KUOPION YLIOPISTON JULKAISUJA D. LÄÄKETIEDE 329
KUOPIO UNIVERSITY PUBLICATIONS D. MEDICAL SCIENCES 329

SATU KÄRKKÄINEN

Genetics of Dilated Cardiomyopathy in the Eastern Finnish Population

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium L21, Snellmania building, University of Kuopio, on Friday 7th May 2004, at 1 p.m.

Department of Medicine
University of Kuopio
Kuopio University Hospital

KUOPION YLIOPISTO
KUOPIO 2004

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ISBN 951-781-449-6
ISBN 951-27-0026-3 (PDF)
ISSN 1235-0303

Kopijyvä
Kuopio 2004
Finland

Kärkkäinen, Satu. Genetics of dilated cardiomyopathy in the Eastern Finnish population. Kuopio University Publications D. Medical Sciences 329. 2004. 106 p.
ISBN 951-781-449-6
ISBN 951-27-0026-3 (PDF)
ISSN 1235-0303

ABSTRACT

Dilated cardiomyopathy (DCM) is a primary myocardial disease characterized by dilatation of the left or both ventricles and impaired systolic function. DCM is associated with sudden cardiac death and it is the leading cause for heart transplantation. Approximately 20-35% of idiopathic DCM cases are familial. Although genetic factors seem to play an important role in the pathogenesis of DCM, so far only isolated mutations have been found in seventeen different genes coding for cardiac actin, desmin, dystrophin, δ-sarcoglycan, troponin T, β-myosin heavy chain, α-tropomyosin, taffazin, titin, lamin A/C, metavinculin, myosin-binding protein-C, cardiac muscle LIM protein, telethonin, phospholamban, α-actinin and troponin I.

In this study we screened nine candidate genes for DCM in 32-90 well-characterized DCM patients from Eastern and Southern Finland by polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) and sequencing method. We detected four novel mutations in three different genes in our basic study group (52 DCM patients). One novel mutation, Ser143Pro, in the lamin A/C gene was detected in five DCM families and in one sporadic case in the whole population. This mutation was associated with a similar phenotype; progressive conduction system disease, often requiring pacemaker therapy, atrial fibrillation, heart failure and elevated risk for sudden cardiac death. According to haplotype analysis, the Ser143Pro mutation in the lamin A/C gene is a founder mutation. Two novel mutations in the β-myosin heavy chain were found in two DCM probands. The Arg1053Gln mutation caused variable phenotypes of cardiomyopathies, presenting mainly as hypertrophic cardiomyopathy (HCM) and leading to a transition from HCM to DCM in one patient. The Arg1500Trp mutation was found in a patient with a classical DCM. One novel mutation in the δ-sarcoglycan gene, Arg71Thr, was detected in two members of a small DCM family. The Arg71Thr mutation seems to cause a relatively mild DCM with late onset of disease. It seems that mutations in the lamin A/C gene are quite common among Finnish patients with DCM, whereas DCM-associated mutations in the β-myosin heavy chain and δ-sarcoglycan genes seem to be uncommon.

These four novel mutations found in the basic study group explained the disease in 11.5% of all cases. The Ser143Pro mutation could be suitable for common screening at least in patients with a conduction system disorder. The main clinical significance of the other mutations is that diagnosing these mutations offers the possibility to focus follow-up on the right family members and to start prognosis-improving treatment in time.

National Library of Medicine Classification: QH 455, QU 58.5, QZ 50, WG 205, WG 280
Medical subject headings: cardiomyopathy, congestive/ genetics; death, sudden, cardiac; DNA mutational analysis; genetic markers; genetic screening; myosin heavy chains; polymorphism, single-stranded conformational; Finland

To Petri

ACKNOWLEDGMENTS

This study was carried out in the Department of Medicine and the Clinical Research Unit, University of Kuopio in 1999-2004. This study is a part of a larger research program investigating pathogenesis and prognosis of DCM in the Finnish population led by Professor Keijo Peuhkurinen, MD, PhD, Department of Medicine, University of Kuopio.

I express my deepest gratitude to my principal supervisor Professor Keijo Peuhkurinen, MD, PhD, who organized this study. Professor Peuhkurinen offered me the challenging and interesting topic of this thesis. I am especially thankful for his professional guidance, enthusiasm and great encouragement during this work. I feel that I am privileged, that Keijo has been my supervisor. He has also given me opportunities to participate in stimulating scientific meetings. I am equally grateful to my supervisor Professor Markku Laakso, MD, PhD, for his support and guidance during my work. I owe much to his knowledge and his great enthusiasm towards science. I am also grateful for him for providing me excellent working facilities in his well-organized unit.

I express my gratitude for collaborators Tiina Heliö, MD, PhD, Docent Johanna Kuusisto, MD, PhD, Professor Markku S, Nieminen MD, PhD, Docent Lauri Toivonen MD, PhD, Maija Kaartinen, MD, PhD, Petri Tuomainen MD, Docent Kari Ylitalo, MD, PhD, Juha Rummukainen, MD, and Eeva Reissel, MD, PhD, for the successful collaboration and valuable help.

I am grateful to the official reviewers of this thesis, Docent Markku Kupari, MD, PhD, and Docent Paavo Uusimaa, MD, PhD, for their valuable and constructive comments in improving this thesis.

During the years of this thesis several persons have helped me in my work. I express my warmest thanks to Raija Miettinen, MSc, for the sequencing analysis and all help in laboratory work and to Pertti Jääskeläinen, MD, PhD, for teaching me the laboratory methods. I am equally grateful to Paula Peltola, MSc, Minna Kinnunen, MSc, Hanna Huopio, MD, PhD, Laura Viitanen, MD, PhD, Teemu Kuulasmaa, MSc, Päivi Kärkkäinen, MSc, and Docent Jussi Pihlajamäki, MD, PhD, for their valuable contributions to this study. I wish to thank Medical laboratory technologists Leena Uschanoff, Kaija Eirola, Raija Räisänen, Eila Ruotsalainen, Tarja Heikkinen, Tiina Sistonen, Annamari Aura and Heli Saloranta, for their help. I also want to thank all fellow researchers in Clinical Research unit during these years for the pleasant working atmosphere in the Laboratory of Internal Medicine.

I am also thankful to research nurses Helena Ollikainen, Sini Puustinen, Satu Nenonen and Marjut Huovinen for the good companionship and help during these years.

I owe my sincere thanks to Docent David Laaksonen, MD, PhD, MPH, for the linguistic revision of this thesis. I am thankful to Tuija Nenonen and Eeva Oittinen for their help during these years.

I owe my warmest gratitude to my parents Sinikka and Pauli Kärkkäinen for their endless love and support during all phases of my life. They have created me excellent possibilities for life. I also warmly thank my sister Sirpa and her husband Matti for their friendliness, sympathy and encouragement.

I wish to thank Aila and Kalevi Kivinen and Jouni, Sonja and Tommi Kivinen for their support during this thesis. I have spent refreshing moments with you. I also wish to thank all my friends for their friendship and encouragement.

My warmest and greatest thanks belong to my husband Petri. His endless love and support has given me so much. Without you this study would not have been possible.

This work was financially supported by Finnish Foundation for Cardiovascular Research, Finnish Cardiac Society, Aarne Koskelo Foundation, Ida Montin foundation, The North-Savo Fund of the Finnish Cultural foundation, Kuopio University Hospital (EVO grant no. 5140) and Helsinki University Hospital (EVO grant no. T1010K0002).

Kuopio, May 2004

Satu Kärkkäinen

SELECTED ABBREVIATIONS

ACE	angiotensin I-converting enzyme
ACTC	cardiac actin gene
ACTN2	α-actinin gene
ARVD	arrhythmogenic right ventricular dysplasia
ATP	adenosine triphosphate
AV	atrioventricular
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CLP	cardiac muscle LIM protein
DAG	dystrophin associated glycoprotein complex
DCM	dilated cardiomyopathy
dCTP	deoxycytidine 5`-triphosphate
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
ECG	electrocardiogram
ECM	extracellular matrix
EF	ejection fraction
HCM	hypertrophic cardiomyopathy
ICD	implantable cardioverter-defibrillator
IVS	interventicular septum
LV	left ventricle/ ventricular
LAH	left atrial hypertrophy
LIM	named according to closely related Lin-11, Isl-1 and Mec-3 genes
LVEDD	left ventricular end-diastolic diameter
LVH	left ventricular hypertrophy
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes
MYBPC-3	myosin-binding protein-C gene
MYH7	β-myosin heavy chain gene
β-MyHC	β-myosin heavy chain
PCR	polymerase chain reaction
PLN	phospholamban gene
SSCP	single-strand conformation polymorphism
TCAP	telethonin gene
TNNC1	cardiac troponin C gene
TNNI3	cardiac troponin I gene
TNNT2	cardiac troponin T gene
TPM1	α-tropomyosin gene
TTN	titin gene

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals:

I Kärkkäinen S, Peuhkurinen K, Jääskeläinen P, Miettinen R, Kärkkäinen P, Kuusisto J, Laakso M. No variants in the cardiac actin gene in Finnish patients with dilated or hypertrophic cardiomyopathy. American Heart Journal 2002; 143: 11-14.

II Kärkkäinen S, Heliö T, Miettinen R, Peltola P, Tuomainen P, Rummukainen J, Ylitalo K, Kaartinen M, Kuusisto J, Toivonen L, Nieminen M, Laakso M, Peuhkurinen K. A Novel mutation Ser143Pro in the lamin A/C gene is common in Finnish patients with familial dilated cardiomyopathy. European Heart Journal, in press.

III Kärkkäinen S, Heliö T, Jääskeläinen P, Miettinen R, Tuomainen P, Rummukainen J, Ylitalo K, Kaartinen M, Reissell E, Toivonen L, Nieminen M, Kuusisto J, Laakso M, Peuhkurinen K. Two novel mutations in the β-myosin heavy chain gene associated with dilated cardiomyopathy. Submitted.

IV Kärkkäinen S, Miettinen R, Tuomainen P, Kärkkäinen P, Heliö T, Reissell E, Kaartinen M, Toivonen L, Nieminen MS, Kuusisto J, Laakso M, Peuhkurinen K. A novel mutation, Arg71Thr, in the δ-sarcoglycan gene is associated with dilated cardiomyopathy. Journal of Molecular Medicine 2003; 81:795-800.

CONTENTS

1. INTRODUCTION

DCM is a primary myocardial disease characterized by dilatation of the left or both ventricles and impaired systolic function (1). DCM causes considerable morbidity and mortality, and it is one of the major causes of sudden cardiac death. DCM can manifest itself at any age, but most commonly after middle age. DCM is clinically a very heterogeneous disease, and patients having this disease may be symptomless but may also have severe heart failure.

The World Health Organization (WHO) has classified DCM according to etiology into four groups; idiopathic, familial/ genetic, viral/ autoimmune or alcoholic/ toxic (1). There are also many other etiological factors, that can not be included into these four groups. DCM is familial in approximately 20-35% of cases of idiopathic DCM. Autosomal dominant inheritance is the most common inheritance form (2-4). Although genetic factors play an important role in the pathogenesis of DCM, hitherto reported mutations explain only a minority of familial DCM. The first familial cardiomegaly was characterized in 1949, but the first DCM-associated mutation (in the dystrophin gene) was described as late as in 1993 (5-7). Since the beginning of 1990´s, the genetics of DCM has been investigated more intensively, especially during the last few years. To date, approximately 60 DCM-associated mutations in seventeen different genes (cardiac actin, desmin, dystrophin, δ-sarcoglycan, troponin T and β-myosin heavy chain, α-tropomyosin, taffazin, titin, lamin A/C, metavinculin, myosin-binding protein-C, cardiac muscle LIM protein, telethonin, phospholamban, α-actinin and troponin I genes) have been reported (6-29), but still these mutations explain only a minority of the etiology of DCM.

Although the prognosis of DCM has improved during the last decades, it still causes considerable morbidity and mortality. Identification of mutations in genes associated with this disease adds not only information on the pathogenesis of DCM, but also on their prognostic significance with respect to cardiac outcome. In this study, I investigated the genetic background of DCM in Eastern Finland by screening several candidate genes for DCM. Also, associations between the mutations and different phenotypes and prognosis were investigated.

2. REVIEW OF THE LITERATURE

2.1 Dilated cardiomyopathy

2.1.1 Epidemiology

DCM is the most common cardiomyopathy explaining about 60% of all cardiomyopathies (30, 31). The incidence of dilated cardiomyopathy has been increasing, which is explained by more accurate diagnosis and a higher index of suspicion among clinicans (32). The incidence of DCM has been suggested to be 5-8/ 100 000/ year in the United States and in European populations (32-35), and the prevalence 40/ 100 000 in the United States (32, 36). In Sweden the incidence rate was 29/ 1 000 000 and the estimated prevalence 131/ 1 000 000 persons per year in inhabitants 16-65 years old (37). In Finland there is epidemiological data of DCM only in children. Arola et al. (38) reported that the prevalence of dilated cardiomyopathy in Finnish children (under 20 years old) is 2.6/ 100 000.

Cardiomyopathy and coronary artery disease are leading causes for heart transplantation (39, 40). Cardiomyopathy (mainly DCM) is the reason for heart transplantation in 45-50% of all cases (39). In 2001 there were over 3100 heart transplantations in the world, and most of the transplantations were done in North-America and Europe (41). In Finland the first heart transplantation was done in 1985. Between 1985 and 1998 altogether 236 heart transplantations had been done for the adults. The leading cause for heart tranplantation in Finland is dilated cardiomyopathy.

2.1.2 Pathophysiology of DCM

DCM is characterized by depressed systolic function, cardiomegaly and ventricular dilatation. Reduced left ventricular contractile force leads to decreased cardiac output, resulting in increased residual volumes in end-systole and end-diastole. Low cardiac output causes upregulation of the sympathetic nervous system and the renin-angiotensin axis. Stimulation of these hormonal tracts results in vasoconstriction and thus further decreases cardiac output (42).

Disturbances in the myocardium lead to the activation of compensatory mechanisms, which include hypertrophy of myocytes, remodelling, activation of baroreceptor reflex

and release of natriuretic peptides, prostaglandins and nitric oxide (42, 43). These compensatory mechanisms try to balance heart failure. In hypertrophy the size of the myocytes is increased without increase of amount of myocytes. The hypertrophy process is activated by mechanical stress and by different growth factors and neurohumoral factors (44). Same mechanisms also activate fibroblasts leading to increase in the amount of collagen and fibrosis. The collagen framework is altered in the remodeling process leading to increased fibrosis (amounts of collagens I, III and IV are increased) (45-47). However, it is still unclear whether fibrosis is the primary or secondary phenomenon (48). Dilatation of the left ventricle is initially a reasonable compensatory mechanism of heart failure. Length of myocytes increases and myofibrils slide by each other due to broken perimycium. Later dilatation of left ventricle is harmful and leads to increase of loading conditions of left ventricle.

Dysfunction of myocytes is assumed to result in decreased sarcoplasmic reticulum Ca^{2+} uptake, reduced β-receptor density and concentration of phospholamban. Degenerated myocytes are not capable of normal function (48). Myocyte loss is one of the etiologic factors of wall thinning and chamber dilatation in DCM (47). Injury of myocardial cells is the trigger of this process, which leads to cell death (49). Myocardial injury may be caused by apoptosis, oncosis, necrosis or autophagic cell death (48, 50). Multiple different factors, e.g. increased cytosolic free calcium, hypoxia, muscle stretch, catecholamines and oxygen free radicals can initiate apoptosis (44, 51-53).

There are some typical histopathological features in DCM, but most of them are unspecific. Increased nuclear size of myocytes, myofibrillary loss within myocytes, focal myocyte necrosis, and an increase in interstitial T lymphocytes, macrophages and interstitial fibrosis are the most typical histopathological changes encountered (54). Fibrosis in DCM is more commonly reactive (interstitial/ perivascular) than reparative (replacement) in nature, which suggests that the pathological mechanism is primarily generalized dysfunction rather than myocardial damage (55, 56). Also substantial myocyte hypertrophy with large, irregularly shaped, hyperchromatic nuclei and an increased number of mitochondria are typical for DCM (57, 58).

2.1.3 Clinical presentation

The clinical presentation of patients with DCM is variable. DCM patients can remain asymptomatic for a long period of time, and the diagnosis may become apparent in routine medical examination due to abnormal ECG or chest x-ray. Unfortunately, the first sign of the disease can also be sudden cardiac death. DCM can be manifested at any age, but most commonly after middle age. Typical symptoms are dyspnoea, fatigue, chest pain, palpitation and exercise intolerance (36, 59, 60). Uncommon manifestations of the disease are systemic and pulmonary emboli and syncope (61, 62). Symptoms can be manifested gradually or suddenly due to triggering factors, such as a respiratory infection (63).

The ECG is usually abnormal in DCM patients, but there are no specific findings. Typical abnormalities are isolated T wave changes and Q waves in the septal leads (60). Left ventricular branch block (LBBB) and the prolongation of atrioventricular (AV) conduction are also common findings. Left ventricular hypertrophy (LVH) and left atrial hypertrophy (LAH) may also occur (63). Different arrhythmias, mainly sinus tachycardia and supraventicular arrhythmias, particularly atrial fibrillation, are found in DCM patients (60, 63). Approximately 20-30% of DCM patients have episodes of non-sustained ventricular tachycardia and a small number of patients present with sustained ventricular tachycardia (60).

2.1.4 Diagnosis

2.1.4.1 Index patients

The cornerstone in the diagnosis of DCM is echocardiography. Echocardiography reveals the dilatation of the left ventricle or both ventricles and impaired systolic function, but also mitral and tricuspid regurgitation. Mural thrombi are sometimes present in the left ventricle (36). The thickness of heart wall is normal or reduced. The diagnosis of DCM can also be based on findings in radionuclide scanning or cardiac catheterization (58, 64). Physical examination and chest x-ray can lead to suspision of the disease, but their accuracy is not very high. Echocardiography, a non-invasive, easily available method, is the best method in the diagnosis of DCM (58). High

accuracy can also be reached by cardiac catheterization, but as an invasive method it is not easily available (58). The diagnostic criteria for DCM are presented in Table 1.

Table 1. Diagnostic criteria for index patients of familial DCM according to Manolio et al. (58), Mestroni et al. (64) and Elliot et al.(60).

Inclusion criteria	**Exclusion criteria**
1) Ejection fraction < 45% (> 2 SD) and/ or fractional shortening < 25% 2) LVEDD > 27 mm/m^2 or LVEDD > 112-117% of the predicted value corrected for age and body surface area, which correspond to 2 SD of the predicted normal limit + 5% or cardiothoracic ratio > 0.50 to 0.55 and 3) Exclusion of secondary causes	1) hypertension *> 160/100mmHg* 2) coronary heart disease *stenosis >50% in a major branch* 3) chronic excess of alcohol consumption *> 40g/day for females and > 80g/day for males for more than 5 years* 4) rapid supraventricular arrhythmias of long duration 5) systemic diseases *LED, rheumatoid arthritis* 6) active myocarditis, pericardial disease 7) congenital heart diseases 8) cor pulmonale 9) storage diseases *sarcoidosis, hemochromatosis* 10) primary valvular diseases 11) metabolic diseases *diabetes, pheochromosytoma, thyroidal diseases* 12) drugs *anthracyclines, corticosteroids, cocaine* 13) nutritional *selenium, calcium and thiamine deficiency hypophosphatemia*

LVEDD, left ventricular end-diastolic diameter; SD, standard deviation

2.1.4.2 Family members

DCM is classified as familial if at least two family members are affected or at least one first-degree relatives has suffered an unexplained sudden death before the age of 35 years (64). Diagnostic criteria for family members are divided into major and minor criteria (Table 2). A family member is classified as affected if he/she fullfils major

criteria (left ventricular dilatation and systolic dysfunction) or left ventricular dilatation exceeds 117% of the predicted value, and additionally one minor criterion is present. A family member is also classified as affected, if three minor criteria are present (64). The affected status can not be reliably verified, when one or two minor criteria are present.

Table 2. Diagnostic criteria for family members of familial DCM according to Mestroni et al. (64).

Major criteria	**Minor criteria**
Same as index patients (Table 1)	1) unexplained supraventricular or ventricular arrhythmias before the age of 50 years 2) LVEDD > 112% of the predicted value 3) LV dysfunction; EF < 50% or fractional shortening < 28% 4) unexplained conduction disease; II° or III°AV block, LBBB, sinus nodal dysfunction 5) unexplained sudden death or stroke before 50 years of age 6) segmental wall motion abnormalities

AV, atrioventricular; EF, ejection fraction; LBBB, left bundle branch block; LV, left ventricle; LVEDD, left ventricular end-diastolic diameter

2.1.5 Prognosis

The prognosis of DCM is variable. Some patients have a benign disease with mild symptoms and low morbidity. Spontaneous healing may occasionally occur, mainly in patients with myocarditis, alcohol-related DCM or peripartum DCM. Typically DCM patients develop progressive heart failure, which may lead to heart transplantation in the most severe cases. Usually patients respond to appropriate drug treatment, their symptoms diminish and echocardiographic findings improve. Sometimes patients on treatment do not fulfill the diagnostic criteria. This stable period of the disease usually lasts from one to ten years. Thereafter, congestive heart failure gradually worsens and

the response to medication can be poor (63). Some patients suffer from sudden cardiac death due to malignant ventricular arrhythmias or death from severe heart failure (59). Dec and Fuster (36) reviewed a series of 15 studies and found that approximately 12% of DCM patients died suddenly, and sudden death accounted for 28 % (ranging from 8% to 50%) of all deaths among DCM patients. The prognosis of DCM has improved during the last decades. The 5-year survival ranges between 50-80% (61, 62, 65, 66). Several different factors have been shown to associate with poor prognosis (Table 3).

Table 3. Prognostic factors of DCM.

Association with poor prognosis	Reference
Male gender	(58, 67)
Old age	(58, 67)
Low ejection fraction	(58)
Decreased wall thickness	(58)
Cardiac index < 2.5 liters/ min/ m^2	(58)
S3	(36, 68-70)
LBBB	(36, 68-70)
Atrial fibrillation	(36, 68-70)
Atrioventricular block	(36, 68-70)
Ventricular arrhythmias	(36, 68-70)
Presence of late potentials	(71)
Peak exercise oxygen uptake < 14 ml/ kg per min	(72, 73)
Mitral or tricuspid regurgitation	(74-77)
Alcohol consumption	(78, 79)
Elevated levels of PIIINP, ICTP, laminin, ANP	(80-82)
Elevated levels of IL-6 and TNF-α	(83-85)

ANP, plasma atrial natriuretic factor; ICTP, type I collagen telopeptide; IL-6, interleukin-6; LBBB, left bundle branch block; PIIINP, procollagen type III aminoterminal peptide; TNF-α, tumor necrosis factor alpha.

2.1.6 Treatment

Drug treatment of DCM is mainly similar to that in systolic heart failure. ACE-inhibitors, β-blockers, AT-blockers, diuretics, digitalis, spironolactone, amiodarone, calcium channel blockers and warfarin are all used in DCM. Of these β-blockers, ACE inhibitors, spironolactone, and possibly also amiodarone have been shown to improve the prognosis of patients with heart failure (86-90). ACE-inhibitors are recommended to all DCM patients with ejection fraction < 40% although asymptomatic (63, 86). All

DCM patients should be treated with β-blockers, but drug treatment should be started with a low dose.

Pacemakers are frequently needed, because conduction system disorders (AV-blocks, left bundle branch block or non-specific interventricular conduction delays) are common in patients with DCM (36). Atrioventricular pacing and resynchronization have shown to improve exercise tolerance and quality of life, but they did not have an effect on survival (91, 92). Automatic cardioverter-defibrillators (ICD) can reduce the risk of sudden cardiac death in patients with previous cardiac arrest. In one recently published study ICD therapy did not demonstrate a survival benefit in patients with DCM (93). Amiodarone and ICD therapy have been shown to be of equal benefit in patients with DCM (94). Recently in the annual congress of the American College of Cardiology (ACC) results of a new study, SCD-Heft (sudden cardiac death in heart failure trial) were presented showing that ICD therapy reduces mortality in patients with DCM (95). According to the new guidelines of the European Society of Cardiology on the prevention of sudden cardiac death in patients with DCM, the use of ICDs for secondary prophylaxis is considered appropriate, but primary prophylaxis with ICDs is recommended only in high-risk patients (96).

When DCM has progressed and the patient develops end-stage heart failure without further response for medication, heart transplantation should be considered. Indications for heart transplantation are NYHA 3-4 symptoms, arrhythmia risk, EF < 20%, cardiac index < 2.1 l/min/m^2, VO_2 max < 12-14 ml/kg/min and recurrent need for inotropic agents. No contraindications are allowed to be present. Also some surgical operations are possible in end-stage DCM. In "latissimus dorsi heart wrap" operation the latissimus dorsi muscle is wrapped to encircle the heart and this strengthens heart muscle contraction. In the Batista procedure part of the left ventricle is removed. Both of these surgical treatments are very seldom used and results have been variable.

New experimental treatments have also been tested e.g. growth hormone, pentoxifylline and immunosuppression. Results in the studies involving these agents have been variable, and none of these agents has penetrated in clinical practice. In one small study, treatment with recombinant human growth hormone increased myocardial mass and reduced the size of left ventricular chamber resulting in improvement in hemodynamics and clinical status (97). Later in a larger randomized and double-blind

placebo-controlled study these findings were not confirmed, however (98). The role of growth hormone in the treatment of DCM is not yet established (99). Pentoxifylline has been shown to improve left ventricular function and symptoms in patients with dilated cardiomyopathy in a couple of small studies (100-102). Later differences between pentoxifylline and placebo in patients with idiopathic or ischemic cardiomyopathy have not been observed (103). Experimental treatment with prednisone also failed to demonstrate improvement in systolic function or symptoms (104, 105). Immunosuppression therapy has been shown to improve symptoms and systolic function in patients with DCM, but no differences in severe endpoints (death, heart transplantation or hospital readmission) have been detected (106, 107). Investigations with intravenous immunoglobulin have given promising results, but larger trials are needed before further conclusions can be made (108, 109).

2.2 Myocardial structure

The heart wall has three structural layers, the endocardium, myocardium and epicardium. The myocardium consists of striated cardiac muscle fibers. They are formed by cardiac myocytes, which are surrounded by sarcolemma and extracellular matrix. Cardiac myocytes have at least one nucleus, but most commonly two nuclei are present. Intercalated discs located between adjacent muscle cells bind cells together. They are composed of gap junctions, desmosomes and adherens junctions and they participate in the transmission of contraction force. The schematic structure of the myocyte is presented in Figure 1.

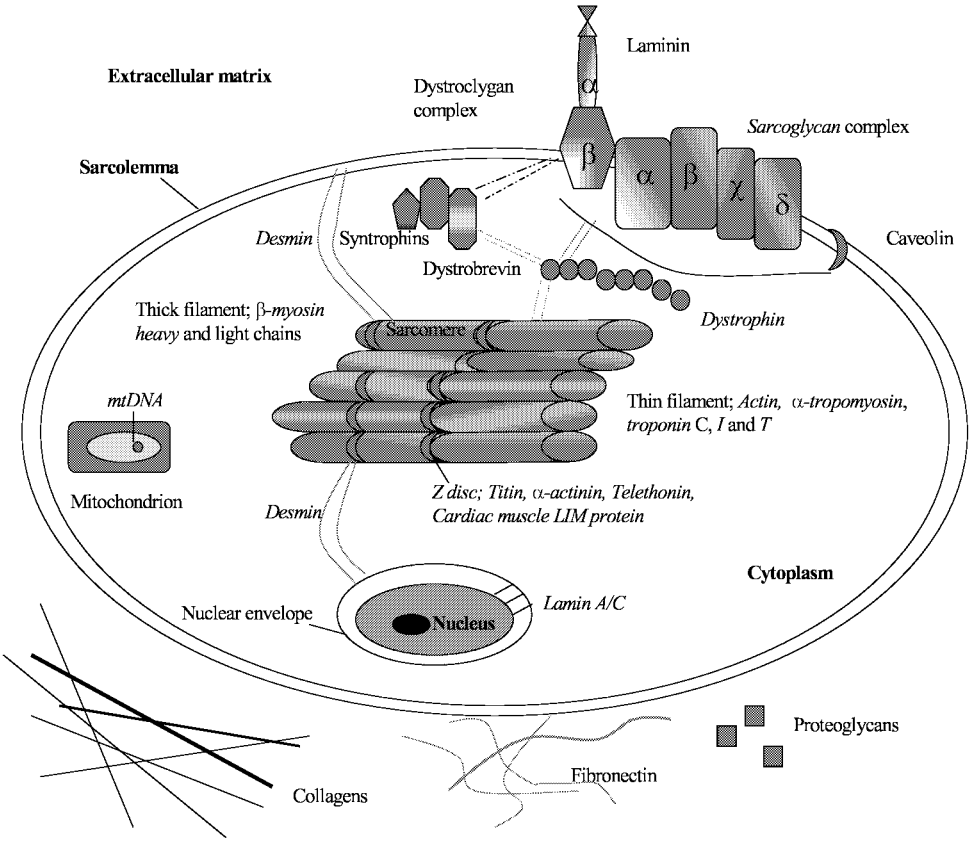

Figure 1. The schematic structure of the myocyte (modified from Dalakas et al. (110)). DCM-associated proteins are marked by italic font.

2.2.1 Sarcomere

The sarcomere is the structural and functional unit of the cardiac muscle cell, and is essential for heart muscle contraction (111). The sarcomere consists of thick and thin myofilaments. The thick filament is composed of myosin heavy chain and essential and regulatory light chains of myosin (112). Each myosin filament has a rod-like tail and two globular heads. Myosin heads have ATPase activity, which is necessary for the initiation of contraction. Myosin-binding protein-C surrounds myosin filaments and bounds different myosin molecules together. The major component of the thin filament is cardiac actin. Other components of the thin filament are α-tropomyosin and troponin complex (troponin C, I and T). Troponin C interacts with Ca^{2+} -ions, troponin I has an inhibitory effect on the contraction and troponin T interacts with tropomyosin. Z discs connect sarcomeres to each other. A schematic illustration of sarcomere is presented in Figure 2.

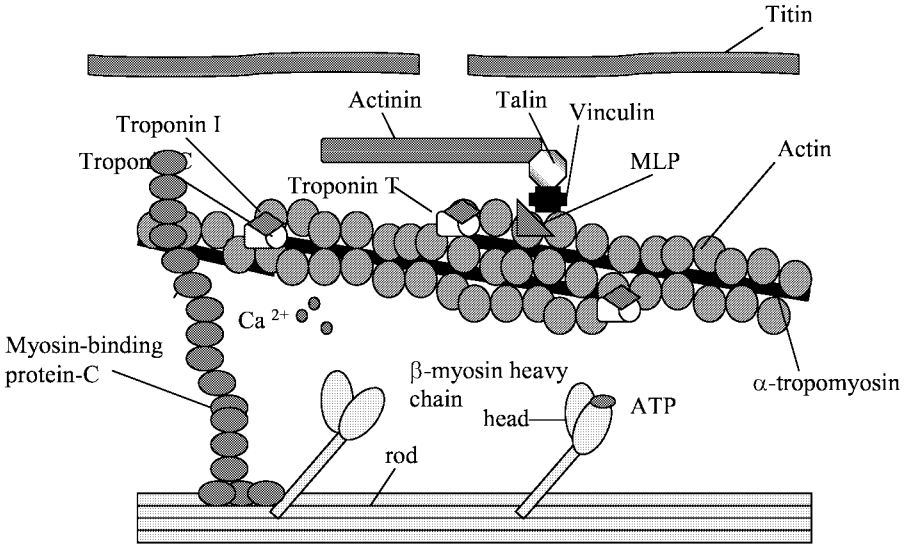

Figure 2. The structure of thick and thin filaments of the sarcomere (modified from Spirito et al. (113)).

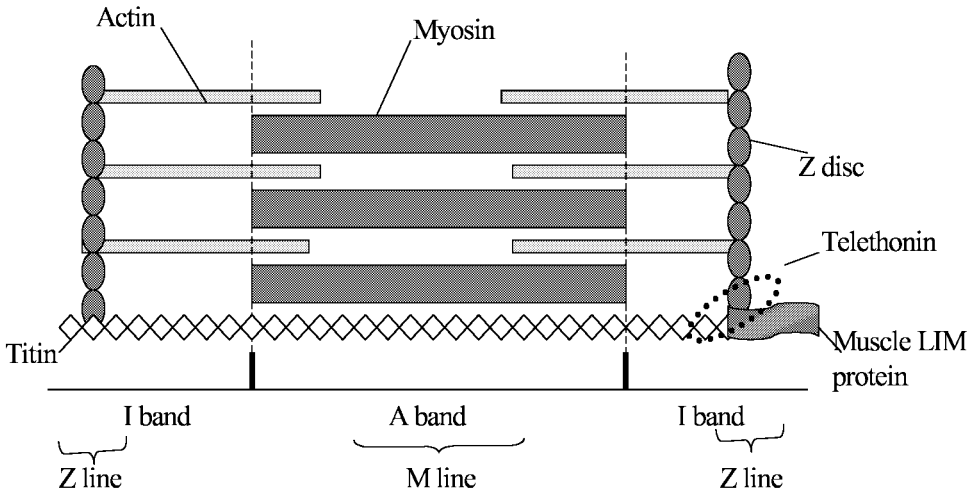

Figure 3. The structure of the sarcomere (modified from Franz et al. (114)).

The sarcomeric cytoskeleton provides a scaffold for the sarcomere (59). The main components of the sarcomeric cytoskeleton are titin, α-actinin and myomesin (115). Titin, also called connectin, is a giant protein, which spans from the Z line to the M line in the sarcomere (Figure 2 and Figure 3). Titin has several functions including the control of thin filament assembly, force transmission, maintenance of resting tension and elasticity of the muscle (116-118). Titin also binds myosin filaments into the Z discs and interacts with myosin-binding protein-C, α-actinin and telethonin (116, 118). Telethonin is actually a substrate of titin. Interaction of titin with telethonin at the Z disc is necessary for proper function of the sarcomere (26). α-actinin is an actin-binding protein, that is also located in Z discs. Its function is to anchor actin filaments and connect actin filaments to the Z disc. Myomesin is located in M line and binds myosin into the central region of the protein and also interacts with titin (119).

Heart muscle contraction is based on the sliding movement of the thin filaments relative to the thick filaments. After the action potential has arrived at motor end plate, acetylcholine is released and sarcolemma and membranes are depolarized. Action potential reaches the sarcoplasmic reticulum via T-tubules, and Ca^{2+} -ions are released from sarcoplasmic reticulum. Ca^{2+} binds to troponin C and then the troponin/Ca^{2+} complex undergoes a conformational change that physically moves tropomyosin into

the center of the helical groove in the actin and reveals the myosin binding sites (111). After that actin-myosin crossbridge formation is able to form. Repeated formation and breaking of crossbridges result in sliding of filaments and sarcomere shortening. During relaxation of the heart muscle cholinesterase is released, and acetylcholine is broken down. After that SR and T-tubules repolarize Ca^{2+}-ions are pumped out of the sarcoplasm into the sarcoplasmic reticulum by sarcoplasmic reticular Ca^{2+}-adenosine triphosphatase (SERCA2a) pump. Actin-myosin crossbridge formation is terminated and tropomyosin covers the actin binding sites. Passive sliding of filaments can occur, and the sarcomere return to the resting state (120-123).

2.2.2 Cytoskeleton

Cytoskeleton provides a mechanical mainstay for the cell. It affects cell stability, anchors subcellular structures and also transmits signals within and between cells (115). The cytoskeletal proteins include actin, desmin and membrane-associated proteins like dystrophin and vinculin. Desmin, which belongs to the group of intermediate filaments, encircles the Z discs and radiates out to connect adjacent myofibrils (Figure 1). It also forms a link between actin and dystrophin-sarcoglycan complex (124). Desmin attaches and stabilizes the sarcomere and connects adjacent myofibrils together and Z bands to plasma membrane and nuclear envelope.

Dystrophin is a large cytoskeletal protein, which is a part of the dystrophin-associated glycoprotein complex (DAG) (Figure 1). The DAG complex includes dystroglycan, sarcoglycan, syntrophin complex and caveolin. The DAG complex forms a transmembrane link between the extracellular matrix and the intracellular cytoskeleton (125). Dystrophin interacts with actin and thereby links the sarcomere to the extracellular space. Dystrophin is thought to play a role in force transduction, membrane stability and intracellular organization. Metavinculin is an isoform of the vinculin and belongs to the group of membrane-associated proteins. Membrane-associated proteins are located in the intercalated discs, costameres and the T tubule system (126, 127). They are thought to have a role in the anchoring of thin filaments, force transmission and stability of the cellular membrane, but their specific function in heart is unknown (23, 59, 128). The cardiac muscle LIM (named according to closely related Lin-11, Isl-

1 and Mec-3 genes) protein (CLP) belongs to cytoskeletal proteins and promotes the assembly of the cytoskeleton (28). It is located in the Z disc and interacts with α-actinin and telethonin. Telethonin in turn interacts with titin. CLP together with telethonin is the key component of the cardiomyocyte stretch sensor machinery (26). CLP is also localized in the nucleus, and its function there is to promote myogenesis (129).

2.2.3 Sarcoplasmic reticulum and T-tubules

Sarcoplasmic reticulum (SR) forms a system of interconnecting tubules in the narrow space between the myofibrils. At the level of the A/I junctions in sarcomere SR forms sac-like swellings called terminal cisternae (Figure 3). T tubules are long invaginations of cell membrane that submerge into cytoplasm and penetrate around myofibrils. The SR controls Ca^{2+} levels in the sarcoplasm. The cisternae of T tubules store Ca^{2+} and release it via Ca^{2+} release channels (ryanodine receptors). T-tubules carry the electrical depolarization produced by the action potential on the muscle membrane to all parts of the interior of the muscle fiber. One of the important proteins in sarcoplasmic reticulum is phospholamban, which participates in regulation of Ca^{2+} levels across the SR membrane by inhibiting the cardiac sarcoplasmic reticular Ca^{2+} -adenosine triphosphatase (SERCA2a) pump and contraction/relaxation cycle of cardiac muscle (130, 131).

2.2.4 Nuclear envelope

The nuclear envelope is a double-layered structure (outer and inner layer) located between the nucleus and cytoplasm (see Figure 1). The outer layer of the envelope is directly connected to endoplasmic reticulum and ribosomes are attached to it. The nuclear lamina, which is composed of lamins A, B and C, is attached to the inner layer of nuclear envelope. Lamins connect the nuclear envelope, nuclear matrix and chromatins together. Lamin A/C is also thought to have a role in cell dividing, nuclear growth and anchorage of nuclear envelope proteins (114, 132, 133). Lamins are major structural components of the lamina network underlying and supporting mechanically the nuclear envelope (134). There are several other nuclear proteins, which interact

directly or indirectly with the lamins, e.g. lamin-associated proteins (LAPs), emerin, otefin and nesprin (132).

2.2.5 Extracellular matrix

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting cells (Figure 1). In addition to serving as a scaffold or support for cells, cellular functions are regulated to some degree by the ECM, which serves as a substrate for cell adhesion, a source of anti-apoptotic signals and a reservoir for growth factors (82). The ECM is composed of three major classes of biomolecules; structural proteins (collagen and elastin), specialized proteins (fibronectin and laminin) and proteoglycans (135).

Collagens are the major protein of the ECM. There are approximately 20 different types of collagens and they are expressed differently in tissues. In the heart muscle the most abundant collagen types are type I and type III. Collagens play a role in tissue stiffness and elasticity (136, 137). Fibronectin belongs to glycoproteins and has a role in cell adhesion and differentiation. Fibronectin contains binding sites for collagen, fibrin and cell-surface receptors (135). Laminin, which belongs to the group of glycoproteins, anchors the cell surface to the basal lamina. It is directly bound to the DAG complex and thereby links intra- and extracellular spaces.

The DAG-complex, which is located in the intra- and extracellular boundary, is constructed by dystroglycans, sarcoglycans, dystrobrevin, syntrophin and caveolin-3 (see Figure 1). The sarcoglycan group consists of the sarcoglycans α, β, γ and δ (138). It stabilizes the plasma membrane, participates in calcium regulation and has a role in organization of membrane proteins and signal transduction (139, 140).

2.3 Etiology and genetics of DCM

2.3.1 DCM-causing mechanisms

The etiology of DCM is very heterogeneous. The etiology of DCM can be classified into primary and secondary DCM. In primary DCM, the etiology remains unknown, and therefore this group is also called idiopathic DCM (IDCM). Approximately 50% of all DCM cases are idiopathic. In secondary DCM, etiological factors are known and can be further divided into familial and non-familial DCM. Only a few specific gene defects causing DCM have been characterized so far and they are included in the familial group of secondary DCM. The mechanisms by which gene defects lead to DCM are largely unknown. However, sarcomere protein and cytoskeletal protein genes are thought to affect force generation or force transmission, and mitochondrial mutations in turn are assumed to affect energy production (141). Recently, a DCM-associated mutation was described in the phospholamban gene, which is believed to cause DCM by disturbing myocellular Ca^{2+} metabolism (27). Also defects in the cardiac muscle LIM protein and telethonin complex in Z disc have been proposed to affect the cardiomyocyte stretch sensory machinery, which in turn could lead to DCM and heart failure (26). Figure 4 shows the complexity of the etiology of DCM and how multiple different mechanisms can lead to DCM.

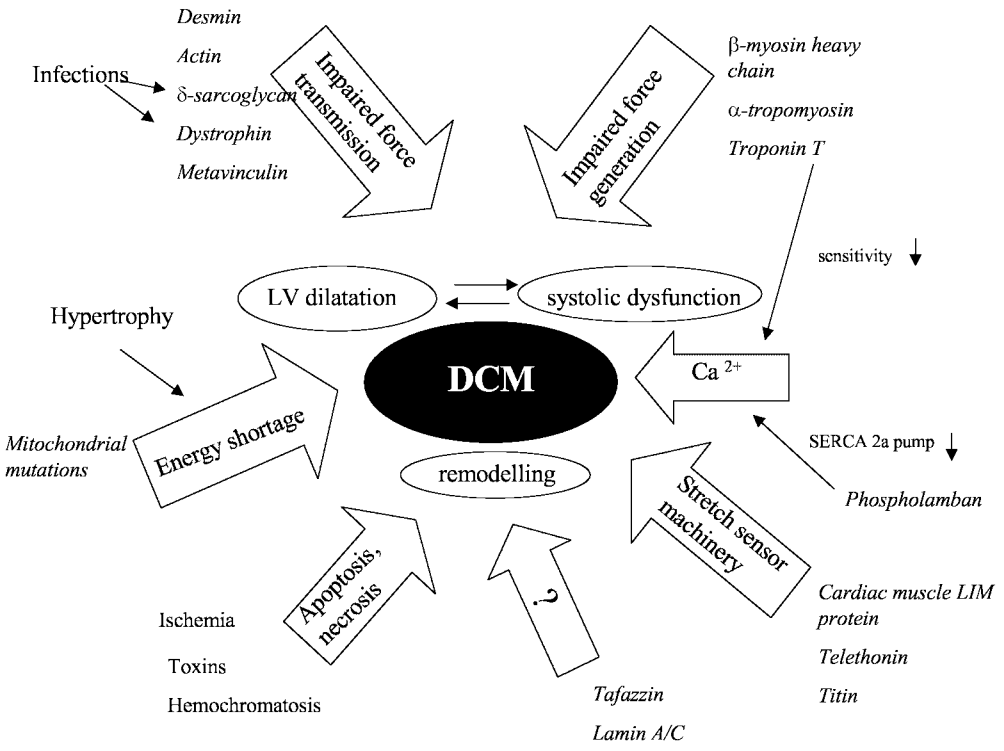

Figure 4. The schematic figure of the DCM-causing mechanisms (modified from Schönberger and Seidman (141) and Fatkin and Graham (59).

2.3.2 Non-familial DCM

Non-familial causes for DCM include active myocarditis, metabolic diseases (diabetes, thyroid disorders and pheochromocytoma), toxics (alcohol, anthracyclines, lithium, cocaine), storage diseases (hemochromatosis) and systemic diseases (sarcoidosis and connective tissues diseases) (1, 58, 60). Coronary artery disease, valvular diseases and hypertension can cause a phenotype similar to DCM. Non-familial causes of DCM and diseases that can cause a similar phenotype have to be excluded before genetic studies. However, these diseases can contribute to characteristics of DCM in familial cases, and they may also affect the prognosis of the disease.

Viruses may have a role in the pathogenesis of DCM. Viral infection of the heart is relatively common, but viral infection does not cause usually major consequences. However, virus infection can lead to cardiomyopathy and heart failure. The results of virus studies in DCM have nonetheless been variable, and therefore their role in the pathogenesis of DCM has not been confirmed. Virus genome has been found also in control patients and in patients with coronary heart disease, and therefore the virus hypothesis remains controversial (142, 143). The diagnosis of viral cardiomyopathy can only be made from endomyocardium biopsy using PCR techniques to detect the viral genome. The most frequently detected viruses are enteroviruses, adenoviruses, cytomegaloviruses, and less frequently Epstein-Barr, influenza, polio, herpes, hepatitis C and varicella zoster viruses (144).

Acute viral infection can lead to cell death by affecting membrane permeability, through disruption of host cell transcriptional and translational machinery or induction of apoptotis (145, 146). In the first phase of viral infection of the heart, infection results in myocyte necrosis and apoptosis with release of intracellular antigens. In the second phase, an autoimmune reaction develops and autoantibodies against cardiac antigens and autoreactive T-lymphocytes are released. Myocardial dysfunction with hemodynamic remodeling and neurohumoral activation takes place months to years after viral infection and leads to chronic heart failure (146). Badorff et al. (147) showed that enterovirus infection can lead to disruption of the cytoskeleton through enteroviral protease cleavage of dystrophin. Cleavage of dystrophin leads to the disruption of the cytoskeleton resulting in myocyte dysfunction, decreased force transmission and increased cell permeability. Furthermore, the sarcoglycan complex can be affected secondarily due to dystrophin cleavage (148).

2.3.3 Familial DCM

The prevalence of familial DCM varies between 10-35%. The lowest prevalence is reported in studies without systematic screening of family members. If family members are systematically screened, the prevalence of familiarity varies between 20-35% (2, 4, 149). The mode of inheritance of DCM can be autosomal dominant (~60%), autosomal recessive (~16%), x-linked (~10%) or mitochondrial (~8%) (150).

Although genetic factors play a role in the pathogenesis of DCM, the genetic background of DCM is largely unknown. Linkage analysis has not been very useful due to a limited size of families and difficulties in the diagnosis of DCM. However, linkage analysis has been applied and several chromosomal loci for DCM have been found. Specific genes have not yet been identified in all loci, but some clinical characteristics are associated with known loci, e.g. skeletal muscle disease and mitral prolapse. Chromosomal loci reported to be associated with DCM are listed in Table 4.

The most commonly used research method in familial DCM is the candidate gene approach. To date, approximately 60 different gene defects have been reported in seventeen genes: cardiac actin, desmin, dystrophin, δ-sarcoglycan, troponin T and β-myosin heavy chain, α-tropomyosin, taffazin, titin, lamin A/C, metavinculin, myosin-binding protein-C, cardiac muscle LIM protein, phospholamban, α-actinin and troponin I genes (6-14, 16-29, 151). These genes can be classified according to their locations or disease-causing mechanisms. Classification according to disease-causing mechanisms is shown in Figure 4, but the details of DCM associated genes and mutations are described below according to classification of locations.

Table 4. Chromosomal loci associated with DCM

Locus	Gene	Clinical characteristics	Reference
1p1-q21	Lamin A/C	CSD, atrial fibrillation, SCD	(8, 10, 13)
1q32	Troponin T	DCM	(16, 18, 20, 152)
1q42-q43	α-actinin		(28)
2q14-q22	?	CSD	(153)
2q31	Titin	DCM	(15, 154)
2q35	Desmin	DCM, muscular dystrophy	(19)
3p22-p25		CSD	(155)
5q33	δ-sarcoglycan	DCM	(25)
6q12-q16	?	DCM	(156)
6q22.1	Phospholamban		(27)
6q23	?	Sensorineural hearing loss, skeletal myopathy, CSD	(157)
9q13-q22	?	DCM	(158)
10q21-23	?	Mitral valve prolapse	(159)
10q22-q23	Metavinculin		(23)
11p11	Myosin-binding protein-C	DCM	(11)
11p15	Cardiac muscle LIM protein	DCM	(26), (28)
14q12	β-myosin heavy chain	DCM	(11, 18)
15q14	Cardiac actin	DCM	(21)
15q22	α-tropomyosin	DCM	(22)
17q12	Telethonin	DCM	(26)
19q13	Troponin I	recessive DCM	(29)
Xp21	Dystrophin	Skeletal muscle disease or isolated DCM	(7)
Xq28	Tafazzin		(12)

CSD, conduction system disease; DCM, dilated cardiomyopathy; SCD, sudden cardiac death; ?, not known.

2.4 Genes associated with DCM

2.4.1 Sarcomere protein genes

2.4.1.1 Overview of sarcomere protein genes

The sarcomere proteins have an essential role in heart muscle contraction, force transmission and forming structural scaffold of the muscle cell. Defects in sarcomere protein genes are the most important etiological factors in hypertrophic cardiomyopathy (HCM), and over 150 HCM associated mutations have been found in genes encoding sarcomere proteins (160-162). In the pathogenesis of DCM, the sarcomere protein genes do not seem to have an essential role, although it has been estimated that sarcomere protein genes explain approximately 10% of all familial cases of DCM (18). To date, approximately 20 different mutations in eight sarcomere protein genes have been reported to cause DCM. All these mutations have been found in single or several families or sporadic patients, and it is therefore difficult to estimate the real importance of the sarcomere protein genes in the pathogenesis of DCM. Table 5 summarizes all published DCM-associated mutations in the sarcomere protein genes, their location, the number of probands with the mutation, the number of patients screened, the phenotypes of the mutated patients and probable effects of specific mutations.

2.4.1.2 Cardiac actin gene (ACTC)

The ACTC gene on chromosome 15q14 has 6 exons. The cardiac actin gene was the first sarcomere protein gene reported to be associated with DCM. Olson et al. (21) found two missense mutations, Arg312His and Glu361Gly, in the ACTC gene in two DCM families. These mutations are located in the region that encodes the immobilized end of the actin filament. This region is near the dystrophin-binding site, which binds domain of the protein to the Z bands and intercalated discs (25, 163, 164). These mutations are suggested to affect force transmission to adjacent sarcomeres and myocytes. Other DCM-associated mutations in the cardiac actin gene have not been published since. Consequently the mutations in the cardiac actin gene are thought to be a rare cause for DCM (165-167). Mutations in the ACTC gene have been reported to cause also HCM (164, 168). HCM-associated mutations are located in different regions

of the gene than those causing DCM, and they affect the interaction between actin and myosin, which in turn is linked to force generation (164).

2.4.1.3 α-tropomyosin gene (TPM1)

The TPM1 gene on chromosome 15q22 has 9 exons. Only two DCM-associated mutations have been reported in the TPM1 gene. Olson et al. (22) described two mutations, Glu40Lys and Glu54Lys, in two small DCM families. These two mutations in the TPM1 gene are thought to alter electrostatic interactions between actin and tropomyosin or compromise the structural integrity of tropomyosin (22). The family with the Glu40Lys mutation had a variable phenotype. One family member had severe disease and underwent heart transplantation at the age of 10 years, whereas one family member developed a mild DCM phenotype at the age of 40 years. The Glu54Lys mutation has been associated with sudden cardiac death at young ages. Other DCM-associated mutations in the TPM1 gene have not been published yet, and therefore, TPM1 gene defects also seem to be uncommon in DCM. Elsewhere in the world, mutations in the TPM1 gene are not very frequently found in patients with HCM either, but in Finland mutations in this gene are the second most common cause for HCM (169, 170).

2.4.1.4 β-myosin heavy chain gene (MYH7)

The MYH7 gene on chromosome 14q11 has 38 coding exons. The β-myosin heavy chain protein has two different domains, a heavy meromyosin domain, which consists of two subfragments (S1 and S2) and a light meromyosin (LMM) domain (171). The S1 locates in the head domain and has actin and ATP binding regions (171). The S2 joins the heads at the neck region (171). The LMM portion forms binding sites for myosin-binding protein-C and titin and is essential for maintaining assembly of the filament (171).

Four DCM-causing mutations have been detected in the MYH7 gene. Kamisago et al. (18) found two mutations, Ser542Pro and Phe764Leu, in the MYH7 gene in two different DCM families. The Ser542Pro mutation was associated with sudden death and heart transplantation at young ages and the Phe764Leu mutation manifested at birth or

at the age of two months. These mutations are thought to disrupt interactions between myosin and actin or diminish the efficiency of contraction, respectively (18). Daehmlow et al. (11) reported two mutations, Ala223Thr and Ser642Leu, in the MYH7 gene. These mutations were found in two sporadic DCM patients. The Ala223Thr mutation is thought to affect thermostability and protein folding and the Ser642Leu mutation in turn conformational structure of protein near the actin-binding site (11). Mutations in the MYH7 gene are known to be the most common causes for HCM accounting generally approximately 30% of all cases, but in Finland only one HCM-causing mutation in this gene has been found so far (162, 169). All these previously described DCM-associated mutations are located in the S1 domain of the protein. The major part of the HCM-associated mutations are also located in the region coding the globular head and head-rod junction of the protein (171).

2.4.1.5 Troponin T gene (TNNT2)

The TNNT2 gene on chromosome 1q32 has 17 exons. Two DCM-causing mutations in this gene have been reported. Kamisago et al. (18) found one deletion, ΔLys210, in two DCM families. Later Hanson et al. (16) detected this same deletion in another DCM family. The Lys210 deletion is associated with sudden cardiac death, heart failure and conduction system disease. This deletion reduces ionic interactions between troponin C and troponin T and decreases the activation of calcium-stimulated actomyosin-ATPase. These changes are thought to lead to a decrease in power stroke (18). One single mutation, Arg141Trp in the troponin T gene, has been found in one DCM family (20). The mutation Arg141Trp is located within the tropomyosin-binding domain of cardiac troponin T and alters the charge of the residue. Morimoto et al. (172) explored the effect of the ΔLys210 mutation under physiological conditions in cardiac muscle fibers of the rabbit. The ΔLys210 mutation was found to decrease Ca^{2+} sensitivity of force generation in the sarcomere, which could be one of the primary mechanisms for the pathogenesis of DCM. Several HCM-associated mutations have been reported in the TNNT2 gene and mutations in the TNNT2 gene are the third most common cause of HCM (173). Robinson et al. (174) compared the changes in thin filament function caused by the mutations in DCM and HCM. The DCM-associated mutation, ΔLys210, decreases

ATPase activity, which in turn speeds filament sliding, and Ca^{2+} activation becomes non-cooperative. The HCM-causing mutation increases ATPase activity, speed of sliding and Ca^{2+} sensitivity (174).

2.4.1.6 Troponin I gene (TNNI3)

The troponin I gene on chromosome 19q13 has eight exons. Murphy et al. (29) recently found the first recessive DCM-associated mutation, Ala2Val, in this gene. This mutation was shown to impair the interaction between troponin I and troponin T and leading to diminished myocardial contraction. The phenotype of the Ala2Val mutation seemed to be severe.

2.4.1.7 Myosin-binding protein-C gene (MYBPC3)

The MYBPC-3 gene on chromosome 11p11 has 34 exons. The protein product of this gene has multiple immunoglobulin C2-like and fibronectin type 3-like domains, as well as a cardiac specific region, a phosphorylation region, and overlapping regions of myosin and titin (59). Only one DCM-associated mutation, Asp948Thr, in one sporadic DCM patient has been reported for this gene (11). The specific effect of this mutation is not known, and because only one patient with the mutation has been reported, the clinical phenotype remains unclear. Mutations in the MYBPC-3 gene are common causes for HCM, accounting approximately 20% of all HCM cases (162).

2.4.1.8 Titin gene (TTN)

The TTN gene on chromosome 2q24 has 362 exons. Gerull et al. (15) found two DCM-associated mutations, 2-bp insertion and Trp930Arg, in the TTN gene. The 2-bp insertion is predicted to truncate the protein and the Trp930Arg mutation affects the Z disc/I band transition zone (15). Itoh-Satoh et al. (116) reported four DCM-associated mutations in the titin gene. Two of these mutations, Val54Met and Ala743Val, are located in the Z line region of titin. These mutations are thought to decrease binding affinities of titin to Z line proteins. Two other mutations, Glu4053ter and Ser4465Asp,

are located in the cardiac specific N2-B domain of titin. The Glu4053ter mutation is thought to encode truncated nonfunctional protein.

2.4.1.9 Telethonin gene (TCAP)

The telethonin gene on chromosome 17q12 probably has two exons. TCAP is a Z disc protein, which is essential for proper sarcomeric function. Knöll et al. (26) recently reported one mutation, Arg87Gln in the TCAP gene in one DCM patient. The mutated telethonin gene leads to a defect in the interaction between cardiac muscle LIM protein and telethonin.

2.4.1.10 Phopholambam gene (PLN)

The phospholamban gene on chromosome 6q22 has two exons. Phospholamban has an essential role in Ca^{2+} metabolism, which in turn is related to proper sarcomere function and heart muscle contraction. Schmitt et al. (27) detected one DCM-causing mutation, Arg9Cys in the phopholamban gene in one DCM family. This Arg9Cys mutation is thought to cause DCM by directly disturbing myocellular Ca^{2+} metabolism due to constitutive SERCA2a inhibition. The authors also made a transgenic mouse with this mutation, which developed a similar type of disease as mutation carriers (heart failure with premature death). Family members with the Arg9Cys mutation had severe disease with frequent heart transplantation and premature death.

Table 5. DCM-associated mutations in the sarcomere protein genes.

Gene exons	Mutation	Location	No of probands with mutation/ no of screened patients	Phenotype of the mutation and clinical consequences	Suggested mechanism of the mutation	Ref
ACTC 6 exons	Arg312His	15q14	1/ 2 F	DCM	defect in force transmission	(21)
	Glu361Gly		1			
TPM1 9 exons	Glu40Lys	15q22.1	1/ 350 S	Variable phenotype, early onset, HT	alters the interaction between actin and tropomyosin or compromise structural integrity	(22)
	Glu54Lys		1			
MYH7 38 exons	Ser532Pro	14q11	1/ 21 F	DCM, HT, SCD, early onset	disrupts interactions between actin and myosin	(18)
	Phe764Leu		1		alters the magnitude or polarity of transmitted movement → efficiency of contraction ↓	
	Ala223Thr		1/ 46	DCM, ICD, early onset	affects the ATP-binding site	(11)
	Ser642Leu		1		affects the actin-myosin interaction	
TNNT2 17 exons	ΔLys210	1q32	2/ 21 F 1/ 61 F+ 53 S	CSD, AF, SCD	affects troponin C binding and diminish activation of calcium-stimulated actomyosin ATPase	(18) (16)

	Arg141Trp	1q32	1/ 1 F	Variable phenotype, SD	affects tropomyosin-binding domain	(20)
MYBPC3 34 exons	Asn948Thr	11p11.2	1/ 46	DCM, ICD	?	(11)
TTN 340 exons	2 bp insertion in exon 326	2q24.3	1/ 2F	?	truncates the A band	(15)
	Trp930Arg		1		disrupts core sequence of immunoglobulin fold near z disc- I-band transition zone	
	Val54Met		1/ 31 F + 89 S	CSD, arrhythmias	decreases the binding affinity of titin to z-lines proteins telethonin	(116)
	Ala734Val		1		decreases the binding affinity of titin to α-actinin	
	Gln4053ter		1		mutations in the cardiac specific region of titin → truncated nonfunctional molecule	
	Ser4465Asp		1			
TNNI3 8 exons	Ala2Val	19q13	2/ 235	DCM, HT	impaired interaction between troponin I and troponin T→contraction ↓	(29)
TCAP 2 exons	Arg87Gln	17q12	1/ 380	?	loss of interaction between cardiac muscle LIM protein and telethonin	(26)

ACTC, cardiac actin gene; AF, atrial fibrillation; CSD, conduction system disease; DCM, dilated cardiomyopathy; F, patient with familial disease; HT, heart transplantation; ICD, automatic cardioverter-defibrillator; MYBPC3, myosin binding protein-C; MYH7, β-myosin heavy chain gene; S, sporadic patient; SD, sudden death; SCD, sudden cardiac death; TCAP, telethonin; TNNI3, troponin I; TNNT2, troponin T; TPM1, α-tropomyosin; TTN, titin; ?, not known or not mentioned

2.4.2 Cytoskeletal protein genes

2.4.2.1 Overview of cytoskeletal protein genes

Cytoskeletal proteins have an essential role as the mechanical mainstay of the cell and in the force transmission. Gene defects in the cytoskeletal protein genes are known to cause various skeletal muscle diseases, but some of them can also be responsible for isolated DCM. To date, over 20 different mutations in six cytoskeletal protein genes have been reported to cause DCM. Three mutations (in the desmin, dystrophin and LIM protein genes) have been detected in more than one DCM patient or family (26, 175, 176). All other mutations have been found only in isolated cases. Table 6 summarizes all DCM-associated mutations in the cytoskeletal protein genes.

2.4.2.2 Desmin gene (DES)

The desmin gene on chromosome 2q35 has 9 exons. The desmin protein has three parts, a central rod domain, and head and tail domains. In the desmin gene, one DCM-associated mutation, Ile451Met, has been reported (19, 175). The Ile451Met mutation is located in a region of the gene encoding the tail domain of the protein, the specific function of which is unknown. It has been speculated that DCM-causing mechanism in the desmin gene is related to ineffective force transmission (19, 141). Clinically, the Ile451Met mutation has been shown to be associated with a classical type of DCM and sudden death. Gene defects in the desmin gene have also been shown to cause skeletal muscle disease combined with cardiomyopathy and desmin-related myopathy, which is caused by abnormal accumulation of desmin in skeletal muscle (110, 177, 178). Generally, mutations in the desmin gene are thought to be rare causes for DCM, accounting at most for about 2% of the cases (167, 175).

2.4.2.3 Dystrophin gene (DMD)

The dystrophin gene on chromosome Xp21 has 79 exons. Dystrophin mutations are better known to cause Duchenne and Becker muscular dystrophies (179, 180). In a few cases, gene defects in the dystrophin gene have been shown to be associated with isolated cardiomyopathy without skeletal muscle disease, however. This disease is

called X-linked dilated cardiomyopathy (XLCM)(181). XLCM often presents in young male patients with heart failure and no signs of skeletal muscle disease. XLCM mutations in the dystrophin gene can be divided into two groups according to localization, 5`end region mutations and midrod domain region mutations (182). The majority of DCM-associated mutations are located in the 5`end region and are malignant in nature (6, 7, 24, 183-186). Several mutations, mainly deletions, have been reported in the midrod domain of the protein. Usually these mutations cause a more benign disease (182, 184, 185, 187, 188). Badorff et al. (147) showed that an enterovirus infection can lead to cleavage of dystrophin. This leads to disruption of cytoskeleton resulting in myocyte dysfunction, decreased force transmission and increased cell permeability

2.4.2.4 Metavinculin gene (VCL)

The metavinculin gene on chromosome10q22 has 22 exons. Metavinculin is an isoform of vinculin, encoded by an additional insert of genome (68 amino acids) in the C-terminal end. Maeda et al. (189) demonstrated an association between metavinculin deficiency and dilated cardiomyopathy due to a defect in alternative mRNA splicing. Olson et al. (23) have found one missense mutation, Arg975Trp and one 3-bp deletion (Leu954del) in the metavinculin gene in two DCM patients. These authors suggested that the mechanism probably causing DCM was the disruption of force transmission at the thin filament –intercalated disc interface.

2.4.2.5 δ-sarcoglycan gene (SGCD)

The SGCD gene on chromosome 5q33 has 8 exons. δ-sarcoglycan has three domains; intracellular, transmembrane and extracellular domains (190). Tsubata et al. (25) detected two DCM-causing mutations, Ser151Ala and ΔLys238 in the SGCD gene in single DCM cases. Both mutations are thought to alter secondary structure of the protein and the DCM-causing mechanism may be a defect in force transmission. The DCM-associated mutations are located in the extracellular domain. Gene defects in the SGCD gene can also lead to limb girdle muscular dystrophy, which is sometimes associated with DCM (191). In animal studies, Nigro et al. (192) showed that a deletion

of the first exon caused a complete absence of the δ-sarcoglycan protein in Syrian hamster. This deletion was associated with skeletal muscle disease, ventricular hypertrophy and dilatation and premature death.

2.4.2.6 Cardiac muscle LIM protein gene (CLP)

The cardiac muscle LIM protein gene on chromosome 11p15 has four exons. The CLP protein has two LIM domains, and these domains are highly conserved (59). The CLP protein is located in the Z disc, and interacts with telethonin and titin (26). Knöll et al. (26) found one DCM-associated mutation, Trp4Arg, in the CLP gene in ten index patients from European subjects. The Trp4Arg mutation results in a defect in interaction with telethonin, and interferes with the cardiac mechanical stretch sensor machinery (26). Clinically the Tpr4Arg mutation causes typical DCM with a low ejection fraction, but only a slightly dilated left ventricle. Mohapatra et al. (28) has found this same mutation in an European family, but the mutation did not co-segregate with DCM. Therefore, it is possible that the W4R mutation is a modifier mutation rather than a disease-causing mutation. Mohapatra et al. (28) detected another DCM-associated mutation, Lys69Arg, in the CLP gene. The Lys69Arg mutation abolishes the interaction of telethonin with CLP and α-actinin and changes the localization of CLP. It is associated with early onset of DCM.

2.4.2.7 α-actinin gene (ACTN2)

The α-actinin gene on chromosome 1q42 has 21 exons. It consists of an N-terminal actin binding domain, a central rod domain, and a C-terminal domain and functions as a homodimer to cross-link actin filaments. The rod domain determines the distance between cross-linked actin filaments and also serves as an interaction site for several cytoskeletal and signaling proteins (193). Recently, Mohapatra et al. (28) detected one mutation, Gln9Arg, in the α-actinin in one DCM patient with severe disease. The Gln9Arg mutation was predicted to alter secondary structure of the protein.

Table 6. DCM-associated mutations in cytoskeletal protein genes

Gene	Mutation	Location	No of probands with mutation/ no of screened patients	Phenotype of the mutation and clinical consequences	Suggested mechanism of the mutation	Ref
DES 9 exons	Ile451Met	2q35	1/ 44 F 3/ 265 F+S	DCM, early onset	?	(19) (175)
DYS 79 exons	Thr279Ala	Xp21	1/ 3 F	DCM + CK↑, poor survival	changes polarity → alters the secondary and tertiary structure of dystrophin	(24)
	deletion in the 5`end		1/ 1 F	DCM with or without SMD	affects dystrophin expression in the heart	(6, 194)
	deletion of exons 48-49 deletion of exons 49-51		1/ 2 F	DCM with or without SMD DCM, normal CK levels	?	(188)
	G→T transversion at the first exon-intron boundary		1/ 1 F	DCM with or without CK↑	inactivates the conversed 5`splice site consensus sequence	(183)
	deletion of exon 48 deletion of exons 48-51 deletion of exons 45-48 deletion of exons 48-53		1/ 201 1 1 1	DCM, no SMD, HT DCM, no SMD DCM, no SMD DCM, no SMD	affects the structural and functional integrity of myocyte sarcolemma	(182)

	Lys18Asn		1/ 22 S	DCM, HT, later CK↑	?	(184)
	Phe3228Leu		1	DCM, early onset		
	IVS 5+1 G→T		1	DCM, early onset		
	Asn1672Lys		1/ 8 F	DCM, early onset, SCD, CK↑		(185)
	IVS 1+ 1 G→T		1	Early onset, SCD, CK↑		
	IVS 1+ 6 T→C		1	Early onset, SCD		
	L1 insertion in 5`end		2/ 3 F	DCM+ SMD	affects transcription or stability of the muscle form of dystrophin	(176)
VCL 22 exons	Arg975Trp	10q22	1/ 350 S	DCM, HT	disrupts force transmission at the thin filament-intercalated disc interface	(23)
	ΔLeu954		1	DCM		
SGCD 8 exons	Ser151Ala	5q33	1 / 50 S + 1 F	DCM, SCD, early onset	alters the secondary structure of the protein	(25)
	ΔLys238		2	DCM, early onset		
CLP 5 exons	Trp4Arg	11p15	10/ 821 patients	DCM	defect in the CLP/telethonin interaction → interferes stretch sensor machinery	(26)
	Lys69Arg			DCM, early onset	abolishes interaction between MLP and α-actinin, changes localization of MLP	(28)
ACTN2 21 exons	Gln9Arg	1q42		DCM, early onset	disrupts the interaction with MLPand inhibits α-actinin function in cultured cells	(28)

ACTN2, α-actinin; CK, creatine kinase; CLP, cardiac muscle LIM protein; DCM, dilated cardiomyopathy; DES, desmin; DYS, dystrophin; No, number of ; F, familial; HT, heart transplantation; S, sporadic; SCD, sudden cardiac death; SGCD, delta-sarcoglycan; SMD, skeletal muscle disease; VCL, metavinculin; Δ, deletion; IVS, intron; ?, not known.

2.4.3 Nuclear envelope protein genes

2.4.3.1 Lamin A/C gene

The lamin A/C gene on the chromosome 1p1 contains 12 exons. Lamin A and C have an identical sequence for the first 566 amino acids in exons 1-10, but differ in the 3′ end of the gene. The lamin A/C protein has three different domains, an N-terminal head, a coiled-coil rod and a C-terminal tail (133). Lamin A/C gene has several different binding domains, for example lamin B, chromatin, emerin and lamin-associated protein binding domains (195). Defects in the lamin A/C gene have been shown to be responsible for seven different diseases; Emery-Dreifuss muscular dystrophy, limb girdle muscular dystrophy type 1B, Dunnigan-type familial partial lipodystrophy, Charcot-Marie-Tooth disease, mandibuloacral dysplasia, Hutchinson-Gilford progeria and dilated cardiomyopathy with conduction abnormalities or mild skeletal muscle disease (8, 10, 13, 196-201).

Mutations in the lamin A/C gene seem to be the most frequent causes of familial DCM. During the past two years several independent research groups have reported different or similar DCM-associated mutations in this gene. Most of the mutations associated with cardiac abnormalities are located in the central rod domain. Only a few mutations are in the tail domain of the protein, which has a role in lamin assembly (132, 133). The mutations in the lamin A/C gene have been shown to cause a loss of integrity of the myocyte nuclei with blebs of the nuclear membrane, herniations and delamination of the nuclear lamina and nuclear pore clustering (8, 202). The clinical disease is very similar in all lamin A/C mutations. The main clinical characteristics are the conduction system disease, a need for a pacemaker, atrial fibrillation and elevated risk for sudden cardiac death. DCM-associated mutations in the lamin A/C gene and their phenotypes are presented in Table 7.

Table 7. DCM-associated mutations in the nuclear envelope protein genes

Gene	Mutation	Location	No of probands with mutation/ no of screened patients	Phenotype of the mutation and clinical consequences	Suggested mechanism of the mutation	Ref
LMNA 12 exons	959delT	1p1-q21	1/ 1F	CSD, LBBB, SVT, slight SMD	affects dimerization through changes in the rod domain	(10), (203)
	Arg60Gly Leu85Arg Asn195Lys Glu203Gly		1/ 11F 1 1 1	CSD, AF, PM, HT, SCD	alters the residues in the α-helical rod domain	(13)
	Arg571Ser		1		alters a residue spesific to the carboxyl tail of the lamin C isoform	
	Lys97Glu Glu111Stop Arg190Trp Glu317Lys		1/ 40 F, 33 S 1 1 1	CSD, LBBB, PM, HT	affects lamin B interaction and tetramere formation	(8)
	Ins ctgc at 2869 cDNA		1		affects the LMNA-emerin interaction	
	Arg644Cys 1397delA		1/ ? 1	? ?	affects the central domain of the protein locates in the carboxyterminal domain of lamin A	(14)

Glu203Lys	1	CSD, SCD		(17)
Arg225Stop	1			
Leu215Pro	1	CSD, AF, PM; SCD	affects the rod domain of the protein	(204)
Arg89Leu	1/ 40 F + 9 S	CSD, SCD, SVT, VT,		(203)
Del 959T	1	HT		
Arg377His	1			(205)
Ser573Leu	1			
Glu161Lys	1/ 66	AF	intermediate filament disorganization	(205)
28insA	1/ 66	CSD	haploinsufficiency	
Arg89Leu	1/ 9F	CSD, PM, HT	leads to loss of integrity of the myocyte nuclei, delamination of the nuclear lamina, loss of protein expression in the selective compartment of non-cycling myocyte nuclei	(202)
165delC	1/ 9F			
908delCT	1/ 1F	CSD, PM		(206)

AF, atrial fibrillation; CSD, conduction system disease; Del, deletion; F, familial; HT, heart transplantation; ins, insertion; LBBB, left bundle branch block; LMNA, lamin A/C; PM, pacemaker; S, sporadic; SCD, sudden cardiac death; SMD, skeletal muscle disease; SVT, supraventricular tachycardia; VT, ventricular tachycardia; ?, not known,

2.4.4 Mitochondrial mutations

The mitochondrial genome is a double-stranded circular molecule. The mitochondrial genome has some specific features compared with genomic DNA. Mitochondria do not have introns and histones and they lack an effective DNA repair system. Mutation rate is higher in mtDNA than in nuclear DNA partially due to ineffective DNA repair and free radical formation (207). Maternal inheritance is typical for mitochondria, but also inheritance according to Mendelian laws can occur. Mitochondria play an important role in energy generation and thus in contractile function, and disturbances in these functions may lead to DCM in some cases. Gene defects in the mitochondrial genome can cause syndromes like MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes), Kearns-Sayre syndrome and MERFF (myoclonus epilepsy with ragged red fibers), which are caused by point mutations and deletions in the mtDNA. Different cardiac abnormalities are accompanied with these syndromes, especially HCM. In few cases DCM has been detected in patients with MELAS and Kearns-Sayre syndrome (208-213).

2.4.5 Susceptibility and modifier genes

2.4.5.1 Susceptibility genes

Susceptibility genes have a role in the development of DCM, whereas modifier genes have a role in the evolution or prognosis of the disease. In DCM susceptibility and modifier genes are not well known and some of the results are contradictory. In most of the studies investigating the role of susceptibility and modifier genes, the sample sizes have been limited, and therefore results have been only suggestive (214).

Tiret et al. (215) investigated the role of eight candidate genes for DCM. The polymorphisms of the ACE, angiotensinogen (AGT), angiotensin-II type receptor (AGTR1), aldosterone synthase (CYP11B2), tumor necrosis factor-alpha (TNF-α), transforming growth factor $beta_1$ (TGFB1), endothelial nitric oxide synthase (NOS3) and brain natriuretic peptide (BNP) genes were studied, but there were no apparent association with DCM. Hiroi et al. (216) described higher frequencies in polymorphisms of the HLA–DR and superoxide dismutase (SOD2) genes in patients with DCM than in control subjects, indicating potential susceptibility affect. Also the

G/T polymorphism of the platelet-activating factor (PAF) acetylhydrolase has been shown to have an association with nonfamilial DCM (217). This polymorphism is also related to left ventricular mass and LVEDD, and the authors suggested that this polymorphism could be a susceptibility gene for non-familial DCM or be related to the progression of the disease.

2.4.5.2 Modifier genes

The angiotensin-converting enzyme (ACE) polymorphism has been commonly studied with variable results. Andersson et al. (218) reported a clear effect of ACE polymorphism on survival in patients with DCM. The patients with a DD genotype had poorer prognosis than other genotypes (5-year survival rate 49 vs. 72%, p=0.0011) (218). The DD genotype was also associated with an increase in left ventricular mass. However, in other studies the association between the ACE polymorphism and the severity of DCM has not been detected (215, 219). Liggett et al. (220) detected poor survival and a high risk for heart transplantation in patients with the Ile164 polymorphism of the β_2-adrenergic receptor (β_2-AR) gene. Also β1-adrenoreceptor gene polymorphism has been shown to affect the survival of patients with DCM (221). Herrmann et al. (222) found that the T allele of the ET_A receptor gene polymorphism (H323H) was associated with a worse survival than non-carriers of this polymorphism, whereas Charron et al. (223) did not find any relationship between the polymorphisms of the genes coding endothelin 1, endothelin type A and type B receptors and DCM. However, the C/T polymorphism in exon 8 of the ET_A receptor gene was a risk factor for idiopathic DCM in patients who were homozygous for the T allele. Loh et al. (224) showed an association between a mutant adenosine monophosphate deaminase 1 (AMPD1) and a longer duration of heart failure symptoms.

Tiago et al. (225) reported that aldosterone synthase (CYP11B2) gene variant predicted an improvement in ejection fraction after initiating medical therapy (diuretics, digoxin and ACE inhibitors). This gene variant was not associated with DCM, but the researchers speculated that the aldosterone synthase locus could regulate the progression of heart failure (225). In the same study, there was no association between the ACE and angiotensinogen polymorphisms and DCM. Kupari et al. (226) investigated polymorphisms of the CYP11B2 gene in young healthy individuals and showed an

association between a polymorphism of the CYP11B2 gene and left ventricular size and mass. The prevalence of different polymorphisms in the hemochromatosis gene have been shown to be variable among DCM patients (227-229). Mahon et al. observed that the frequency of the H63D polymorphism is increased in patients with DCM, but other research groups did not find such an association (227-229). Mahon et al. (228) reported that DCM patients with the C282Y polymorphism of the same gene have less ventricular dilatation and better ejection fraction. Therefore, this polymorphism might have a modifier effect, but this hypothesis should be confirmed in a larger study.

3. THE AIMS OF THE STUDY

This study is part of a larger study project, the purpose of which is to investigate the etiopathogenesis and prognosis of DCM in the Finnish population. The primary goal was to collect all patients with idiopathic DCM from the Kuopio University Hospital region and to investigate the genetic and clinical characteristics of DCM in the Eastern Finnish population. Furthermore, the association between potential mutations and prognosis were investigated. We used a candidate gene approach, because most of the families were too small for linkage analysis. The following specific questions were studied:

1) Are there mutations in the ACTC gene that are associated with DCM in patients from Eastern Finland? (Study I)

2) Are there mutations in the lamin A/C gene that are associated with DCM in patients from Eastern and Southern Finland, and if so, what are the clinical manifestations? (Study II)

3) What is the role of variants in the previously reported sarcomere protein genes (MYH7, TPM1, TNNT2) and new candidate genes (TNNI3 and TNNC1) in patients with DCM from Eastern Finland, and what is the phenotype of patients having mutations in these genes? (Study III)

4) Do mutations in the δ-sarcoglycan, desmin and metavinculin genes cause DCM in patients from Eastern Finland? (Study IV)

4. SUBJECTS AND METHODS

4.1 Study subjects

4.1.1 Subjects in Study I

In the first study we included 32 DCM and 42 HCM patients from the Kuopio University Hospital region in Eastern Finland. Commonly approved diagnostic criteria, LV (left ventricular) ejection fraction < 45 to 50% and LV end-diastolic diameter > 27mm/m^2 (58) were used in diagnosis of DCM. All patients having secondary causes for DCM were excluded from the study. The diagnostic criteria for HCM were LV wall thickness ≥15 mm, blood pressure ≤160 /100 mmHg and no other causes for ventricular hypertrophy, e.g. primary valvular diseases.

4.1.2 Subjects in Study II

The study group consisted of 52 DCM patients from the Kuopio University Hospital region. The clinical characteristics of the study subjects (n=52) are given in Table 8. In addition to this study group, an additional group of 38 patients from the Helsinki University Hospital region were studied.

4.1.3 Subjects in Studies III-IV

The patient group in Studies III and IV consisted of 52 DCM patients from the Kuopio University Hospital region.

4.1.4 Additional study group in Studies III-IV

An additional study group (n=104) consisted of 38 DCM patients from Southern Finland (same patients as in Study II) and 66 DCM patients that had undergone heart transplantation. We screened this additional study group for the mutations found in the β-myosin heavy chain and δ-sarcoglycan genes.

4.1.5 Control subjects

Control subjects were healthy unrelated subjects from our previous population studies (230, 231). Control subjects did not have any chronic diseases such as diabetes, coronary heart disease or hypertension. Altogether 82 of them had undergone echocardiography. The number of control subjects varied between 37 and 172 in different studies. All mutations were screened in at least 150 control subjects.

4.1.6 Clinical characteristics of the original study group

The original study population consisted of 52 DCM patients from Eastern Finland. The study population was carefully evaluated by medical history and clinical examination and later by echocardiography and cardiac catheterization. The clinical characteristics presented in Table 8 represent those observed at the time of diagnosis.

Our study patients were mainly middle-aged at the time of diagnosis. Only a few patients were diagnosed with DCM in their childhood. Almost all study patients had cardiac symptoms before diagnosis, dyspnea being the most common symptom. Patients had relatively mild symptoms (NYHA 1-2 functional class mostly). The most frequent findings were left ventricular hypertrophy (LVH), left atrial hypertrophy (LAH) and left bundle branch block (LBBB) on ECG. In echocardiography left ventricular end-diastolic diameter varied from 51 to 90 mm (mean 65 mm) and the mean ejection fraction was 34%. Mild mitral regurgitation was common, approximately two thirds of the patients had some degree mitral regurgitation. Clinical characteristics of 52 DCM patients who formed the original study group are given in Table 8.

Table 8. Clinical characteristics of DCM patients in the original study group.

	N=52
Men/Women	37/ 15
Age (years)	50 ± 2(11-71)
Cardiac symptoms	46 (88%)
Dyspnea	35 (67%)
Chest pain	14 (27%)
Presyncope or syncope	4 (8%)
Palpitation	24 (46%)
NYHA I	15 (2%)
NYHA II	23 (44%)
NYHA III	13 (25%)
NYHA IV	1 (2%)
Systolic BP (mmHg)	135 ± 5
Diastolic BP (mmHg)	87 ± 2
Abnormal auscultation	
S3	19 (37%)
S4	12 (23%)
Systolic murmur	22 (42%)
Diastolic murmur	3 (6%)
Abnormal ECG	51 (98%)
LVH	15 (29%)
LAH	22 (42%)
LBBB	17 (33%)
Pathologic Q waves	2 (4%)
CSD	12 (23%)
Arrhythmias	19 (37%)
LVEDD (mm)	65 ± 7.3 (51-90 mm)
EF (%)	34 ± 9.3
Aortic regurgitation	2 (4%)
Mitral regurgitation	40 (74%)
Grade I	21 (53%)
Grade II	14 (35%)
Grade III	4 (10%)
Tricuspid regurgitation	20 (39%)

BP, blood pressure; CSD, conduction system disease; ECG, electrocardiogram; EF, ejection fraction; LAH, left atrium hypertrophy; LBBB, left bundle branch block; LVH, left ventricular hypertrophy; NYHA, New York Heart Association. Data are means (%) ± SD.

4.2 Methods

4.2.1 Evaluation of the index patients

All DCM patients in this study lived in the referral areas of the Kuopio or Helsinki University Hospitals. First, all medical records of patients with the diagnosis of DCM were carefully evaluated. Patients who had some signs or a suspicion of secondary DCM were excluded from the study. Patients, who were classified as idiopathic DCM patients, were included into further evaluation. Diagnostic criteria for DCM were ejection fraction under 45% and left ventricular end-diastolic diameter > 27 mm/ m^2. The patients underwent a careful cardiological examination including two-dimensional and Doppler echocardiography. Laboratory tests for the exclusion of secondary DCM were also taken. In cases of no secondary causes for DCM, the patients were included in genetic studies. Patients also filled out a family questionnaire. Whenever there was any suspicion of a family history, the family members were also examined. Altogether, 88% of the study patients underwent diagnostic coronary angiography. The rest of the study population was young and did not have any risk factors for coronary heart disease.

For the exclusion of secondary causes of DCM, several blood tests were taken from all index patients: erythrocyte sedimentation rate, C-reactive protein, blood count, sodium, potassium, creatinine, creatine kinase, glucose, thyroid function tests, ACE, iron, transferrin, transferring saturation, antinuclear antibody (ANA), rheumatoid factor, Waler-Rose and virus antibodies.

4.2.2 Genetic studies

4.2.2.1 Polymerase chain reaction (PCR)

Genomic DNA was prepared from peripheral blood leukocytes either by the proteinase-K phenol-chloroform or salt-precipitation method. Primers of the genes were planned according to GenBank sequences or as described earlier by others (Table 9). PCR was performed in a volume of 6 μl containing 40 ng of genomic DNA, 3 pmol of each primer, 10 mmol/liter Tris-HCl (pH 8.8), 50 mmol/ liter KCl, 1.5 mmol/ liter of $MgCl_2$, 0.1% Triton X-100, 200 μmol/ liter dNTP (200μmol/ liter dATP, dGTP, dTTP; 160 μmol/liter dCTP), 0.15 units of DNA polymerase (Dynazyme DNA polymerase, Finnzymes, Finland) and 0.3-0.6 μCi of alpha-[^{33}P] dCTP. The PCR conditions were denaturation at 94 °C for 3-5 min, followed by 35 cycles of denaturation at 94 °C for 20-40 s, annealing at 48-72 °C for 20-45 s and extension at 72 °C for 20-45 s with final extension at 72 °C for 2-4 min. The PCR product was labeled by incorporation of ^{33}P-dCTP during amplification. PCR products were digested with restriction enzymes if the length of the fragments was > 290 base pairs for facilitating the detection of sequence variants, but most of the fragments were under 250 base pairs.

4.2.2.2 Single strand conformation polymorphism (SSCP)

The SSCP analysis was performed essentially according to Orita et al (232). PCR products were first diluted 4-16-fold with 0.1% sodium dodecyl sulfate (SDS), 10 mmol/ liter ethylenediaminetetraacetic acid (EDTA) and then diluted (1:1) with loading mix (95% formamide, 20 mmol/ liter EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). After denaturation at 98 °C for 3 min, samples were immediately cooled on ice, and 3 μl of each sample was loaded onto 6% nondenaturing polyacrylamide gel (acrylamide/N,N-methylene-bis-acrylamide ratio 49:1) containing 10% of glycerol. Each sample was run at two different gel temperatures: 1) at 38 °C for approximately 4h and 2) at 29 °C for approximately 5h. The gel was dried on filter paper and autoradiographed at –20 °C

Table 9. Genes screened in Studies I-IV, and references or accession numbers of the GenBank sequences used in the primer design

Study	Gene	Exons	GenBank sequences used in primer design
I	ACTC	6/ 6	Ref. (21) or AH005305, J00071, J00072, J00073
II	LMNA	12/ 12	L12399, L12400, L12401
III	MYH 7	38/38	X52889, M57965 or http://genetics.med.harvard.edu/~seidman/primersequence.b-MHC.html
III	TPM1	9/ 9	Ref. (233)
III	TNNT2	16/16	AY044273
III	TNNI3	8/ 8	X90780
III	TNNC1	6/ 6	M37984
IV	SGCD	9/ 9	Ref. (25)
IV	DES	9/ 9	M63391
IV	VCL	3/ 22	NM_014000

ACTC, cardiac actin; LMNA, lamin A/C; MYH 7, β-myosin heavy chain; TPM1, α-tropomyosin; TNNT2, troponin T; TNNI3, troponin I; TNNC1, troponin C; SGCD, δ-sarcoglycan; DES, desmin; VCL, metavinculin. Exons: number of exons screened/number of existing exons.

4.2.2.3 Sequencing

Genomic DNA from individuals with different SSCP patterns was reamplified and sequenced with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits using ABI Prism 310 or 3100 Genetic Analyzers (Applied Biosystems, Foster City, California, USA). The sequence homology of the mutated samples was compared with the sequences of phylogenetically lower animals, e.g. Mesocricetus auratus, Mus musculus and Gallus Gallus.

4.2.2.4 Restriction fragment length polymorphism analysis (RFLP)

Non-radioactive PCR samples were digested with specific restriction enzymes and separated by electrophoresis in 5% nondenaturing polyacrylamide or 3% agarose gel (NuSieve; FMC BioProducts, Rockland, ME, USA) containing 1.0 μl/ml ethidium bromide.

4.2.2.5 Haplotype analysis

Primers for microsatellite markers (dinucleotide repeats) located near at the region of the gene of interest were synthesized (two markers before and two after the LMNA gene). One of the primers was labeled with fluorescein during synthesis. The polymorphic DNA fragments were amplified with PCR and analyzed with an automated laser fluorescence DNA sequencer (ALFexpress, Amersham Pharmacia Biotech). Haplotypes were constructed manually.

4.2.2.6 Statistical analyses

Data are given as means ± SD, or percentages. Statistical analyses were performed with statistical software packages (SPSS Win 9.0., SPSS Inc., Chicago, IL, USA). Survival analyses were estimated by the Kaplan-Meier method. A value of $p< 0.05$ was considered significant. P values were derived by Log Rank.

4.3 Approval of Ethics Committee

The study protocol was approved by the Ethics Committee of the University of Kuopio and Helsinki and was in accordance with the Helsinki Declaration.

5. RESULTS

5.1 Cardiac actin gene (Study I)

The cardiac actin gene was the first gene that has been reported to be responsible for two different types of cardiomyopathies, DCM and HCM. Therefore, we screened the ACTC gene for variants by the PCR-SSCP method in 32 DCM and 42 HCM patients from Eastern Finland. Previously described diagnostic criteria were used. We did not find any previously reported variants or any new variants in either of two study groups.

5.2 Lamin A/C gene (Study II)

We screened the lamin A/C gene in 18 confirmed familial DCM cases and 72 sporadic cases from Eastern and Southern Finland by the PCR-SSCP method. All available first-degree relatives of the 18 families were evaluated by physical examination, 12-lead ECG and transthoracic echocardiography. Additionally, haplotype analysis with four dinucleotide markers (D1S498, D1S305, D1S506 and D1S484) was performed.

5.2.1 Screening results

We found one novel mutation, Ser143Pro, in the lamin A/C gene in 24 subjects from five unrelated DCM families and in one sporadic case with DCM. The Ser143Pro mutation co-segregated with the 5-5-5-3 or 5-5-5-2 or 5-5-5-1 haplotypes and the mutation was not found in more than 344 chromosomes of clinically healthy persons, indicating that the mutation is not common polymorphism in the Finnish population. Haplotype analysis also suggested that the mutation was a founder mutation (Figure 5). The Ser143Pro mutation is located in a highly conserved region of the gene, which suggests that the mutation causes disease.

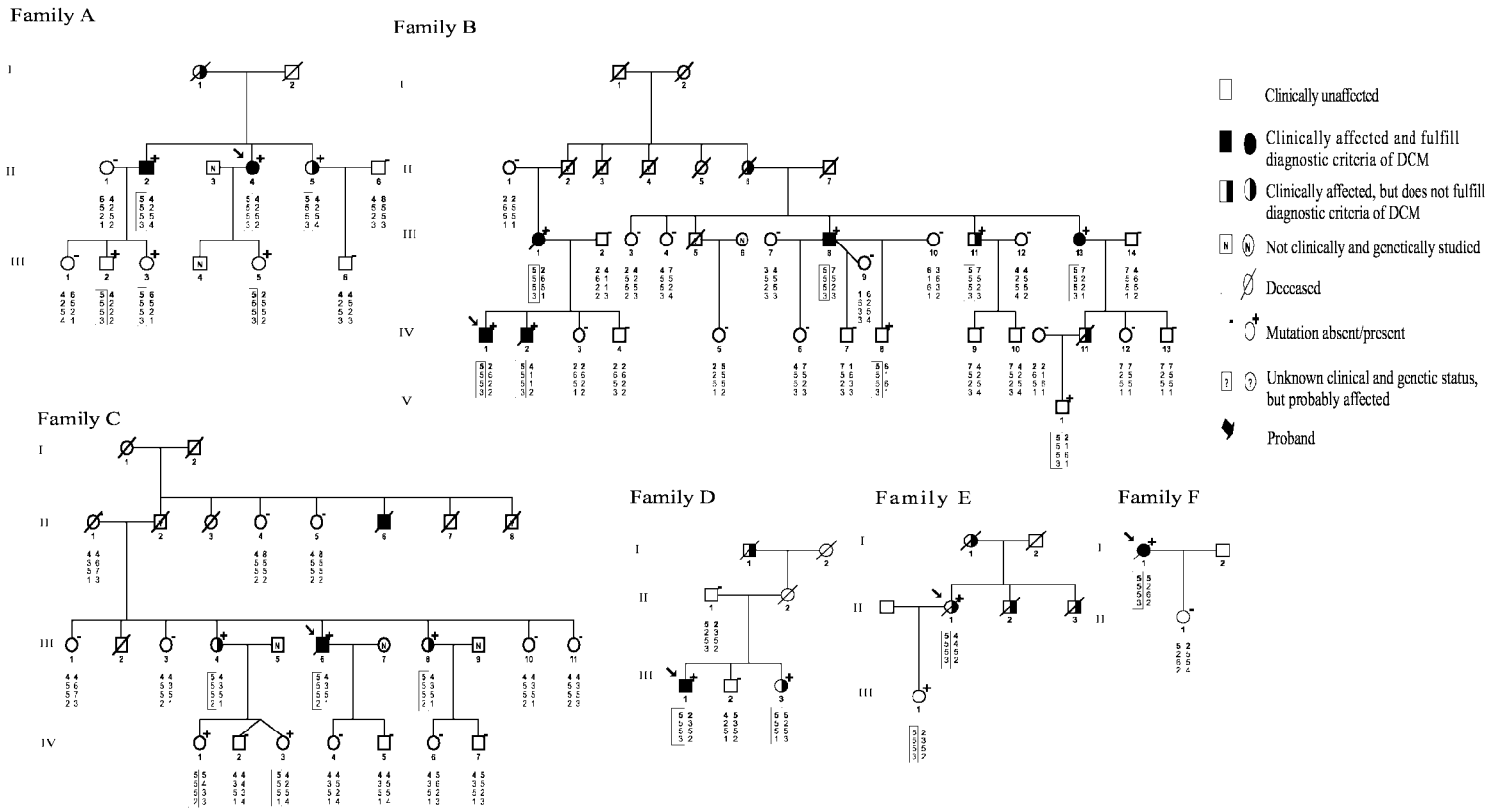

Figure 5. Pedigrees of the families with the Ser143Pro mutation. Haplotypes constructed from the markers D1S498, D1S305, D1S506 and D1S484 are depicted under each individual. The haplotypes co-segregating with the Ser143Pro mutation are boxed. Squares indicate male family members, circles female family members and symbols with slash deceased members. Solid symbols indicate family members, who fulfilled diagnostic criteria for DCM, open symbols indicate unaffected members, and half-filled circles and squares indicate members, who do not yet fulfill all diagnostic criteria for DCM, but are clinically affected. N means unknown clinical and genetic status. Mutation carrier are marked by a + sign. A – sign means that the family member is not mutation carrier.

The Ser143Pro mutation is located in the rod domain of the protein, and almost all DCM-associated mutations in the lamin A/C gene have been located in this same region. The exact molecular mechanisms of the Ser143Pro mutation remain unclear. Several other variants in the lamin A/C were also detected, but these were silent mutations and not causally related to DCM. All variants found in the lamin A/C gene are shown in Table 10.

Table 10. All variants found in the lamin A/C gene in the study group

Gene	Location	Amino acid change	No. of mutation carriers in index patients / family members
LMNA	Exon 2	Arg119Arg	
	Exon 2	**Ser143Pro**	6 / 24
	Exon 3	Leu204Leu	
	Exon 5	Ala287Ala	
	Exon 7	Asp446Asp	
	Exon 10	His566His	

LMNA, lamin A/C gene; number of family members also includes the index patients

5.2.2 Phenotype of the Ser143Pro mutation carriers

The mutation carriers of the Ser143Pro mutation have a very similar phenotype. The main characteristic features are progressive conduction defect, need for a pacemaker, atrial fibrillation, sudden death and heart failure leading to heart transplantation in some cases. All mutation carriers over 40 years old had some manifestation of DCM and only the young mutation carriers under 30 years old were free from clinical disease. The penetrance of the disease is almost 100% in subjects over 40 years old. Two-thirds (66%) of the mutation carriers had some degree of atrioventricular conduction defect. The conduction defect seemed to be progressive in nature and a need for pacemaker became common at older ages. The conduction defect was also clinically manifested as

slow atrial fibrillation. The Ser143Pro mutation can be classified as a relatively malignant mutation, because several sudden cardiac deaths occured in these families carrying the mutation. Three mutation carriers have undergone heart transplantation and one family member is presently under consideration. According to Kaplan-Meier analyses, the cumulative survival was reduced, and severe endpoints were more common among the S143P mutation carriers. Only index patients with the S143P mutation were included in statistical analyses to avoid the potential bias created by familiarity. The index patients with the S143P mutation had a significantly poorer prognosis than non-carriers (2 deaths in 6 subjects vs. 11 deaths in 84 subjects, p= 0.0004) (Figure 6A). The analysis of a composite endpoint of death, heart transplantation, malignant arrhythmias, resuscitation or AICD shocks showed a statistically significant difference between the mutation carriers and non-carriers (4 events in 6 subjects vs. 18 events in 84 subjects, p=0.0004) (Figure 6B). When heart failure or implantation of pacemaker were included in the composite endpoint, the prognosis of the carriers of the S143P mutation did not differ from that of the non-carriers (p=0.1166) (Figure 6C).

The Ser143Pro mutation explained 7% of all DCM cases in Study II and it is the most frequent mutation described in the lamin A/C gene in patients with DCM so far. The pedigrees of the Ser143Pro mutation are shown in Figure 5. The clinical characteristics of the mutation are shown in Table 11.

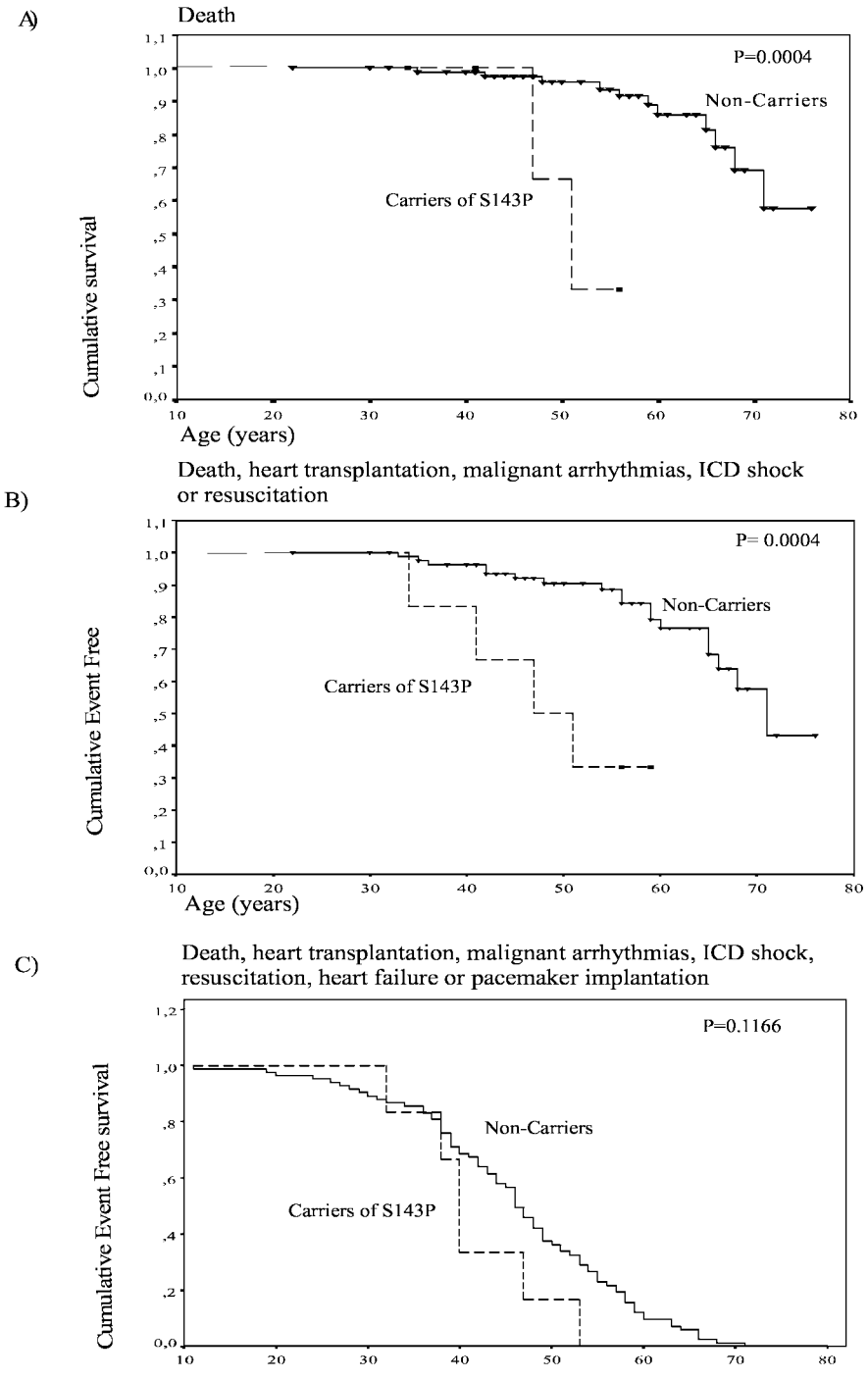

Figure 6. Kaplan-Meier cumulative survival curves for different endpoints in patients with the S143P mutation (dashed line) and in patients with DCM, but no lamin A/C mutation (solid line). P values are derived by the log rank test.

Table 11. Clinical characteristics of proven or likely Ser143Pro mutation carriers

	n (% of proven or likely carriers)
All considered affected	32
Mutation detected or obligatory carrier	25 (78%)
Age of mutation carriers with normal phenotype	20 years (range 11-29)
Age at recognition or first manifestation of disease	43 years (range 27-53)
Age at death due to cardiomyopathy	50 years (range 34-68)
Conduction system disorder	21 (66%)
Pacemaker	13 (41%)
Atrial fibrillation	8 (25%)
Ventricular dysfunction or congestive heart failure	13 (41%)
Heart transplantation	3 (9%)
Known or probable cardiac death	11 (34%)

Data are presented as mean and range or percentage. All considered affected family members means member, who has diagnosis of DCM, atrioventricular block, pacemaker or heart failure, but whose DNA sample was not available.

5.3 β-myosin heavy chain, α-tropomyosin, troponin C, troponin I and troponin T genes (Study III)

In Study III, the study population consisted of 52 DCM patients from Eastern Finland. The focus of interest in this study was in the sarcomere protein genes, because previously several isolated DCM associated mutations have been reported in the sarcomere protein genes. We screened the coding regions of the five sarcomere protein genes (β-myosin heavy chain, α-tropomyosin, troponin C, troponin I and troponin T genes) for variants.

5.3.1 Screening results

Two novel mutations, Arg1053Gln and Arg1500Trp, in the β-myosin heavy chain gene were found in this study. The Arg1053Gln mutation is located in exon 25, which is part of the S2 domain. The S2 domain is thought to affect assembly of the thick filament and have binding sites for myosin-binding protein-C. The Arg1500Trp mutation is located in exon 32, which belongs to the rod domain of the protein called light meromyosin (LMM). The LMM domain has a role in the assembly of thick filament and has binding sites for titin and myosin-binding protein-C. The residues 25 and 32 are located in the conserved regions of the gene. The Arg1053Gln and Arg1500Trp mutations were not detected in 150 control subjects or in the additional study group of DCM patients (n=104).

Several variants in all screened genes were detected, but none of the variants were clearly associated with the clinical disease. All variants in the β-myosin heavy chain, α-tropomyosin, troponin C, troponin I and troponin T genes are presented in Table 12.

Table 12. Variants found in the β-myosin heavy chain, α-tropomyosin, troponin C, troponin I and troponin T genes

Gene	Location	Amino acid or nucleotide change	No. of mutation carriers in index patients/family members
MYH7	Exon 3	Thr63Thr	
	Exon 8	Phe244Phe	
	Exon 12	Gly354Gly	
		Lys365Lys	
	Exon 21	Thr786Thr	
	Exon 24	Ile989Ile	
	Exon 25	**Arg1053Gln**	1/ 6
	Exon 31	Ser1412Ser	
	Exon 32	**Arg1500Trp**	1/ 1
		Ala1702Ala	
	Exon 33	Thr1522Thr	
	Exon 34	Asp1602Asp	
	Exon 35	Ala1702Ala	
TPM1	Exon 4	Ala151Ala	
		Tyr162Tyr	
TNNC1	Exon 6	A→ C	
	3`UTR + 83 bp		
TNNI3	Exon 5	Arg68Arg	
TNNT2	Intron 6	G→ T/A	
	- 50 bp		
	Exon 9	Ser79Ser	
	Exon 10	Ile116Ile	
	Intron 11		
	+8 bp	C→ A	
	+102 bp	G→ A	
	Exon 15	Lys263Arg	

MYH7, β-myosin heavy chain gene; TPM1, α-tropomyosin gene; TNNC1, troponin C gene; TNNI3, troponin I gene; TNNT2, troponin T gene

5.3.2 Phenotypes of the Arg1053Gln and Arg1500Trp mutation carriers

The family screening revealed that the Arg1053Gln mutation caused primarily a phenotype resembling HCM and obviously the phenotype resembling DCM was a later manifestation of the disease. We were not able to confirm that a transition from HCM-type to DCM-type of the disease took really place in our index patient, because the first sign of disease, cardiomegaly, was noticed at the time when there was no possibility for echocardiography. During the follow-up his clinical disease fulfilled the diagnostic criteria for DCM, and only slight hypertrophy was detected. The DCM phenotype in the mutation carriers was characterized by atrial fibrillation, need for pacemaker and sudden cardiac death. The HCM phenotype was characterized by septal hypertrophy and myocardial bridging in one mutation carrier. The Arg1053Gln mutation co-segregated with the clinical disease, either HCM or DCM. A pedigree of the family with the Arg1053Gln mutation is shown in Figure 7.

The Arg1500Trp mutation was found in a single DCM patient, who had only one living family member. We could not confirm the inheritance of the mutation. The mother of the index patient suffered from heart failure in her 50s, but there were no echocardiographic data available. The daughter of the index patient was healthy and had normal echocardiographic findings, and she did not carry the mutation. These findings support the notion of a disease-causing mutation, but do not confirm it. The Arg1500Trp mutation caused a classical type of DCM with symptoms of heart failure, palpitations and chest pain. A pedigree of the family with the Arg1500Trp mutation is shown in Figure 7.

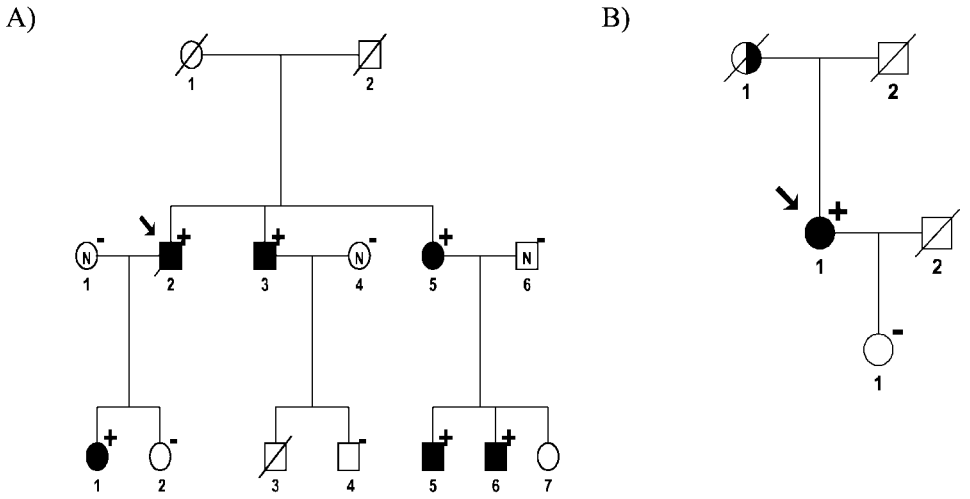

Figure 7. A) Pedigree of the family with the Arg1053Gln mutation. Only the proband had a phenotype of DCM; all other affected family members presented with HCM. B) A pedigree of the Arg1500Trp mutation. Squares indicate male family members, circles female family members and symbols with slash deceased members. Solid symbols indicate affected family members and open symbols unaffected members. The half-filled circle indicates a family member who had symptoms and signs of DCM, but the diagnosis of DCM was not confirmed. The mutation carrier is indicated by a + sign; subjects without mutation are indicated by a – sign. N means not clinically studied.

5.4 δ-sarcoglycan, desmin and metavinculin genes (Study IV)

In Study IV, we screened all the coding regions of the δ-sarcoglycan and desmin genes and the metavinculin-specific part of the vinculin gene in the same DCM population as in Study III.

5.4.1 Screening results

We found one novel mutation, Arg71Thr, in the δ-sarcoglycan gene in two family members of a small DCM family (Figure 8). The mutation was not detected in the one healthy family member, in 150 control subjects or in the additional study group of DCM patients (n=104). The Arg71Thr mutation is located in the extracellular C-terminal domain of the protein. Residue 71 is located in a conserved region of the gene. Several

other variants were identified in three screened genes, but they were not causally related to DCM. All variants in the δ-sarcoglycan, desmin and metavinculin specific parts of the vinculin gene are shown in Table 13.

A)

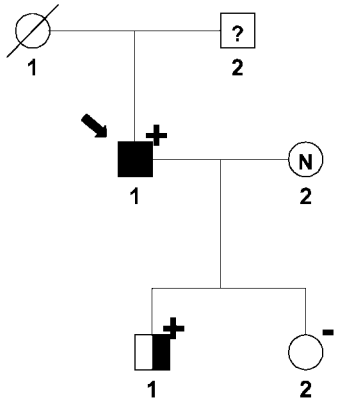

Figure 8. A) A pedigree of the family with the Arg71Thr mutation in the δ-sarcoglycan gene. Squares indicate male family members, circles female family members and symbols with slash deceased members. Solid symbols indicate family members who fulfilled diagnostic criteria for dilated cardiomyopathy (DCM), open symbols indicate unaffected members, and the half-filled square indicates a member who does not yet fulfill all diagnostic criteria for DCM, but has abnormalities on echocardiography. N means unknown clinical and genetic status and ? means unknown status of the individual. Mutation carrier is marked by + sign, and non-carrier is marked by – sign.

5.4.2 Phenotype of the Arg71Thr mutation carriers

The Arg71Thr mutation was found in only two family members of one small DCM family. Therefore, no definite conclusions of the phenotype can be drawn. However, the Arg71Thr mutation seems to cause a relatively mild DCM with late onset disease and carriers of this mutation have a good response to medication. One of the mutation carriers had paroxysmal atrial fibrillation and a presyncopal attack. Additionally, he suffered NYHA II dyspnea during exercise. On echocardiography all diagnostic criteria for DCM were fulfilled (LVEDD 73 mm and EF 45%). On medication, symptoms diminished and findings on echocardiography normalized (EF 53% and LVEDD 57 mm). His son also carries the same mutation, but he has only a slightly dilated left ventricle (LVEDD 61 mm) with a well-preserved systolic function (EF 67%). One

family member without the mutation is clinically healthy and has normal findings on echocardiography.

Table 13. Variants found in the δ-sarcoglycan, desmin and metavinculin genes

Gene	Localization	Amino acid change	No. of mutation carriers in index patients/ family members
SGCD	Exon 3	Tyr28Tyr	
	Exon 4	**Arg71Thr**	1/ 2
	Exon 4	Arg97Gln	
DES	Exon 4	Asp275Asp	
	Exon 5	Leu336Leu	
	Exon 6	Ala376Ala	
VCL		Gly85Gly	

SGCD, δ-sarcoglycan; DES, desmin; VCL, metavinculin

6. DISCUSSION

6.1. Study subjects

6.1.1 Representativeness of study subjects

The study group of the DCM patients in this study is a representative sample of DCM patients from Eastern Finland. All patients were collected from the medical records of the Kuopio University Hospital. From the start of the research project in 1999 all newly diagnosed patients have been included in our analyses. DCM patients are also followed up at Kuopio University Hospital. Therefore, it is likely that only asymptomatic patients or patients with mild disease and patients who have died or undergone heart transplantation before 1999 are not included in this series. Thus study patients included in our studies were selected without a major referral bias. Clinical characteristics, ECG and echocardiographic findings of our study subjects are in accordance with other studies including DCM patients (2, 79, 237).

6.1.2 Exclusion of secondary causes for DCM

All study patients were diagnosed with idiopathic DCM, and they underwent a careful evaluation including non-invasive and invasive clinical examination and several laboratory tests before they were accepted in the study. Exclusion of all secondary causes for DCM or diseases that can cause a similar phenotype to DCM is very difficult and, therefore, it is possible that we have missed some of these diseases in some study patients. However, this is not a problem, because secondary causes do not exclude the possibility of a genetic disease. A secondary cause for DCM can modify and accelerate the clinical manifestation of the disease. The most important diseases that must be excluded are coronary heart disease and primary valvular disease. We excluded both of these diseases carefully in our study. Echocardiography was performed on all patients and 88% of study patients underwent also coronary angiography. Only young study patients without any risk factors for coronary heart disease did not undergo coronary angiography.

The most difficult disease to exclude as an underlying cause is myocarditis. We measured basic infection laboratory tests and antibodies for viruses, but endomyocardial biopsies were not taken due to the elevated risk for perforation of thin ventriclar walls. On the other hand, endomyocardial biopsy is not necessarily a reliable technique for diagnosing myocarditis (58).

The study patients did not have any symptoms or signs of skeletal muscle disease. Skeletal muscle biopsies were not taken, but creatine kinase levels were normal whenever measured. Therefore, coexistence of clinically significant skeletal muscle disease is not very likely. Other laboratory tests were taken to exclude the main metabolic, systemic and storage diseases.

6.1.3 Determination of familiarity

The prevalence of familial DCM varies between different studies. It has been estimated that DCM is familial in 10% of cases, if only known a positive family history is taken into account (59). If family members are systematically evaluated, the prevalence of familial DCM can be as high as 30-50% (2, 4, 149). In our study, the prevalence of familial cases is most probably underestimated, because we screened systematically families of only eight probands in our basic study group of 52 DCM patients, and in the rest of the cases only a few family members have been clinically examined. If we could have screened all family members of the index patients, some more familial cases might have been identified.

It is possible to miss familial DCM for several reasons, even if all family members were examined by echocardiography. First, there is a wide variation in the phenotype among family members. Typically, DCM is manifested in middle age and before middle age a definitive diagnosis is difficult (64). Second, DCM families tend to be quite small, because usually only few family members can be classified as affected. Therefore, linkage analysis is not a very useful and helpful method in DCM compared with other monogenetic diseases (64). Third, the penetrance of the disease is variable and typically age dependent (64, 238). Fourth, diagnostic criteria for family members have not been commonly approved before Mestroni et al. published guidelines for studies of familial DCM (64).

6.2 Clinical and molecular methods

6.2.1 Echocardiography

In this study, the primary diagnosis was based on commonly approved echocardiographic diagnostic criteria for DCM (64). All published guidelines prefer echocardiography in making the diagnosis of DCM (36, 64). Echocardiography has many advantages. It is a readily available, non-invasive and cheap method, and provides data on wall thickness, chamber dimension, secondary valvular diseases and intracavitary thrombi (58). In this study, experienced cardiologists performed all echocardiographic studies. In typical cases, the diagnosis of DCM is fairly easy to do echocardiographically, but there are also limitations. Normally, the above-mentioned parameters can be measured reliably, but sometimes limited visibility can interfere with the measurement. Another disadvantage of echocardiography is that the repeatability of the method is not very good.

In this study we used WHO criteria for DCM. The WHO criteria were commonly accepted in those days when our study was initiated. Later Mestroni et al. (64) have published more specific criteria for DCM. The WHO criteria have been criticized, because left ventricular end-diastolic diameter (LVEDD) > 27mm/m^2 is not specific for DCM. When LVEDD is combined with reduced ejection fraction, the diagnosis becomes more specific. In our study all study patients fulfilled the LVEDD criteria clearly, and the ejection fraction was also reduced. Therefore we assume that all our patients have DCM. Nowadays the criteria of mestroni et al. should be preferred due to specificity.

6.2.2 Single strand conformation polymorphism (SSCP) analysis

The SSCP analysis that we used in the screening of our studies is a frequently used method for screening of unknown gene variants, with a relatively high sensitivity (~80-90%) and specificity (239, 240). The sensitivity of this method depends on various factors, e.g. the number, type and position of substitution(s) on a particular fragment, length and GC content of a fragment, ingredients and their concentrations in the gel matrix, and conditions of electrophoresis (240). We used standardized gel running

conditions with two different temperatures and a gel matrix that has performed well in previous studies (240). Our SSCP conditions have been validated against the known variants of the lipoprotein lipase gene (241, 242). Additionally, we were able to find several previously reported polymorphisms in the screened genes. Therefore, it is unlikely that we have missed a significant number of variants in screened genes. All abnormally migrated samples in the SSCP films were sequenced and all detected mutations were confirmed with two separate methods, e.g. sequencing with forward and reverse primers or using sequencing and restriction enzyme.

6.3 Prevalence and genetic background of DCM in Eastern Finland

In our study we found approximately 100 patients with idiopathic DCM (of whom 52 patients were included in the genetic studies) at the area of Kuopio University Hospital (250 000 inhabitants). Therefore, the prevalence of DCM is at least 40/ 100 000, which is similar to that reported elsewhere (32). The incidence of DCM can not be estimated reliably because of the short follow-up period, small background population and relative uncommones of DCM. We screened all coding regions of nine candidate genes and a part of the tenth gene in Finnish patients with DCM. Four novel mutations in three different genes were detected. Altogether these mutations accounted for 11.5% of the etiology of DCM for 52 patients. The Ser143Pro mutation was found to be quite common in Finnish patients with familial DCM, and is the most frequent mutation reported in DCM so far. The haplotype analysis strongly suggests that the Ser143Pro mutation is a founder mutation, probably the first founder mutation described for DCM.

Other mutations, Arg1053Gln and Arg1500Trp in the β-myosin heavy chain gene and Arg71Thr in the δ-sarcoglycan gene, seem to be less common in Finnish DCM patients. These mutations were not found in an additional sample of 104 DCM patients (38 DCM patients from Southern Finland and 66 DCM patients with heart transplantation from allover Finland). Therefore, their prevalence seems to be quite low, approximately 1% in this study.

It is difficult to compare our genetic findings with findings from other DCM populations. Because almost all reported mutations are from single families phenotypes can not reliably be compared. Only a few previously reported mutations have been

found in more than one family or in more than sporadic cases (10, 16, 18, 26, 175, 176, 203). Overall, mutations in the lamin A/C gene are the most frequent mutations reported, but in most of these reports, study populations have been selected e.g. patients have had conduction sytem disorder. In our unselected population, we found one founder mutation in the lamin A/C gene in several unrelated families.

The Finnish population has descended from only a small number of founders, who migrated from the south over the Gulf of Finland approximately 2000 years ago (243, 244). This genetic isolation is related to different distribution of diseases and disease-causing mutations in the Finnish population. Founder mutations are frequently found in monogenetic diseases in Finland, such as hypertrophic cardiomyopathy, congenital hyperinsulinism, familial hypercholesterolemia and long QT syndromes (245-248).

6.4 DCM and the sarcomere protein genes

6.4.1 DCM-associated gene defects in the sarcomere protein genes

Gene defects in the sarcomere protein genes are better known for causing disease in HCM. Over 150 mutations in eleven different sarcomere protein genes have been reported. These genes are thought to explain the disease in more than two-thirds of all HCM cases (160-162). The situation is not as clear in DCM, but approximately 20 different mutations in eight sarcomere protein genes have been detected until now (11, 16, 18, 21, 22, 26, 29, 116, 151). It has been estimated that approximately 10% of all familial cases in DCM are caused by mutations in the sarcomere protein genes (18). However, single genes do not seem to play a very important role in the pathogenesis of DCM (167). The DCM-causing mechanisms in the genes encoding the sarcomere proteins are thought to be related mainly to two different mechanisms: force generation or force transmission (141). In DCM most of the defects in the sarcomere protein genes are thought to alter the secondary structure of the protein and interfere interactions between different proteins.

In this study, we found only two DCM-associated mutations in one sarcomere protein gene, although we screened six different genes. This is not surprising, since more than one or two mutations have been reported only in the β-myosin heavy chain and titin genes (11, 18, 116, 151). The two novel mutations in the β-myosin heavy chain detected

in our study are somewhat different compared with the previously reported DCM-associated mutations in this particular gene. All four previously reported DCM-causing mutations in the β-myosin heavy chain gene cause changes in the S1 domain of the protein (11, 18). The majority of the HCM-causing mutations and the five previously reported mutations in patients with a transition from HCM to DCM are also located in this S1 domain (171, 249, 250). S1 domain has binding sites for actin and ATP and, therefore, it is essential for generation of movement needed in contraction (234). Our two mutations, Arg1053Gln and Arg1500Trp, disturb the S2 and light meromyosin (LMM) domains, respectively. Both of these domains participate in interaction between myosin-binding protein-C and myosin heavy chain. LMM domain has also a binding site for titin (171, 234). It has been speculated that both domains, S2 and LMM, affect assembly of the filament or the overall stability of the protein (234, 235). We do not have a protein model for these mutations, but we can speculate that our mutations affect the secondary structure of the protein and interfere structurally important parts of the protein. Different locations of the mutations may also be related to the unique genetic origin of the Finnish population.

6.4.2 Phenotypes of the mutations in the sarcomere protein genes

So far approximately 20 different mutations in the sarcomere protein genes have been reported in patients with DCM. Only one of these mutations has been found in more than one DCM family. It is therefore difficult to make reliable conclusions on the phenotypes caused by these mutations. Phenotypes, severity and onset of the disease vary considerably among mutation carriers. According to present knowledge, mutations in the troponin T and α-tropomyosin genes seem to cause a relatively severe disease with early onset disease and a high risk for premature death (18, 20, 22).

Four different mutations in the β-myosin heavy chain gene have been described in isolated DCM families. The onset of the disease in patients with the mutations in the β-myosin heavy chain gene varies (ranging from 2 to 57 years), but most commonly the onset occurs before middle age (11, 18). Two mutations, Ser532Pro and Phe764Leu, in the β-myosin heavy chain gene have been shown to be associated with sudden cardiac death and early onset of the disease (18). Two mutations, Ala223Thr and Ser642Leu,

found in two sporadic cases caused a classical DCM-type of disease and both patients had ICDs implanted (11). In our study, only one index patient carried the Arg1500Trp mutation and therefore, we can not compare the phenotype of this patient with previously reported mutations. Our patient's phenotype resembled classical DCM without any signs of HCM.

Primarily, HCM patients have normal systolic function and no dilatation of the left ventricle, but a small proportion (~10%) of them develop heart failure with a dilatation of the left ventricle and reduced ejection fraction, the phenotype resembling that of classical DCM (251). Several clinical reports on the transition from HCM to DCM type of disease have been published, but only few specific mutations in the β-myosin heavy chain gene, α-tropomyosin, troponin T, mitochondrial genome and myosin binding protein C gene have been reported (249, 250, 252-259). In our study, one of the patients carrying the Arg1053Gln mutation in the β-myosin heavy chain gene presumably had a phenotype that first resembled HCM, but later on DCM. Previously, only two mutations in the β-myosin heavy chain gene with coexistence of mitochondrial mutations have been reported in patients with a transition from HCM to DCM (249). We could not reliably confirm whether this DCM patient with the Arg1053Gln mutation first had an HCM phenotype that later developed into DCM because of a lack of echocardiographic data. However, several factors support a transition from HCM to DCM. Other mutation carriers in the family, including the daughter of the index patient, had an HCM phenotype. The disease had some features similar to those previously described for patients with a transition subtype of HCM. Usually patients with a transition of HCM to DCM are quite young at the onset of the disease (249, 252, 258). Patients with a transition from HCM to DCM have a poor prognosis and they are prone to sudden cardiac death and need for heart transplantation (260, 261).

The family members of the index patient carrying the Arg1053Gln mutation had septal hypertrophy with LVH on ECG. One family member had also myocardial bridging on coronary angiography, which is typical for HCM. Also young family members (under 30 years old) were clinically affected. In HCM, mutations in the β-myosin heavy chain gene are usually associated with an early-onset disease and extensive hypertrophy (262). Some of the HCM-associated mutations in the β-myosin heavy chain gene have a malignant nature and have reduced life expectancy and a high

rate of sudden cardiac death, but also benign mutations with normal life expectance have been detected (263-265). The prognostic significance of the Arg1053Gln mutation can not be yet determined due to the relatively short follow-up period.

6.5 DCM and the lamin A/C gene

6.5.1 DCM-associated mutations in the lamin A/C gene

Gene defects in the lamin A/C gene are the most frequent causes for DCM. So far over 20 different DCM-associated mutations have been reported in the lamin A/C gene (8, 10, 13, 14, 17, 202-206). In our own study we found one founder mutation in our study patients. Our finding agrees with previous data that gene defects in the lamin A/C gene are important in the pathogenesis of DCM, at least in familial cases. The main difference between our study and previous studies is that our mutation is the first mutation that has been found in several unrelated families.

Almost all mutations in the lamin A/C gene are located in a region that encodes the rod domain of the lamin A/C protein. Proper function of the rod domain seems to be critical for the normal conduction system of the heart, because all phenotypes caused by mutations in the lamin A/C gene are characterized by conduction system disorders (8, 13, 203). Only a few DCM-associated mutations cause changes in the tail domain of the protein (13, 203). The lamin A/C mutation found in this study is also located in the rod domain of the protein. The central rod domain of the protein is well-conserved region (132). Recently, Verga et al. (202) showed that different mutations in the lamin A/C gene cause similar damage in the nuclear membrane or a loss of protein expression. Mutations in their study were located near to the S143P mutation found in our study. It is therefore possible that this mutation also has similar consequences for the nuclear envelope. However, exact molecular mechanisms of this mutation remain unclear.

It is not exactly known how gene defects in the lamin A/C gene cause DCM. There is no evidence that lamin A/C participates directly in force generation or transmission. Lamin A/C does not interact directly with cytoskeletal or sarcomere proteins according to the present knowledge (59). It has been speculated that gene defects in the lamin A/C gene could distrupt nuclear function or decompose nuclear structure (59). Arbustini et al. (8) showed that nuclear membrane damage is associated with gene defects in the

lamin A/C gene. It is believed that nuclear membrane damage can lead to disruption of the cell causing myocyte death and tissue damage (8, 195). Verga et al. (202) showed that lamin A/C mutations can cause a loss of integrity of myocyte nuclei with blebs of the nuclear membrane, herniations and delamination of the nuclear lamina and nuclear pore clustering. The authors suggest that gene defects in the lamin A/C gene are associated with a loss of protein expression in the selective compartment of non-cycling myocyte nuclei. Recently, Nikolova et al. (266) reported about lamin A/C deficient mice, which developed rapidly progressive DCM. Lamin A/C-deficiency caused detachment of desmin at the nuclear-cytoskeletal interface, because lamin A/C deficiency is expected to impair binding between lamin B and desmin (266). Thereby lamin A/C deficiency can affect force transmission. Better knowledge of the disease-causing mechanisms of these mutations requires in vitro studies with cell culture or in vivo studies with animal models. Furthermore, mutant lamins might confer increased susceptability to degerative processes such as apoptosis or biochemical stress (59).

6.5.2 Phenotype of the mutations in the lamin A/C gene

The clinical manifestations of DCM caused by the mutations in the lamin A/C gene are well known compared with other DCM-causing genes. In all reports the phenotype caused by mutations in the lamin A/C gene is very similar. The main chraracteristic features are an atrioventricular conduction system disorder, atrial fibrillation, need for permanent pacemaker and an elevated risk for sudden cardiac death (203). The studies have shown that the onset of the disease is normally at early middle age, but it can range from 4 to 69 years (204). The first manifestation of the disease is usually a conduction system disorder, which seems to be typically progressive in nature. The conduction system disorder is present in approximately 30-70% of all cases, and a need for pacemakers is also common, ranging from 20 to 80% (204). Typical findings of DCM, left ventricular dilatation and contractile dysfunction develop later in life. All clinical manifestations caused by the lamin A/C gene mutations develop later in life, and are not present at birth (59).

The phenotype caused by the Ser143Pro mutation is very similar to other DCM-associated mutations in the lamin A/C gene. The phenotype of DCM patients with the lamin A/C mutation is somewhat different from that normally detected in patients with

DCM. The first manifestation of the disease is conduction system disease. Dilatation of the left ventricle and impaired systolic function appear later during the course of the disease. Young carriers of the Ser143Pro mutation usually have only 1° degree AV block on ECG without any signs of DCM. Previous studies and also our study suggest that 1° AV block in younger patients with a family history of DCM can be clinically very significant, and often these patients finally require pacemaker implantation. On the other hand, familial cardiomyopathy should also be suspected in young patients with a conduction system disorder and impaired systolic function, even if there is no significant ventricular dilatation present. Therefore, generally accepted diagnostic criteria for DCM might not be applicable for all patients with mutations of the lamin A/C gene. It is also known that gene defects in the lamin A/C gene are associated with sudden cardiac death, and this was seen also in our families. A possible mechanism for sudden cardiac death is rapid arrhythmias, because pacemakers have not been able to prevent sudden cardiac deaths. It has been suggested that implantation of ICD should be seriously considered in patients with a lamin A/C mutation.

6.6 DCM and the cytoskeletal protein genes

6.6.1 DCM-associated mutations in the cytoskeletal protein genes

Over twenty different mutations in six cytoskeletal protein genes have been reported. The most frequent mutations have been detected in the dystrophin gene, and only few mutations in the desmin, δ-sarcoglycan, metavinculin, cardiac muscle LIM and α-actinin protein genes. A possible founder mutation in the cardiac muscle LIM protein gene has been reported recently in European subjects (German and United Kingdom). Later, another research group found this same mutation in a European family (United Kingdom), but in this family the mutation did not co-segregate with clinical disease (26, 28). We screened the entire coding regions of the desmin and δ-sarcoglycan genes, and only a part of the metavinculin and dystrophin genes. We found one novel mutation in the δ-sarcoglycan gene. Negative findings in the desmin and metavinculin genes were in accordance with previous expectations of a low prevalence of mutations in these genes in DCM patients. Gene defects in the dystrophin gene are usually associated with

elevated CK levels without a clear skeletal muscle disease. Our patients had normal CK levels. It is therefore not surprising that we did not find mutations.

We found one novel mutation, Arg71Thr, in the δ-sarcoglycan gene. This was the third DCM-causing mutation described for this gene. The Arg71Thr mutation and mutations described by Tsubata et al. (25) are located in the extracellular C-terminal domain of the protein and cause changes in the secondary structure. We do not know the exact molecular consequences of the Arg71Thr mutation. However, the Arg71Thr mutation seems to have less harmful effects on protein synthesis than mutations described by Tsubata et al, because the phenotypes of those mutations are more severe.

6.6.2 Phenotype of the mutations in the cytoskeletal protein genes

Gene defects in the cytoskeletal protein genes have been found mainly in isolated cases and, therefore, it is not possible to make reliable conclusions about the main characteristics of the phenotype. Previously, two different mutations have been detected in the δ-sarcoglycan gene in one DCM family and in two sporadic cases. In a family having the Ser151Ala mutation, the phenotype was early onset and quite malignant (25). The Trp4Arg substitution in the cardiac muscle LIM protein was found in ten index patients from German and United Kingdom (26). The authors did not describe the clinical characteristics of mutation carriers in detail, but phenotype seems not to be very malignant. LVEDDs of the mutation carriers were only slightly increased and ejection fractions varied between 38-47% and obviously there were no sudden cardiac deaths in the families with the mutation. Another research group has found the same mutation, but it did not co-segregate with the clinical disease (28). Therefore, the Trp4Arg mutation is probably a modifiying mutation rather than a disease-causing mutation. In our family having the Arg71Thr mutation the clinical disease was very different. The Arg71Thr mutation was associated with a late onset of the disease and had a relatively benign phenotype. Only two family members carried this mutation. The older mutation carrier had clear DCM, and his son (32 years) carrying the same mutation had subclinical disease with some left ventricular dilatation and well-preserved systolic function.

6.7 Concluding remarks

During the last years genetics of DCM has been under intensive investigation and thereby the knowledge on the genetic basis of DCM has increased. DCM is genetically a very heterogeneous disease and mutations identified so far explain a minority of cases. The purpose of this study was to investigate the genetics of DCM in Eastern Finland. We investigated several candidate genes for DCM, and found four novel DCM-associated mutations in lamin A/C, β-myosin heavy chain and δ-sarcoglycan genes. These four DCM- associated mutations explain 11.5% (6/ 52) of cases in our study population.

The most important mutation of this study is the Ser143Pro in the lamin A/C gene. This mutation seems to be a founder mutation and quite frequent in Finnish patients with DCM. Therefore, this mutation should be screened when evaluating DCM patients having the conduction system disease. Three other mutations in the β-myosin heavy chain and δ-sarcoglycan genes caused classical DCM or HCM. These mutations are not very common in Finnish patients with DCM, and therefore they are not suitable for DNA screening in DCM patients. The main importance of these mutations is that the confirmation of the mutation offers a possibility to follow up family members and start adequate treatment early.

The future research should be focused on further investigations of the lamin A/C gene, and particularly factors determining the prognosis among these patients. Echocardiographic parameters (LVEDD and EF) are not the best possible prognostic markers, and therefore other indicators are needed for progressive heart failure and sudden cardiac death. Taken into consideration that approximately 20-35% of the DCM cases are familial in origin, undetected mutations must be present in the Finnish population. There are several interesting candidate genes that should be investigated in the future, e.g. cardiac muscle LIM protein, myosin-binding protein-C, phospholamban, laminin and α-actinin.

7. SUMMARY

Study I: In this study, 32 DCM and 42 HCM patients were screened for variants in the cardiac actin gene. Disease-causing gene defects were not found in either group.

Study II: One novel mutation, Ser143Pro, in the lamin A/C gene was found in six unrelated index patients from Eastern and Southern Finland. The haplotypes 5-5-5-3, 5-5-5-2 and 5-5-5-1 co-segregated with the mutation suggesting a founder effect for the mutation. The Ser143Pro mutation is probably the first founder mutation and the most frequent single mutation reported in DCM so far. This single mutation explained the disease in ~7% of all study cases and 28% of confirmed familial cases. The Ser143Pro mutation resulted in a relatively malignant disease.

Study III: Two novel mutations in the β-myosin heavy chain gene, Arg1053Gln and Arg1500Trp, were detected in single DCM patients. The Arg1053Gln mutation was primarily a HCM-causing mutation, but led to a transition from HCM to DCM in the index patient. Both of these mutations were quite uncommon causes for DCM, altogether accounting for ~ 4% of all cases. No DCM-associated mutations were found in the α-tropomyosin, troponin C, troponin I and troponin T genes.

Study IV: One novel mutation, Arg71Thr, in the δ-sarcoglycan gene was detected in a small DCM family. This mutation caused a relatively benign disease with a late-onset. No DCM-causing gene defects were found in the desmin and metavinculin genes.

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