving patient care, it is of utmost importance to involve and motivate cardiologists in regional hospitals so that both the researchers and the patients who might suffer from an heritable cardiac disease may benefit.

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