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GENOTYPE-PHENOTYPE CORRELATIONS IN DILATED CARDIOMYOPATHY

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ACADEMIC DISSERTATION

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ABSTRACT

Cardiomyopathies are heart diseases, which are categorized according to morphology and function into hypertrophic, dilated, restrictive and arrhythmogenic right ventricular subtypes with significant genetic and phenotypic overlap. Dilated cardiomyopathy (DCM) is a heart disease characterized by left ventricular dilatation and systolic dysfunction in the absence of abnormal loading conditions or coronary artery disease sufficient to cause the phenotype. The diagnosis is based on echocardiography or other imaging modalities. The prevalence of DCM is estimated to be at least 1:2500. The proportion of familial or genetic DCM is currently estimated to be 30-50%. The mode of inheritance is typically autosomal dominant, but also recessive, X-linked, and mitochondrial inheritance patterns have been identified. Truncating titin variants, i.e. mutations leading to a truncated form of the protein, are the most common genetic cause of DCM. However, over 40 genes have been related to DCM, and about a hundred to cardiomyopathies in general, which is why the genetic etiology has often remained unknown even in familial cases using traditional Sanger sequencing.

Next generation sequencing methods allow the sequencing of all known cardiomyopathy-related genes simultaneously. As a result many DCM patients without a genetic diagnosis are receiving one. On the other hand more and more patients are found to carry a variant of unknown significance (VUS), a rare genetic variant, which could be disease causing, insignificant, or possibly a modifying variant. Even with modern sequencing methods thorough variant classification is as important as ever, relying on disease co-segregation with the variant combined with several other factors. Dilated cardiomyopathy is not a homogeneous disease entity, and some genetic causes produce a distinct phenotype. These genotype-phenotype correlations can help to identify familial disease, lead to successful genetic testing and guide clinical decision-making. In clinical practice genotype information still quite rarely affects treatment. As genotype-phenotype information accumulates it is likely that the management of dilated cardiomyopathy becomes more individualized taking the genotype in account.

The aims of this thesis were the identification of new cardiomyopathy-causing variants, and the description of genotype-phenotype correlations in dilated cardiomyopathy.

LMNA mutations are the second most common cause of genetic dilated cardiomyopathy. In this thesis new *LMNA* mutations were reported, and *LMNA* mutation carriers were studied in detail to further analyse the phenotype.

In study I four cardiomyopathy-causing *LMNA* variants were identified. One of the mutations was studied in more detail due to its atypical phenotype affecting primarily the right side of the heart. In study II 26 individuals carrying DCM-causing *LMNA*-mutations were studied repeatedly using spiroergometry. The symptomatic mutation carriers showed a lower maximal working capacity, maximal oxygen uptake and fraction of end-tidal CO_2 ($FetCO_2$), and an increased slope of ventilation/carbon dioxide exhaled, or VE/VCO_2 slope during exercise, namely, changes seen typically in heart failure patients. The findings of

the asymptomatic mutation carriers concerning maximal working capacity and oxygen uptake were similar to those of healthy controls. However, the asymptomatic mutation carriers had higher VE/VCO2 slope and lower FetCO2 levels than the healthy controls suggesting inefficient ventilation during incremental exercise. The results in study II suggest that signs of inefficient ventilation during exercise might imply evolving cardiomyopathy in *LMNA* mutation carriers.

In study III clinical follow-up results of 27 *LMNA* mutation carriers were reported and a new ECG entity common in *LMNA* mutation carriers and rare in other cardiomyopathy patients was introduced. In the clinical follow-up men exhibited cardiomyopathy symptoms earlier than females. Due to rigorous follow-up a very high non-sustained ventricular tachycardia (NSVT) prevalence, 77.8%, was found in *LMNA* mutation carriers highlighting the need for accurate risk-assessment schemes for clinical decision-making concerning ICD implantation. Septal remodeling, i.e. ECG abnormalities present in standard ECG leads V1-V3 were very common in *LMNA* mutation carriers, quite rare in DCM patients without *LMNA* mutations and absent in healthy controls. This suggests that septal pathology is typical to cardiolaminopathy.

In study IV a targeted sequencing panel using Os-Seq technology and covering 101 genes associated with cardiomyopathy was used to study 145 Finnish DCM patients. Study IV confirmed the significance of truncating titin variants as the most significant known cause for DCM. Truncating titin variants were found in 20.6% of familial DCM patients and 14.6% of non-familial DCM patients. The overall diagnostic yields were 47.6% for patients with familial disease and 25.6% for those without a family history of cardiomyopathy. Among other findings a likely Finnish founder mutation *DSP* c.6310delA p.(Thr2104Glnfs*12) was found in 6 non-related probands. Study IV also showed that a panel-based next generation sequencing approach is useful in the genetic diagnostics of cardiomyopathy.

TIIVISTELMÄ

Kardiomyopatiat eli sydänlihassairaudet jaotellaan morfologian perusteella ja toiminnallisesti hypertrofiseen, dilatoivaan, restriktiiviseen, oikean kammion arytmogeeniseen ja luokittelematomiin kardiomyopatioihin. Kardiomyopatioiden ilmiasuissa ja genetiikassa on kuitenkin merkittävää päällekkäisyyttä. Dilatoivassa kardiomyopatiassa (DCM) sydämen vasen kammio laajentuu ja sen pumppausteho heikkenee. Diagnoosi perustuu sydämen ultraäänitutkimukseen tai muuhun kuvantamismenetelmään, kuten sydämen magneettikuvaukseen. Muut samankaltaiseen ilmiasuun johtavat tilat tulee sulkea pois ennen diagnoosin asettamista. DCM:n esiintyvyydeksi arvioidaan vähintään 1:2500, ja suvuttaisen taudin osuudeksi 30-50%. Tyypillisin periytymismalli on autosomaalinen dominantti, mutta myös resessiivinen, X-kromosomaalinen ja mitokondriaalinen periytymistapa ovat mahdollisia. Trunkoivat, eli tynkäproteiinin muodostukseen johtavat, titiinimutaatiot (*TTNtv*) ovat yleisin DCM:n geneettinen syy. Muita DCM-tautigeenejä tunnetaan yli 40, ja kardiomyopatiaan yleisesti liitettyjä geenejä noin sata, mistä johtuen DCM:n genetiikan tutkiminen on ollut työlästä perinteisillä menetelmillä.

Uuden polven sekvensointimenetelmät (NGS, next generation sequencing) mahdollistavat kaikkien kardiomyopatioihin liitettyjen geenien samanaikaisen sekvensoinnin. Menetelmien kehityksestä johtuen yhä useammat DCM-potilaat voivat saada tarkan geneettisen diagnoosin kliinisen diagnoosin lisäksi. Toisaalta myös epäselvien varianttien (VUS, variant of unknown significance) määrä lisääntyy. Tämän vuoksi tarkka varianttianalyysi, joka perustuu kosegregaation arvioimiseen ja muihin kriteereihin, on tärkeää. DCM:n kliininen kuva on vaihteleva, ja jotkut geenivirheet aiheuttavat tyypillisen, muista eroavan ilmiasun. Genotyyppi-fenotyyppi-analyysistä saattaakin olla hyötyä (suvuttaisen) sairauden tunnistamisessa sekä yksilöllisissä hoitoratkaisuissa. Käytännössä genotyypin mukaan räätälöity hoito ei ole vielä arkipäivää, vaan mahdollistunee genoyyppi-fenotyyppi-tiedon lisääntyessä tulevaisuudessa.

Tämän tutkimuksen tavoitteena oli tunnistaa uusia kardiomyopatiaa aiheuttavia geenivirheitä sekä kuvata niitä mahdollisimman tarkasti. Osatyöt I-III käsittelivät *LMNA*-geenivirheitä ja niiden kliinistä kuvaa. Osatyössä I kuvattiin neljä *LMNA*-mutaatiota, joista yhtä tarkasteltiin tarkemmin, sillä sen ilmiasu oli tavallisesta poikkeava. Kyseisen mutaation kantajilla kardiomyopatia affisioi erityisesti sydämen oikeaa puolta. Osatyössä II tutkittiin 26 *LMNA*-mutaationkantajaa toistetuilla spiroergometriatutkimuksilla. Oireisten mutaationkantajien spiroergometrialöydökset olivat sydämen vajaatoimintaan sopivia; kokonaissuorituskyky, maksimi hapenkulutus ja uloshengitysilman hiilidioksidipitoisuus loppu-uloshengityksessä ($FetCO_2$) olivat alentuneet, kun taas minuuttiventilaation ja hiilidioksidintuotannon suhteen kulmakerroin (VE/VCO_2 slope) oli koholla. Oireettomien mutaationkantajien tulokset olivat samankaltaisia kuin terveiden verrokeiden, mutta heilläkin VE/VCO_2 käyrän kaltevuus oli koholla ja $FetCO_2$ oli alentunut viitaten epätaloudelliseen ventilaatioon rasituksen aikana. Rasituksenaikainen

ventilaation epätaloudellisuus saattaakin olla merkki subkliinisestä kardiomyopatiasta. Osastyössä III raportoitiin 27 *LMNA*-mutaationkantajan kliinisen seurannan tuloksia. Päälöydöksinä oli, että *LMNA*-mutaation aiheuttamat sydänpoikkeavuudet ilmaantuvat aikaisemmin miehille kuin naisille. Lisäksi havaittiin, että lyhyet kammiotakykardiapyrähdykset (NSVT) ovat hyvin yleisiä *LMNA*-mutaationkantajilla. Tämän vuoksi vakavien kammiotakykardioiden ilmaantumisriskin arvioimista tulee kehittää näillä potilailla, jotta ennaltaehkäisevät rytmihäiriötahdistimet osataan kohdistaa potilaille, jotka niistä hyötyvät. Osatyössä raportoitiin myös EKG-poikkeavuus, englanniksi "septal remodeling", joka on yleinen *LMNA*-mutaationkantajilla, mutta harvinainen DCM-potilailla, joilla ei ole *LMNA*-mutaatiota. Kyseinen EKG-poikkeavuus viitannee paikallisiin poikkeavuuksiin septumissa.

Osatyössä IV tutkittiin 145 suomalaista DCM-potilasta NGS-teknologiaa hyödyntävällä Os-Seq-menetelmällä, joka kattoi 101 kardiomyopatioihin liitettyä geeniä. Tutkimus vahvisti trunkoivien titiinimutaatioiden merkityksen DCM:ssä; familiaalisessa DCM:ssä 20,6%:lta löytyi *TTNtv* ja sporadisessakin 14,6%:lta. Kaiken kaikkiaan todennäköisen geenidiagnoosin sai 47,6% familiaalista ja 25,6% sporadista tautia sairastavista. Muista löydöksistä merkittävin oli *DSP* c.6310delA p.(Thr2104Glnfs*12) variantti, joka löytyi kuudelta DCM-indeksihenkilöltä, jotka eivät olleet sukua toisilleen. Tutkimus osoitti myös Os-Seq-menetelmän käyttökelpoisuuden kardiomyopatioiden geenidiagnostiikassa.

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ABBREVIATIONS

AF	allele frequency
AFib	atrial fibrillation
AHA	American Heart Association
AMPK	adenosine monophosphate-activated kinase
ARVC	arrhythmogenic right ventricular cardiomyopathy
AV block	atrioventricular block
CMR	cardiac magnetic resonance imaging
CPET	cardiopulmonary exercise testing
CRT	cardiac resynchronization therapy
CRT-D	cardiac resynchronization therapy defibrillator
DCM	dilated cardiomyopathy
DNA	deoxyribonucleic acid
DSC2	desmocollin-2 gene
DSG2	desmoglein-2 gene
DSP	desmoplakin gene
ECG	electrocardiogram
ESC	European Society of Cardiology
ESP	NHLBI Exome Sequencing Project
ExAC	Exome Aggregation Consortium
FetCO2	Fraction of end-tidal CO2
FS	fractional shortening
gnomAD	Genome Aggregation Database
HCM	hypertrophic cardiomyopathy
HGMD	Human Gene Mutation Database
ICD	implantable cardioverter-defibrillator
iPS cell	induced pluripotent stem cell
JUP	plakoglobin gene
LMNA	*LMNA* gene
LOD	logarithm of odds
LVEDD	left ventricular end-diastolic diameter
LVEF	left ventricular ejection fraction
LVH	left ventricular hypertrophy
MYBPC3	myosin-binding protein C gene
MYPN	myopalladin gene
MYH6	alpha-myosin heavy chain gene
MYH7	beta-myosin heavy chain gene
NGS	next-generation sequencing
NHLBI	National Heart, Lung, and Blood Institute
NSVT	non-sustained ventricular tachycardia
NYHA	New York Heart Association

Os-Seq	oligonucleotide-selective sequencing
PCR	polymerase chain reaction
PKP2	*PKP2* gene
PRKAG2	protein kinase AMP-activated non-catalytic subunit gamma 2 gene
PSI	proportion spliced in
RBM20	RNA binding motif protein 20 gene
RCM	restrictive cardiomyopathy
RQ	respiratory quotient (VCO2/VO2)
SCD	sudden cardiac death
SCN5A	sodium voltage-gated channel alpha subunit 5 gene
SD	standard deviation
SiSu	Sequencing Initiative Suomi
TCAP	telethonin/titin cap gene
TGFβ3	transforming growth factor-β3 gene
TMEM3	transmembrane protein 3 gene
TNNT2	cardiac troponin T type 2 gene
TTN	titin gene
TTNtv	truncating titin variant
V̇EO2	ventilatory equivalent for oxygen
V̇ECO2	ventilatory equivalent for carbon dioxide
V̇E/V̇CO2 slope	slope of ventilation/CO2
V̇O2	oxygen consumption
VUS	variant of unknown significance

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles:

I. Ollila L, Kuusisto J, Peuhkurinen K, Kärkkäinen S, Tuomainen P, Kaartinen M, Raheem O, Udd B, Magga J, Rapola J, Lahtinen A. M, Lehtonen E, Holmström M, Kivistö S, Widén E, Saksa M, Heliö T. Lamin A/C mutation affecting primarily the right side of the heart. Cardiogenetics 2013;3:e1

II. Ollila L, Heliö T, Sovijärvi A, Jalanko M, Kaartinen M, Kuusisto J, Kärkkäinen S, Jurkko R, Reissell E, Palojoki E, Piirilä P. Increased ventilatory response to exercise in symptomatic and asymptomatic *LMNA* mutation carriers: a follow-up study. Clinical Physiology and Functional Imaging. 2017; 37:8-16. doi: 10.1111/cpf.12260.

III. Ollila L, Nikus K, Holmström M, Jalanko M, Jurkko R, Kaartinen M, Koskenvuo J, Kuusisto J, Kärkkäinen S, Palojoki E, Reissell E, Piirilä P, Heliö T. Clinical disease presentation and ECG characteristics of *LMNA* mutation carriers. Open Heart 2017;4:e000474. doi: 10.1136/openhrt-2016-000474

IV. Akinrinade O*, Ollila L*, Vattulainen S, Tallila J, Gentile M, Salmenperä P, Koillinen H, Kaartinen M, Nieminen M. S, Myllykangas S, Alastalo T-P, Koskenvuo J. W, Heliö T. Genetics and genotype-phenotype correlations in Finnish patients with dilated cardiomyopathy. European Heart Journal 2015; 36 (34): 2327-2337.

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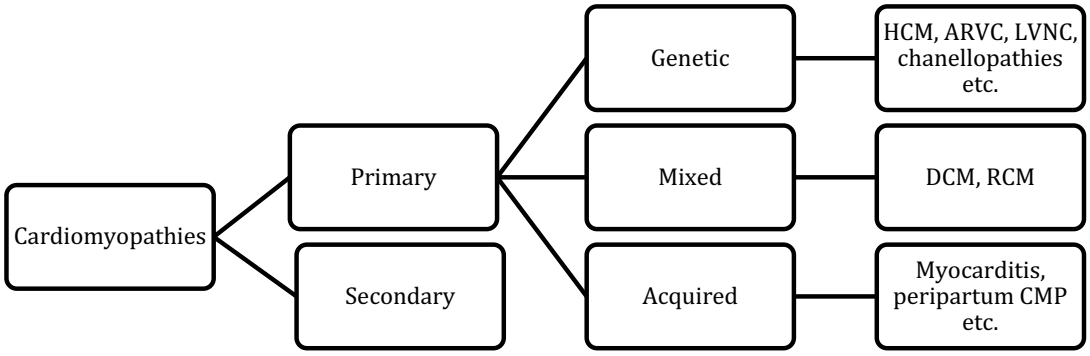
LITERATURE REVIEW

1.1 CLASSIFICATION OF CARDIOMYOPATHIES

The term cardiomyopathy was first introduced in mid-1900s. Due to the variable phenotypes, evolving diagnostics, and understanding of etiology, multiple classification systems have been proposed over the years. (1) The 1980 World Health Organization (WHO) Task Force defined cardiomyopathies as "heart muscle diseases of unknown cause" highlighting the then limited understanding of etiology, and divided cardiomyopathies according to morphology and histology into dilated (DCM), hypertrophic (HCM) and restrictive (RCM) subtypes. (2) The 1995 version introduced arrhythmogenic right ventricular cardiomyopathy (ARVC) as a distinct cardiomyopathy subtype. (3) The most recent classification systems are the American Heart Association (AHA) classification from 2006 and European Society of Cardiology (ESC) working group statement from 2008. (1, 4) Both statements aimed to fuse the traditional classification to the advances made in understanding etiology, especially genetics.

The 2008 European Society of Cardiology working group statement defines cardiomyopathies as myocardial disorders with structural and functional abnormalities in the absence of coronary artery disease, hypertension, valvular disease, or congenital heart disease sufficient to cause the phenotype in question. Furthermore, cardiomyopathies are divided into familial and non-familial forms; familial meaning that the phenotype or a phenotype possibly caused by the same gene defect is present in a family member. Figure 1a shows the basis of this classification system. The working group statement continues to divide non-familial cardiomyopathy to idiopathic, in which the cause is unknown, and acquired, in which the cardiac phenotype is a complication of a known disorder, such as amyloidosis or myocarditis. Familial, on the other hand, is divided into subtypes based on the genetic cause and whether it is known. (4) The AHA classification takes a slightly different approach by first making the division into primary and secondary cardiomyopathy, primary affecting only the heart, and secondary being a part of a systemic disease with myocardial involvement. (1) Figure 1b presents a schematic interpretation of the 2006 AHA classification. One of the main differences in the European and American classification systems is the inclusion of channelopathies in the AHA classification (1), and the purposeful exclusion of them in the ESC classification. (4, 5) The division into primary and secondary cardiomyopathies in the AHA classification can be criticized as somewhat arbitrary as, for instance, many genetic muscular syndromes include cardiac manifestations, and conversely many genetic cardiomyopathies have extra-cardiac manifestations. (6-9) Also the division into genetic, mixed and acquired is a bit problematic, and the allocation of cardiomyopathies into these subtypes is in some cases

already out-dated. For instance, peripartum cardiomyopathy is listed as an acquired condition, but it has later been showed that gene variants causing DCM may also predispose to peripartum cardiomyopathy. (10) It can also be questioned why genetics wasn't more strongly incorporated into these classification systems. A feasible reason for this is that most cardiomyopathy patients are still recognised based on phenotype as opposed to genotype. (5) However, this is already changing as more and more family members of cardiomyopathy patients are identified based on genotype. The rapid development of genetic variant classification and recognition of disease-causing genetic variants will likely affect the next propositions for cardiomyopathy classification systems.



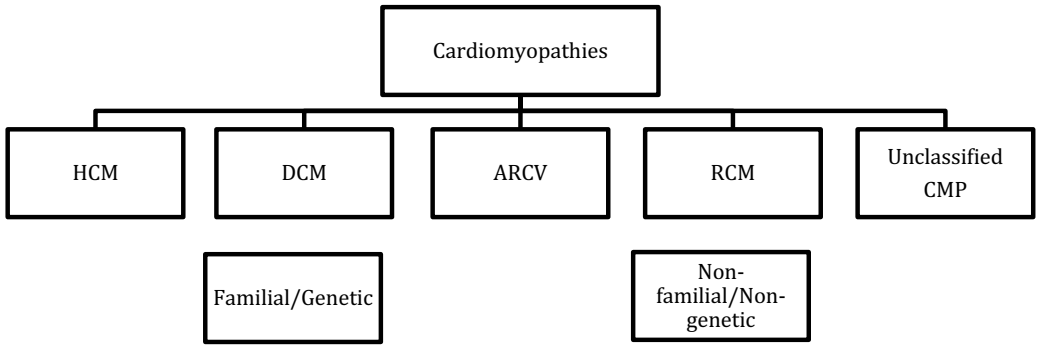


Figure 1 a. (Above) Classification of cardiomyopathies according to the American Heart Association. Adapted from Maron et al. 2006. HCM, hypertrophic cardiomyopathy; ARVC, arrhythmogenic right ventricular cardiomyopathy; LVNC, left ventricular non-compaction cardiomyopathy; DCM, dilated cardiomyopathy; RCM, restrictive cardiomyopathy; CMP, cardiomyopathy. b. (Below) Classification of cardiomyopathies according to the European Society of Cardiology. Adapted from Elliot et al. 2008. (4)

1.2 DILATED CARDIOMYOPATHY

1.2.1 DEFINITION AND EPIDEMIOLOGY

Dilated cardiomyopathy is defined as left ventricular dilatation and left ventricular systolic dysfunction in the absence of abnormal loading conditions or coronary artery disease sufficient to cause the phenotype. (4) Traditionally, the diagnosis has been based on echocardiography, but cardiac magnetic resonance imaging (CMR) is an emerging diagnostic entity. (11) Neither the ESC nor the AHA classification systems define specifically the level of dilatation or systolic dysfunction required for diagnosis, and variation exists in the criteria used. Mestroni et al. suggested left ventricular ejection fraction (LVEF) <45% and/or fractional shortening (FS)<25%, both corresponding to >2 standard deviations (SD) and left ventricular end-diastolic diameter (LVEDD) >117% of the predicted value corrected for age and body surface area corresponding to >2 SD of the predicted normal limit +5%. (12) These were an adaptation from the Manolio criteria (13) from 1992 with a slightly different criterion for LVEDD, >27 mm/m^2, criticized for worse specificity. (12) However, in clinical and research practices looser criteria are often used, sometimes with specific limits given only for LVEF. (14, 15) The Mestroni et al. (12) recommendation also lists exclusion criteria, which can cause a similar phenotype; systemic arterial hypertension with specific limits, coronary artery disease with >50% obstruction in a major branch, history of chronic excessive use of alcohol, again with specific limits, clinical sustained and rapid supraventricular arrhythmias, systemic diseases, pericardial diseases, congenital heart disease, and cor pulmonale. (12)

The prevalence of dilated cardiomyopathy has previously been estimated to 1:2500-3000 (12, 16), however these figures are based on rather old data, and it is now accepted that the prevalence is likely much higher, (17) estimated even up to 1:250.(18) This revised prevalence estimate is important when evaluating the frequencies of DCM-associated variants in population studies or reference populations. However, one should remember that the figure 1:250 is not obtained from a population-based study. Instead, it is a rough revised estimate based on the fact that the hallmark Olmstead county prevalence study from 1989 (16) underestimated the prevalence of HCM ten-fold, and might, thus, have similarly underestimated the prevalence of DCM.(18) As there is a lack of up-to-date large-scale population-based studies on the prevalence of DCM it is particularly difficult to compare the prevalence numbers between different populations. A Japanese study from 2002 reported an estimated prevalence of 1:7000 for DCM in Japan (19) while an English study from 1985 reported a prevalence of 1:12 000. (20) Since these studies are from an era when the availability of cardiac imaging was very different from today, it is difficult to draw conclusions from them, and newer population-based studies from different ethnicities are needed.

1.2.2 DIAGNOSIS

The ESC Working group on myocardial and pericardial diseases has given a structured recommendation on the diagnostic process in cardiomyopathies. (11) The recommendation describes an approach based on detailed personal and family history, continued with thorough clinical assessment and combined with targeted molecular genetic testing in select cases. It emphasizes the significance of each step with the idea that information gathered should direct further assessment, and the aim should be a specific diagnosis, as it can influence the management of the individual patient as well as family members.

The assessment should begin with the exclusion of common causes for ventricular dysfunction, such as hypertension, myocardial ischemia, valve dysfunction, and prior exposure to toxins. The detailed personal history should include general medical history as well as cardiac, bearing in mind the possibility of multisystem, syndromic, neuromuscular, or metabolic disease. (1, 4, 11) Family history ought to be covered in a systematic way using a three- or four-generation pedigree as a tool. Additionally to cardiac disease, signs suggestive of genetic disorder or skeletal muscle disease, and stroke, especially at an early age should be queried. The personal and family histories are mainly targeted towards identifying genetic cardiomyopathy. However, history or signs of autoimmune disease, such as diabetes mellitus type I, or inflammatory disease, such as sarcoidosis and giant cell myocarditis, should not be forgotten. (11) Regarding cardiac sarcoidosis improved diagnostics has lead to a marked increase in its detection rate. (21)

Standard electrocardiogram (ECG) can guide towards specific diagnosis in certain cases, such as atrioventricular block (AV block), low amplitude P waves, or sinus bradycardia in laminopathy caused by *LMNA* mutations (22, 23), or ventricular pre-excitation or AV-block in storage diseases. (24, 25) Some laboratory parameters, such as creatine kinase, kidney and liver function tests, haemoglobin and white blood count, ferritin, calcium, phosphate, thyroid function tests, should be taken from all DCM patients, and some, such as autoantibodies, titres for infectious agents, thiamine, urine or plasma catecholamines, and angiotensin converting enzyme, based on clinical evaluation and the suspicion of specific diagnoses. (11) Coronary angiography is often performed to rule out coronary artery disease as a cause for the DCM phenotype. In some cases CMR might be sufficient, and due to its non-invasive nature preferable, as a first-line diagnostic tool in determining the cause of heart failure of unknown aetiology. (26) However, controversy remains on the ability of CMR to rule out ischemic heart disease. (27) A non-invasive imaging modality better suited for the study of coronaries is coronary CT. (28) When inflammatory cardiomyopathy is suspected 18-F-fluorodeoxyglucose positron emission tomography (18F-FDG PET/CT) is the preferable imaging modality to date due to its ability to detect inflammatory processes in the myocardium. (29)

The frequency of the usage of endomyocardial biopsy in the diagnostic process of dilated cardiomyopathy varies. The procedure is not free from risk, but can be useful in differential diagnosis, in particular, identifying myocarditis or cardiac sarcoidosis patients who can benefit from immunosuppressive therapy. (30, 31) Inflammatory cardiomyopathy, or myocarditis, is myocardial disease caused by an inflammatory process of varying aetiology, e.g. viral, autoimmune, or toxic. Also the clinical presentation varies as the condition can be asymptomatic and self-limiting, or can progress to DCM. A definite

diagnosis requires histological confirmation. (32) Due to these reasons inflammatory cardiomyopathy is difficult to study, and estimates of its significance as a cause of idiopathic DCM vary. (32-34) Two studies, using endomyocardial biopsy and the Dallas criteria (35) for the histopathologic diagnosis of myocarditis reported similar prevalences of 9.2% (36) and 9.6% (37) of myocarditis among patients with idiopathic DCM.

1.2.3 ECG IN DILATED CARDIOMYOPATHY

An abnormal ECG can often be the first sign suggestive of dilated cardiomyopathy. However, the ECG abnormalities seen in DCM are usually not specific to DCM or familial DCM. (38, 39) Instead they can raise the clinical suspicion of heart disease leading to further examination, usually echocardiography, and diagnosis. (11) Yet some ECG abnormalities are typical to certain genetic etiology. In cardiolaminopathy common ECG abnormalities include low-voltage P wave, progressive conduction system disease, and both atrial and ventricular arrhythmias. (22, 23, 40) In laminopathy ECG abnormalities tend to precede structural disease. (23) Additionally to *LMNA* mutation carriers conduction system disease is also seen more often in DCM patients with RNA binding motif protein 20 (*RBM20*), beta-myosin heavy chain (*MYH7*) (41) and sodium voltage-gated channel alpha subunit 5 (*SCN5A*) mutations (15) than in other DCM patients. In phospholamban mutation carriers low voltage is prevalent and ventricular arrhythmias are more common than in other DCM patients. (41) In conclusion some genetic forms of DCM have typical ECG characteristics that can be suggestive of the genetic etiology, but no ECG abnormalities are specific to a certain genetic etiology.

1.2.4 CLINICAL PRESENTATION AND MANAGEMENT

The clinical presentation of DCM ranges from asymptomatic to end-stage heart failure. Disease onset occurs typically in adulthood, but can take place already in infancy. (15) In contrast to adult DCM, paediatric cardiomyopathy has worse prognosis (42) and is a lot more rare, with an incidence of 1.24: 100 000 in Australia in 1987-1996, (43) 1.13: 100 000 in the United States in 1996-1999, (44) and 0.34: 100 000 in Finland in 1980-1991. Symptomatic DCM patients often present with symptoms of heart failure, such as dyspnoea, exercise intolerance, fatigue, or hepatomegaly and peripheral oedema when also right ventricular dysfunction is present. If valvular regurgitation is present, a heart murmur can be heard in auscultation. Other auscultation abnormalities include a gallop and tachycardia. (15, 45) Arrhythmias, such as atrial fibrillation or other atrial arrhythmias and ventricular tachycardia, can also be the main symptom leading to cardiac assessment and diagnosis. Other electrophysiological or ECG abnormalities include atrioventricular blocks, ST-T abnormalities, bundle-branch block, and Q waves. Sometimes DCM diagnosis is reached following a thromboembolic complication, or the chance finding of cardiomegaly in a routine chest x-ray. (45) Sudden cardiac death (SCD) may also be the first disease presentation of DCM. (15)

The treatment of DCM follows the general guidelines of heart failure management. The use of angiotensin converting enzyme inhibitors delays the onset of symptoms and reduces the risk of death or hospitalization due to heart failure in asymptomatic patients with reduced LVEF. Angiotensin receptor blockers can be used instead in patients who have adverse effects to angiotensin converting enzyme inhibitors. Beta-blockers are also recommended, although the prognostic advantage in asymptomatic patients is not as clear. In symptomatic patients ACIs and beta-blockers should be used as they favour prognosis. (46) The newest addition to the treatment of heart failure is an antihypertensive drug sacubitril, which inhibits neprilysin, an enzyme degrading atrial and brain natriuretic peptides. The combination of sacubitril and valsartan in comparison to enalapril has been shown to reduce the risk of death and hospitalization due to heart failure. (47)

Diuretics and dietary salt and fluid restriction is recommended to patients with fluid retention. Drugs known to be harmful, such as non-steroidal anti-inflammatory drugs should be avoided. Digoxin can be used to alleviate symptoms of heart failure. Aldosterone antagonists can improve prognosis in symptomatic patients and can be used with close serum potassium and renal function monitoring. (46, 48)

Tachycardia can worsen the cardiomyopathy phenotype or even cause it, (49) and consequently, the ventricular response in atrial fibrillation should be controlled or sinus rhythm restored in DCM patients. (48) Anticoagulation is currently recommended only to those DCM patients with atrial fibrillation (50) following the general recommendations, and using the CHA2DS2-VASc (51) and HASBLED (52) risk stratification tools for thromboembolic events and major bleeding, respectively.

The risk of SCD is high among DCM patients. (53) SCD can result from either ventricular tachycardia, ventricular fibrillation, or bradyarrhythmia. (54) Implantable cardioverter-defibrillators (ICDs) are used both in primary and secondary prevention of ventricular arrhythmias in DCM patients. In secondary prevention, ICDs reduce the risk of mortality, and are indicated in patients with otherwise good prognosis. (46) The patient selection for primary prevention is based on LVEF, and ICD implantation is recommended to symptomatic patients with an LVEF less than or equal to 35% and otherwise good prognosis. (46) However, SCD often occurs in DCM patients who do not meet the LVEF criterion, and, on the other hand, many who do never undergo an appropriate ICD event. Thus, it is recognised that better tools for risk-stratification and other preventive measures are needed for optimal SCD prevention. (54)

Sinus node dysfunction and AV blocks resulting in symptomatic bradycardia are treated with pacemaker therapy in DCM as in other heart disease. (55) Cardiac resynchronization therapy (CRT) is used to treat ventricular dyssynchrony resulting in suboptimal ventricular filling and greater severity of mitral regurgitation. Prognostically ventricular dyssynchrony leads to increased mortality, (56) and treatment with CRT in turn decreases mortality in appropriately selected patients. (57)

The survival rates of heart failure patients have improved in the last decades. (58) There has also been advancement in the prognosis of acute decompensated heart failure requiring hospitalization, but in a recent population-based American study the five-year post-discharge survival in patients hospitalized in 2004 was still under 30%. (59) Patients with comorbidities had a worse prognosis. It is likely that patients with heart failure due to

coronary heart disease have more comorbidities than DCM patients, so the prognosis for heart failure due to DCM might not be as grim. Heart transplantation is a treatment option for end-stage heart-failure patients, and DCM is the most frequent cause for heart transplantation. (1) In Finland, 411 patients had undergone heart transplantation by 2010, and in recent years, some 15-20 heart transplantations have been performed annually. (60) Left ventricular assist devices can be used as a "bridge" to heart transplantation or as destination therapy for select patients who are not eligible for transplantation. (46, 48)

Current treatment strategies have altered the prognosis of DCM. In some cases even improvement in LVEF as well as reduction in LVEDD, referred to as reverse remodelling, is seen. (61) Recently diagnosed disease, non-familial disease, pregnancy-associated disease, left-ventricular hypertrophy (defined as left-ventricular wall thickness of 12mm or more in echocardiography), and an initial LVEF ≤ 25% have been identified as independent predictors of persisting improvement of LVEF defined as LVEF improved by at least 10 % units seen in at least two echocardiography examinations with a minimal time interval of 12 months. (62) Due to the possible improvement with appropriate pharmacological treatment it is recommended that ICD implantation in primary prevention should not be performed before at least 3 to 6 months of appropriate medical therapy. (63) Furthermore, as a nationwide Danish study shows, complications following cardiac implantable electronic devices are more frequent than previously thought, illustrating the need to assess the possible risks as well as the benefits before implanting any cardiac electronic device. (64)

Currently genotype affects treatment and follow-up of individual patients in only a minority of cases, in which the phenotype is clearly distinct. For instance, for *LMNA* mutation carriers ICD implantation under looser LVEF criteria than for other DCM patients can be reasonable. (65-68)

1.2.5 CARDIOPULMONARY EXERCISE TESTING

Cardiopulmonary exercise testing (CPET) is used to measure cardiorespiratory fitness, assess the mechanisms underlying reduced ability to exercise, and make prognostic estimates in various diseases. Prognostic stratification can in turn guide clinical decision-making, for example when assessing eligibility for heart transplantation. In cardiopulmonary exercise testing ventilatory gas exchange is measured non-invasively during progressive exercise. (69)

Cardiopulmonary testing variables with prognostic significance in heart failure are peak oxygen consumption (VO2), slope of ventilation/CO2, i.e. VE/VCO2 slope, exercise oscillatory ventilation, and resting or exercise partial pressure of end-tidal carbon dioxide, the first two being the most established variables. (69-71) Peak oxygen consumption is the most widely used prognostic marker in heart failure with low values, especially below a cut-off value of 14 ml kg^{-1} min^{-1} predicting mortality and traditionally used in heart transplantation eligibility assessment. (71-73) In recent years VE/VCO2 slope, not relying on maximal exertion, has been proposed as a more reliable prognostic marker in heart failure. (71) Variables with prognostic value respond to appropriate pharmacological,

surgical and lifestyle interventions, and can also in that respect be useful in clinical decision-making. (69, 74, 75) During exercise heart failure patients have a tendency to hyperventilate, and a reduced ventilatory efficiency is seen resulting in an elevated VE/ VCO2 slope. (76) CPET studies often comprise of heart failure patients with variable etiology, (71, 77-83) which is a possible limitation when drawing conclusions from them concerning DCM patients. A study addressing the prognostic value of cardiopulmonary exercise testing variables in dilated cardiomyopathy patients identified VE/VCO2 slope > 29 and percent-predicted maximal oxygen consumption (VO2%) <60% as the best predictors for death or urgent heart transplantation. (84)

Multiple mechanisms have been proposed to underlie the inefficient ventilation in heart failure: increased dead space, early development of lactic acidosis caused by an inability to increase cardiac output to correspond to metabolic demand, and changes in breathing control related to changes in both chemoreflex and metaboreflex control. (85, 86)

1.3 ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY

ARVC (previously ARVD for arrhythmogenic right ventricular dysplasia) is a familial heart muscle disorder characterized by fibrofatty replacement, dilatation, and abnormal contraction of the right ventricle. Increasingly also left ventricular and biventricular forms are being recognised. Ventricular arrhythmia is a typical feature of ARVC and can cause SCD already in early adulthood. (87, 88) The mode of inheritance is typically thought of as autosomal dominant, although other forms of inheritance have been identified. (87-89)

Desmosomes, cell-to-cell adhesion junctions found in abundance in the heart and the epidermis, bind cardiomyocytes to each other enabling synchronized contractility. Together with gap junctions, which allow for the fast ion flux within the myocardium, and adherens junctions, desmosomes reside in the intercalated disc of the cardiomyocytes joining adjacent cardiomyocytes together. Desmosomes also play a part in wound-repair during which they forgo their normal hyperadhesiveness for a more dynamic state. Intracellularly desmosomes are connected to the cytoskeleton. (89) Mutations in desmosomal genes were first identified in skin fragility conditions. (90) A homozygous plakoglobin (*JUP*) mutation was then connected to Naxos disease characterized by palmoplantar keratoderma, woolly hair, and ARVC. (91) Since then several other desmosomal genes have been implicated in ARVC, namely, desmoplakin (*DSP*), plakophilin-2 (*PKP2*), desmoglein-2 (*DSG2*), and desmocollin-2 (*DSC2*). Additionally some non-desmosomal genes, such as transforming growth factor-β3 (*TGFβ3*), transmembrane protein 3 (*TMEM3*), and *LMNA* have been connected to ARVC. (87, 91, 92)

The original 1994 Task Force criteria (93) for the diagnosis of ARVC were based on knowledge gathered from symptomatic index cases and SCD victims and, thus, represented the most affected ARVC cases, but lacked sensitivity to identify the less severe phenotypes. (87) The 2010 revision to the criteria was aimed to increase sensitivity.

Among other adjustments it includes pathogenic genotype and recognises family members with looser criteria. (87) The diagnosis is still based on a compilation of major and minor criteria from different areas, including morphology (echocardiography, CMR, angiography), biopsy, ECG, signal averaged ECG, typical arrhythmias, family history, and genetic analysis.

Apart from ARVC, desmosomal mutations have also been linked to DCM. Elliot et al. (94) found desmosomal mutations in *PKP2* and *DSP* from five patients from a cohort of 100 DCM patients who did not fulfil ARVC criteria or have ARVC in family history. The *PKP2* mutation c.419C>T, p.(Ser140Phe) found in three probands had previously been reported in ARVC patients whereas the two *DSP* mutations were novel. Similarly Garcia-Pavia et al. (95) found desmosomal mutations considered pathogenic in 13% (12/89) of DCM patients who had undergone heart transplantation. Again none of the probands fulfilled ARCV criteria nor had ARVC in family history. However, most (9/12) patients presented with at least one minor ARVC diagnosis criterion.

On the other hand, ARVC-associated mutations have also been found in the general population highlighting the need for broad population-based reference datasets. Lahtinen et al. reported ARVC-associated desmosomal mutations at a 0.5% frequency in a Finnish population cohort of over 6000 individuals. (96) The most prevalent was a *PKP2* c.176A>T, p.(Gln59Leu) mutation found in 0.3% of the cohort. The same variant had previously been found in two unrelated ARVC probands and eight family members of whom only one fulfilled the modified ARVC criteria for family members. (97) Functional evidence from epithelial cell lines suggests that the mutation disrupts contact with desmoplakin. (98) These studies combined it seems that the *PKP2* c.176A>T, p.(Gln59Leu) mutation is not harmless, but it is unclear whether it is enough to cause disease on its own. Interestingly in the Garcia-Pavia study of end-stage DCM patients four out of the 12 patients with desmosomal mutations had two mutations. (95)

1.4 FAMILIAL CARDIOMYOPATHY

Up to 30-50% of DCM is currently considered familial or genetic, (18, 99) although estimated proportions vary depending on the specific phenotype and age group studied. (100) Over 40 genes have been related to DCM and about a hundred to cardiomyopathies in general. (99) Mutations in practically any structure or pathway of the cardiomyocyte can result in the DCM phenotype. (99) Recent advancement in the study of the genetics of cardiomyopathies has lead to an understanding about the presence of genetic and phenotypic overlap in DCM, HCM, and ARVC. (18, 100) Familial DCM is typically considered a monogenic disorder, but it is possible that especially in the case of sporadic disease the genetic etiology is more complex involving the interaction of several possibly common variants and environmental effects. (18, 100)

In the 2008 ESC classification of cardiomyopathies familial cardiomyopathy is defined quite loosely as the occurrence of the same disorder or phenotype that is or can be caused

by the same genetic mutation in more than one family member. (4) For research purposes other varying definitions for familial (dilated) cardiomyopathy have also been used. (12, 101) When only oral family history is used to assess whether the disease is familial, the proportion of familial DCM is underestimated as opposed to the situation where family members are examined using echocardiography. (102)

Dilated cardiomyopathy is most often inherited in an autosomal dominant pattern, often with an age-dependent penetrance. (22, 103, 104) However, recessive, (18) X-linked, (6) and mitochondrial (105) forms have been identified. The recessive conditions often represent syndromic or metabolic diseases, (18) and, similarly, in the case of metabolic diseases the mode of inheritance is usually recessive or X-linked. (103) Thus, drawing the pedigree is essential in determining the mode of inheritance and can even give clues as to what the genetic etiology might be. The age-related penetrance makes assessing the presence of a familial cardiomyopathy complicated especially in the case of children and young adults. (18, 103) Other challenges when estimating whether cardiomyopathy is familial are small family size, especially in Western countries, (106) and the tendency among patients and even clinicians to refer to any cardiac event as "heart attack". (18) These obstacles put together make it understandable that cardiomyopathy appearing to be sporadic can often have a genetic etiology.

1.5 MOLECULAR GENETICS

The majority of individual genes thought to cause DCM explain only a small fraction of the disease burden. (18, 107) On the other hand truncating mutations of titin (*TTN*), the most prevalent genetic cause of DCM, explain 11-18% of sporadic and up to 19-25% of familial DCM with variation depending on disease severity. (18, 101, 106, 108, 109) Mutations of *LMNA*, encoding Lamins A and C of the nuclear lamina, are the second most prevalent cause of DCM explaining 5-8% of familial DCM. (107) In the Finnish population *LMNA* mutations have been found in 9% of heart transplant recipients with DCM, (110) while in an other study the Finnish founder mutation c.427T>C, p.(Ser143Pro) was found in 7% of Finnish DCM probands. (111) Mutations in sarcomeric proteins, such as alpha-myosin heavy chain (*MYH6*), beta-myosin heavy chain (*MYH7*), myosin-binding protein C (*MYBPC3*), myopalladin (*MYPN*), and cardiac troponin T (*TNNT2*), have been thought to explain 2-4% of DCM each with some genetic overlap to HCM. (18, 112) Several cytoskeleton proteins have been thought to cause up to 1% of DCM each. (18) The desmosomal proteins desmoplakin (*DSP*), desmocollin-2 (*DSC2*), and desmoglein-2 (*DSG2*) have been associated with DCM as well as ARVC. (18, 95) *RBM20* is a DCM-gene expressed in striated muscle, especially the heart, functioning in the regulation of splicing of titin and other cardiomyopathy-related genes associated with sarcomere-organization and ion transport in the sarcoplasmic reticulum. (113) It might explain up to 3% of idiopathic DCM. (114, 115) Table 1 lists genes associated to DCM.

ABCC6	ATP-binding cassette subfamily C member 6
ABCC9	ATP-binding cassette subfamily C member 9
ACTA1	alpha-actin 1
ACTC1	alpha-cardiac actin
ACTN2	actinin alpha 2
ALMS1	Alström syndrome 1
ALPK3	alpha-kinase 3
APOA1	apolipoprotein A-I
BAG3	BCL2-associated athanogene 3
DES	Desmin
DMD	Dystrophin
DOLK	dolichol kinase
DSC2	desmocollin 2
DSG2	desmoglein 2
DSP	Desmoplakin
DYSF	Dysferlin
EEF1A2	eukaryotic translation elongation factor 1 alpha 2
EMD	Emerin
EPG5	ectopic P-granules autophagy protein 5 homolog
ETFA	electron transfer flavoprotein alpha subunit
ETFB	electron transfer flavoprotein beta subunit
ETFDH	electron transfer flavoprotein dehydrogenase
FBXO32	F-box protein 32/atrogin 1
FKTN	Fukutin
FLNC	filamin C
FOXD4	forkhead box D4
GATA6	GATA binding protein 6
GBE1	glycogen branching enzyme
GLB1	galactosidase beta-1
HAND1	heart and neural crest derivatives expressed 1
HCN4	hyperpolarization activated cyclic nucleotide-gated potassium channel 4
JPH2	junctophilin 2
JUP	(junction) plakoglobin
LAMP2	lysosomal-associated membrane protein 2
LMNA	lamin A/C
LRRC10	leucine rich repeat containing 10
MLYCD	malonyl-CoA decarboxylase
MYBPC3	myosin binding protein C
MYBPHL	myosin binding protein H-like
MYH6	myosin heavy chain 6
MYH7	myosin heavy chain 7
MYL4	myosin light chain 4

PCCA	propionyl CoA carboxylase alpha subunit
PCCB	Propionyl CoA carboxylase beta subunit
PKP2	plakophilin-2
PLEKHM2	pleckstrin homology and RUN domain containing M2
PLN	Phospholamban
PRDM16	PR domain containing 16
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
RBCK1	RanBP-type and C3HC4-type zinc finger-containing protein 1
RBM20	RNA binding motif protein 20
RMND1	required for meiotic nuclear division 1 homolog
SCN5A	sodium voltage-gated channel alpha subunit 5
SPEG	SPEG complex locus
TAB2	TGF-beta activated kinase 1
TAZ	Tafazzin
TBX5	T-box 5
TBX20	T-box 20
TCAP	telethonin/titin-cap
TNNC1	troponin C type 1
TNNI3	troponin I type 3
TNNI3K	TNNI3 interacting kinase
TNNT2	(cardiac) troponin T type 2
TOR1AIP1	torsin A interacting protein 1
TPM1	Tropomyosin
TTN	Titin
TTR	Transthyretin
VCL	Vinculin
VPS13A	vacuolar protein sorting 13 homolog A

Table 1. *Genes associated with DCM. Well-established DCM-genes in bold. Well-established defined as 2 or more cases of a rare variant in a conserved locus either 1) showing cosegretation with DCM in a family, or 2) a de novo case. (J. Koskenvuo, personal communication)*

1.6 FAMILY SCREENING AND GENETIC TESTING

Clinical screening of first-degree family members of DCM patients is recommended to enable early diagnosis. There is evidence to suggest that early diagnosis allowing early pharmacological interventions and clinical counselling can improve patient outcomes. (15, 116) At least in the case of known familial disease the clinical evaluation of first-degree relatives should happen repeatedly, every 2-5 years in adulthood until age 50-60, by which

age the penetrance of most genetic cardiomyopathies is full, whereas in the sporadic form repeated evaluation might not be necessary. (103) A recent nationwide study from Denmark showed that a family history of sudden death from cardiomyopathy in a first degree relative before the age of 60 increases the risk of cardiomyopathy 30-fold and up to 100-fold if the relative died before age 35. (117) A purpose for genetic testing in dilated cardiomyopathy is that the discovery of a disease-causing mutation allows the genetic testing of relatives freeing those not carrying the disease-causing mutation and not at risk to develop disease from follow-up thus making the allocation of clinical resources to those at risk possible. (103) Genetic testing and a possible genetic diagnosis can also influence clinical decision-making in the individual patient. For instance *LMNA* mutation carriers are at a high risk for malignant ventricular arrhythmias, and can, in the presence of certain risk factors, namely male gender, non-missense mutation (i.e. insertion, deletion, truncating mutation, or a mutation affecting splicing) or non-sustained ventricular tachycardia (NSVT), benefit from ICD implantation even when the ventricular function is somewhat conserved. (68) Therefore receiving a cardiolaminopathy-diagnosis can favour ICD-implantation under less strict criteria than in other DCM patients. Similarly, mutations in the ion-channel gene *SCN5A* have been associated with a somewhat high frequency of ventricular and other arrhythmias. (118) With the emergence of next-generation sequencing (NGS) technologies there are hopes for a more extensive characterization of genotype-phenotype correlations affecting clinical decision-making in DCM. However in the context of more comprehensive genetic testing in larger numbers, it is certain that the number of variants of unknown significance (VUS) found will also rise. (99) In this scenario it is more likely to receive an initial VUS-diagnosis than a conclusive genetic diagnosis. (101) Naturally the discovery of a VUS makes the follow-up of relatives less straightforward as no one is entirely freed from risk of developing the disease regardless of whether they carry the identified VUS or not.

The ESC Working Group for Myocardial and Pericardial Diseases recommends that when possible, genetic testing should be targeted to the likely diagnosis. (11) The current tendency, however, is towards large, comprehensive test panels aimed to cover all cardiomyopathy-related genes. (101, 106) The inevitable consequence of this trend is not only more DCM-patients receiving a genetic diagnosis, but also many receiving inconclusive results from genetic testing with the above-mentioned consequences. (18, 100, 101) When genetic testing is performed the individual with the most severe phenotype should be the one to undergo testing. (18) As the understanding of the genetics of cardiomyopathies is evolving continuously, also the process of genetic counselling in a family with cardiomyopathy patients can and perhaps should be seen as a dynamic process. (99) This is particularly important in families and individuals with inconclusive test results from genetic testing. Genetic counselling should be incorporated into the process of genetic testing. Patients should be informed about the aims, possibilities, limitations and possible consequences of genetic testing before undergoing the testing. (119) In the management of patients and families with hypertrophic cardiomyopathy there is evidence to support cost-effectiveness of genetic testing. (120, 121) Although this is not yet the case in DCM, it likely will be with the rapid advances made in the field of genetics of DCM.

1.7 SEQUENCING

Genetic testing in cardiomyopathies, as in other genetic conditions, previously relied on Sanger sequencing of individual genes, which was time-consuming and lead to only a small fraction of patients receiving a genetic diagnosis even in cases of known familial disease. (38) The development of next-generation sequencing methods have enabled the simultaneous study of all desired genes related to a given condition, the whole exome or even the entire genome in a matter of days and at a reasonable cost. (99, 119)

Oligonucleotide-selective sequencing (Os-Seq) is a targeted sequencing method where target genomic areas are captured and sequenced on the solid Illumina flow cell. (122) The Os-Seq method is based on first synthesizing the target-specific oligonucleotides and immobilizing them on the sequencer flow cell. Then a DNA library is prepared by shearing the sample DNA into fragments and ligating known adapter sequences to the ends of the DNA fragments. The library is then added to the flow cell in which the target areas are captured, amplified and sequenced. The sequence analysis is based on detecting fluorescently labelled nucleotides while they incorporate to the growing DNA strand. (122, 123) Os-Seq has been shown to capture target regions effectively and specifically with a low false-positive rate, and it allows the creation of a customized targeted sequencing platform. This makes the method suitable for the study of diseases in which a large number of genes are implicated. (122)

In exome sequencing the target area for sequencing is the roughly 1% of the genome consisting of exons, whereas in whole genome sequencing the aim is to sequence the entire genome. However, in current reality varying portions of the genome, depending on the sequencing platform and the area in question, are not covered adequately to draw conclusions from. (124) Positive findings acquired by next-generation sequencing methods are still often confirmed with Sanger sequencing. (93)

For all types of next-generation sequencing, namely targeted, exome and whole-genome sequencing, the amount of data generated is enormous, and consequently, expertise in bioinformatics is required for data analysis. (119) Furthermore, next-generation sequencing approaches bring about the issue of distinguishing rare but benign frequencies from actual disease-causing mutations. Strict criteria for variant classification should be used, as classifying a variant pathogenic will most likely affect the treatment and follow-up of the patient carrying the variant as well as both carrier and non-carrier family members. (100, 125)

1.8 VARIANT CLASSIFICATION

Advancements in next-generation sequencing have lead to an increase in the clinical sensitivity of genetic test panels. On the other hand the number of inconclusive test results rises alongside with the number of genes studied in a genetic test panel. (126) In a study by

Pugh et al. the proportion of DCM patients receiving a VUS-diagnosis increased 10-fold, from 4.6% to 51%, as the panel size increased from five to 46 genes. (101) Variant classification is a process taking in account several lines of evidence to come up with a conclusion about the pathogenicity of a variant. In 2015 the American College of Medical Genetics and Genomics gave a recommendation to guide variant interpretation. (125) This guideline answered a clear demand, as without such a reference, discordance between reviewers assessing the pathogenicity of a variant is common. If two reviewers come up with differing views on pathogenicity, it is likelier that the less pathogenic assessment is correct upon revision. (127) The American College of Medical Genetics and Genomics guideline recommends the division of variants into five categories, namely: pathogenic, likely pathogenic, uncertain significance, likely benign, and benign. There are multiple lines of evidence for assessing the pathogenicity of a variant, such as segregation of the disease phenotype with the variant, the allele frequency of the variant in a population database, evidence of pathogenicity from functional assays, assessment of scientific literature, type of mutation, and *in silico* prediction tools. Variant classification should be seen as a dynamic, on-going process taking in account all available evidence favouring or opposing pathogenicity. With increasing evidence initial diagnoses, especially VUS-diagnoses, are likely to change over time. It is thus important to create systems in genetics laboratories to systematically review, and if necessary change, previous classifications. (125, 128)

1.8.1 CO-SEGREGATION

With the detection of large numbers of variants per test, the significance of the segregation of the disease with the variant has, if possible, increased. (129) The logarithm of odds (LOD) score is a mathematical means of assessing co-segregation in a pedigree, with a positive LOD score suggesting linkage. (130) When choosing the threshold for a significant LOD score the context should be taken in account, for example whether the locus or gene of interest is previously known to be associated with the disease. (126, 127) The interpretation of co-segregation is complicated by incomplete or age-dependent disease penetrance, and thus in the case of cardiomyopathies unaffected individuals should not be included in segregation-analysis especially at young age. (126) However, an affected individual in a pedigree not carrying the candidate variant is strong evidence against pathogenicity. In general, the more individuals there are available for analysis preferably from multiple families, the more reliable the assessment of co-segregation. (125) Roughly ten available segregations for assessment are needed to obtain a LOD score >3 traditionally considered sufficient proof of segregation. (126)

1.8.2 *DE NOVO* MUTATIONS

A *de novo* mutation in a disease-associated gene in an individual with the disease not present in either parent is strong evidence of pathogenicity of the variant. However,

parentage must be confirmed, and the possibility of a sampling error must be excluded. (126, 127)

1.8.3 ALLELE FREQUENCY AND REFERENCE POPULATION DATABASES

High allele frequency (AF) in a reference population is a strong indication of a benign variant in disorders of dominant inheritance. However, a founder mutation in a population with a relatively high incidence of the disorder in question might have a slightly higher allele frequency. When assessing the allele frequency, disease prevalence should be taken in account. If the allele frequency is greater than the expected frequency of the disease, the variant is very likely benign. (125) The largest reference population database to date is the Genome Aggregation Database (gnomAD) containing exome sequencing data from over 120 000 individuals, as well as whole genome sequencing data from over 15 000 individuals.

GnomAD is most efficient in filtering candidate variants when population-specific subsets are used. From a Finnish standpoint gnomAD provides a good opportunity for this as it contains the exome data of over 11 000 and whole genome data of 1747 Finns. The Exome Aggregation Consortium (ExAC), containing exome sequencing data from over 60 000 individuals, including a subset of some 3000 Finnish individuals, was the predecessor of gnomAD. On average individuals in ExAC carry 53 variants, which have been reported disease-causing in disease databases. However, 41 of these variants have an AF of >1% in at least one population found in ExAC supporting the idea that there is an abundance of false-positives in disease databases. (131)

1.8.4 FUNCTIONAL ANALYSES

Functional analysis can offer additional proof of pathogenicity of a variant. If a functional assay is performed, the assay used needs to be well correlated with the studied disorder and validated with known variants. Availability issues as well as interpretation difficulty limit the use of functional assays especially in clinical context. (127) Functional data can appear contradictory and should thus be viewed with caution and when possible in a wider context. For instance, protein kinase AMP-activated non-catalytic subunit gamma 2 (*PRKAG2*) mutations cause a cardiac phenotype characterised by electrophysiological disturbances, typically pre-excitation, glycogen-containing storage-vacuoles, and left-ventricular hypertrophy. (132) In an attempt to assess the specific cellular mechanisms behind the phenotype functional studies have been performed with conflicting results; in murine models both increased and decreased activation of adenosine monophosphate-activated kinase (AMPK) have been seen. (133, 134) AMPK is a protein kinase, which normally activates during energy depletion, and *PRKAG2* encodes the Y2-subunit of this protein kinase. An explanation to these apparently contradictory findings seen in murine models is that *PRKAG2* mutations cause an initial increase in the activation of AMPK, but as glycogen accumulates in the cardiomyocyte, AMPK activity is downregulated. (135)

Thus looking at only one functional study would give an incomplete view of the mechanisms behind cardiomyopathy caused by *PRKAG2* mutations.

In the recent paper by Lek et al. introducing the ExAC 192 variants found in ExAC and previously considered pathogenic were reassessed for evidence of pathogenicity. 163 of them were reclassified as likely benign or benign. Furthermore, 18 of these reclassified variants had functional data supporting pathogenicity. (131)

1.8.5 *IN SILICO* PREDICTION

Currently most *in silico* tools predict whether missense mutations are damaging to the protein structure or function or whether there is an effect on splicing. The tools assessing the effect of missense mutations are based on evaluating evolutionary conservation, the location of the variant in the gene, and/or the biochemical consequence of the amino acid change. (125) *In silico* tools that can additionally predict the effects of insertions and deletions (indels) are also starting to appear. (136) As *in silico* tools have varying accuracy evaluating known pathogenic variants and a known tendency to overestimate pathogenicity; they should never be used as a sole line of evidence in variant analysis. (125, 137)

1.9 GENES OF PARTICULAR INTEREST

1.9.1 LAMIN A/C (*LMNA*)

Lamins A and C of the intermediate filament family encoded by the *LMNA* gene locate in the nuclear lamina on the nuclear side of the inner nuclear membrane. Besides structural proteins they are thought to function in transcription, replication and signalling. *LMNA* mutations cause a variety of clinical phenotypes, such as muscular and lipodystrophies, neuropathy, Hutchinson-Gilford progeria syndrome causing premature aging, and cardiomyopathy. The broad spectrum of disease, or laminopathy, caused by *LMNA* mutations seems to be connected to varying expression and functions of lamins in different tissues at distinct developmental stages. (138)

Cardiomyopathy caused by *LMNA* mutations typically follows an age-dependent course first manifesting in early adulthood as electrical abnormalities, such as low amplitude P wave, atrioventricular block, atrial and ventricular arrhythmias, and need for a pacemaker, and later progressing to dilated cardiomyopathy. (15, 22, 23, 139) Although the left ventricular dilatation can appear modest in cardiolaminopathy, the disease can advance to end-stage heart failure requiring heart transplantation. (15, 110) Besides DCM, *LMNA* mutations have also recently been linked to a right-dominant cardiomyopathy. (92) Functional studies suggest that DCM-causing *LMNA* mutations might lead to impaired

assembly of the nuclear lamina and reduced cellular stress tolerance especially in cells, which are under constant mechanical stress. (140)

1.9.2 TITIN (*TTN*)

Titin, the largest known protein with a molecular mass of up to 4 MDa, consists of 363 exons and comprises four distinct regions spanning half the length of the sarcomere. The N-terminal part anchors the protein to the Z-disk playing a role in myofibril assembly and the maintenance of sarcomere structure. (141, 142) The elastic I band, which is a site of extensive alternative splicing, acts as a spring restoring sarcomere length after systole and on the other hand limiting sarcomere length in early diastole. (109, 143) The constitutively expressed A-band binds to myosin and myosin-binding protein and connects to the C-terminal M-band, which contains a strain-sensing kinase and is thought to respond to changes in mechanical strain by partaking in signalling and affecting gene expression. (109, 144) The major cardiac isoforms of titin are the longer N2BA and the shorter and stiffer N2B isoform; additionally a short novex isoform is expressed in low quantities in adult human heart. (141) The expression ratios of these isoforms vary between species but also within the heart resulting in changes in myocyte stiffness and diastolic force generation. (145) A schematic of titin within the sarcomere is shown in Figure 2.

In 2002 two co-segregating *TTN*-mutations in large pedigrees were reported to cause familial DCM. One was a truncating mutation located in the A band and the other a missense mutation in a conserved location in the Z-disc-I-band transition zone speculated to disrupt Z disc architecture. (146) Due to the large size and variation of *TTN* it took another decade and the development of next-generation sequencing methodology to establish its important role in DCM. Herman et al. were the first to report that truncating *TTN* variants (*TTNtv*) are the most important genetic cause of dilated cardiomyopathy. They reported that 25% of familial DCM patients and 18% of sporadic cases harbour truncating *TTN* mutations. However, also 3% of the control group and 1% of the HCM group had *TTNtvs*. Furthermore, each HCM patient harbouring a *TTNtv* also had a mutation in a well-established HCM-related gene *(MYH7 or MYBPC3)*. (108) Several studies confirmed the relevance of *TTN* in dilated cardiomyopathy, but the question of the prevalence of *TTNtvs* in the reference databases remained. Pugh et al. reported a prevalence of 14% of *TTNtvs* in their DCM cohort and 1.65% in the National Heart, Lung and Blood Institute Grand Opportunity Exome Sequencing Project (ESP). (101) Roberts et al. reported a similar prevalence of 13% *TTNtvs* in unselected DCM patients and a higher 22% in end-stage DCM patients. In the three control groups they used, the Jackson and Framingham Heart Studies and a healthy volunteer group, *TTNtv* prevalence was 1.6%, 1.0%, and 2.9%, respectively. They estimated the prevalence of *TTNtvs* in the general population to be roughly 2% based on the ESP and 1000 Genomes databases and figures reported in earlier studies. (108, 109, 147) In a large study based on DCM patient-sets from eight countries Haas et al. found a *TTNtv* prevalence of 11% for sporadic and 19% for familial DCM. (106) Already in the study of Herman et al. it was shown that the DCM-

associated *TTNtvs* were concentrated on the A-band region whereas those observed in the controls were more uniformly distributed. The study by Pugh et al. confirmed this difference in the distributions of *TTNtvs* in DCM cases and controls. (101)

Approaching the issue of evaluating the pathogenicity of *TTNtvs* Hinson et al. used cardiac microtissues engineered from induced pluripotent stem cells (iPS cells) to assess the cellular effects of two A-band *TTNtvs* and a missense mutation located in the Z-I-junction. All the cardiac microtissues produced from mutated iPS cells exhibited less than half the contractile force than the wild type. (148) The authors also tackled the question of I-band *TTNtvs* found in healthy individuals without the DCM phenotype, and concluded that alternative splicing explains this disparity. Roberts et al. reached the same conclusion and used the concept of proportion spliced in (PSI) as a measure of this phenomenon. They also reported that many exons in the I-band area are symmetric and can be excluded by alternative splicing without affecting the overall structure and function of the protein. The mutations located in highly expressed exons, such as the constitutively expressed A-band, are thus likely pathogenic whereas mutations located in areas exhibiting a low PSI are more likely tolerated. (109) Similarly, Akinrinade et al. concluded that *TTNtvs* affecting five or more of seven transcripts should be prioritized in variant analysis, (149) and that this corresponds to PSI>0.9 considered as high level of expression by Roberts et al.

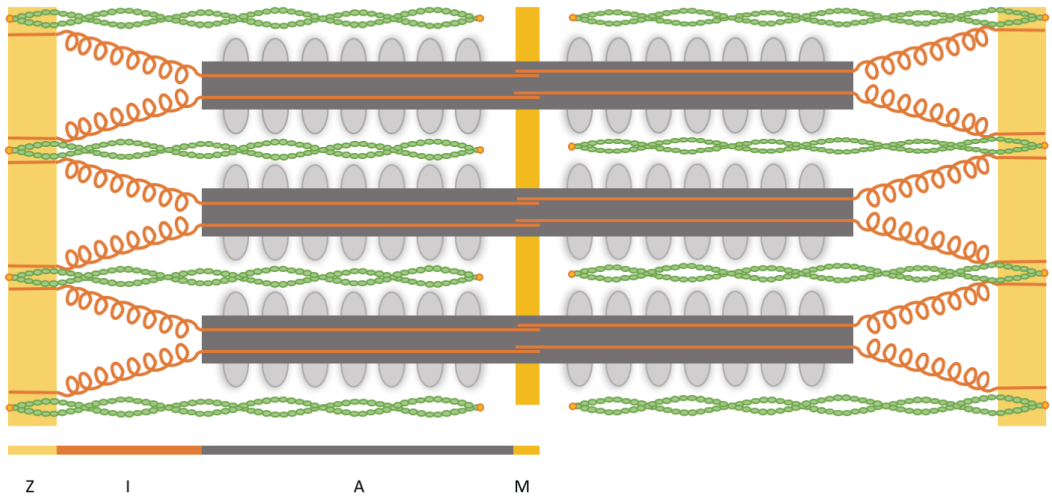


Figure 2 An illustration of the sarcomere. Titin depicted in orange, actin in green, and myosin in grey. Under the sarcomere the schematic structure of the N2BA titin isoform is shown. (Picture by Santtu Ollila. Adapted from Guo et al. 2013. (150))

1.9.3 DESMOPLAKIN (*DSP*)

Desmoplakin (*DSP*) is an important intracellular component of the desmosome anchoring cytoplasmic intermediate filaments to the desmosomal plaques. (89, 151) The first desmoplakin mutation was reported in 1999 and linked to a dominantly inherited skin condition with acral manifestation called striate palmoplantar keratoderma. (152) Then in 2002 Rampazzo et al. reported a *DSP* missense mutation c.897C>G, p.(Ser299Arg) resulting in the classic ARVC phenotype also with autosomal dominant inheritance pattern. They postulated that different *DSP* mutations might lead to different phenotypes with distinct modes of inheritance. (153) In a further phenotyping study of four families with different *DSP* mutations, including the c.897C>G, p.(Ser299Arg) mutation, left ventricular involvement was quite common. (154)

Another recessive *DSP* mutation has been reported to cause Carvajal syndrome with palmoplantar keratoderma, woolly hair and ARVC, although initially the cardiac phenotype was described as dilated cardiomyopathy. (89, 155) Autopsy of a deceased patient showed biventricular, but right dominant, dilatation, myocyte loss and fibrosis. (156) Whittock et al. described two compound heterozygous infants carrying nonsense-missense *DSP* mutations with a severe dermatological phenotype with no apparent cardiomyopathy. However, the cardiovascular assessment was not thoroughly described in the report. (157) The obvious question remains why the phenotype sometimes involves only the skin or the heart and on other occasions both. Varying tissue expression of different *DSP* isoforms has been studied and might offer an explanation, but the answer is still inconclusive. (158)

2 AIMS OF THE STUDY

The aims of this study were to identify cardiomyopathy-causing genetic variants and to describe genotype-phenotype correlations and disease progression in dilated cardiomyopathy. Specific aims were

(I) to search for new *LMNA* mutations among Finnish DCM patients and to describe an atypical laminopathy phenotype

(II) to investigate disease progression in *LMNA* mutation carriers using repeated cardiopulmonary exercise testing

(III) to study disease onset, presentation, progression and gender-specific differences in *LMNA* mutation carriers

(IV) to investigate the genetic background of DCM in a representative cohort of DCM patients referred to a tertiary hospital using a new comprehensive tool for genetic diagnosis

3 PATIENTS AND CONTROLS

3.1 PATIENTS AND CONTROLS IN STUDY I

The study group consisted of 133 probands of Finnish origin with dilated cardiomyopathy from the university hospitals of Helsinki and Kuopio. Patient recruitment took place between 1999 and 2007. Additionally two patients who did not quite fulfil the echocardiography criteria used for diagnosing dilated cardiomyopathy presenting with an incomplete phenotype including ventricular dilatation and systolic dysfunction were included since a genetic cause was suspected.

All available family members of the four probands with *LMNA* mutations reported in this study were included in the study and underwent genetic testing concerning the family's *LMNA* mutation and clinical evaluation including echocardiography.

The control group for the genetic analyses concerning the *LMNA* mutation c.710T>C, p.(Phe237Ser) in exon 7 consisted of 186 healthy control individuals of Finnish origin.

3.2 PATIENTS AND CONTROLS IN STUDY II

The study group consisted of 26 individuals each carrying one of five cardiomyopathy-causing *LMNA* variants identified in previous studies. (110, 111) The mutations and their frequencies are listed in Table 2. The patients were identified and recruited from Helsinki and Kuopio University Hospitals.

The control group for the spiroergometry testing consisted of 23 individuals without known heart disease. The control group was matched for the study group based on age, sex and body mass index. Seventeen of the 23 control individuals underwent echocardiography.

LMNA mutation	Asymptomatic carriers (n=12)	Symptomatic carriers (n=14)
c.394G>C, p.(Ala132Pro)	0	3
c.568C>T, p.(Arg190Trp)	2	2
c.1493delG, p.(Ala499Leufs*49)	0	1
c.427T>C, p.(Ser143Pro)	9	6
c.1085delT, p.(Leu363Trpfs*117)	1	2

Table 2. *The LMNA mutations of the study patients in study II.*

3.3 PATIENTS AND CONTROLS IN STUDY III

The study group consisted of 27 individuals each carrying one of five cardiomyopathy-causing *LMNA* mutations identified in previous studies. (110, 111) The study group comprised 24 of the *LMNA* mutation carriers from Study II and three additional *LMNA* mutation carriers. The recruitment and annual follow-up of these patients took place in 2005-2010. Clinical endpoint data were collected until 31 December 2014.

The control group for clinical endpoints comprised 78 probands with dilated cardiomyopathy diagnosed and recruited before 2010. These control DCM patients were collected retrospectively from our database excluding patients who have been recruited as study patients after possible heart transplant to avoid possible collection bias. An inclusion criterion for the control DCM patients was having been tested for cardiomyopathy-causing mutations using Os-Seq, a next-generation-sequencing method, as described in Study IV (159) to exclude possible *LMNA* mutation carriers from the control group.

Concerning ECG findings the *LMNA* mutation carriers were also compared to an available group of 20 healthy controls (13 females, seven males).

3.4 PATIENTS AND CONTROLS IN STUDY IV

The study group consisted of 145 unrelated probands with dilated cardiomyopathy recruited between 1999 and 2013 in Helsinki University Hospital.

Candidate variants obtained by Os-Seq (method described later) were compared to the ExAC database containing > 60 000 individuals including the Finnish Sequencing Initiative Suomi (SiSu) database.

4 METHODS

4.1 ECHOCARDIOGRAPHY CRITERIA USED TO DIAGNOSE DCM

The echocardiography criteria used to diagnose dilated cardiomyopathy in studies I-IV were left ventricular end-diastolic diameter (LVEDD) > 27 mm/m² and left ventricular ejection fraction (LVEF) < 45% in the absence of abnormal loading conditions such as primary valvular disease or hypertensive heart disease. Patients with the dilated cardiomyopathy phenotype secondary to another known cause, such as coronary artery disease, or sarcoidosis, were excluded from the study. (13, 111) Clinical echocardiography data from hospital records were used, and in many instances echocardiography was done by a clinical researcher for research purposes.

4.2 CLINICAL EVALUATION

4.2.1 CLINICAL ENDPOINT DATA IN STUDY III

The criteria used for the diagnosis of DCM were the ones stated in 4.1. All available hospital records were used for clinical endpoint data collection. Incidence times of atrial fibrillation, non-sustained ventricular tachycardia (NSVT), resuscitation or appropriate ICD therapy, i.e. ICD shock or antitachycardia pacing in response to sustained ventricular tachycardia or ventricular fibrillation, likely cardiogenic embolism and pacemaker/ICD implantations were recorded. NSVT was defined as more than 3 consecutive ventricular beats. Due to less regular follow-up of the DCM control group compared to the *LMNA* mutation carrier group NSVT was not recorded in the DCM control group. Due to the relatively small size of the *LMNA* mutation carrier group, a composite endpoint of resuscitation, appropriate ICD therapy, death and heart transplantation was used in survival analysis.

4.2.2 CLINICAL EVALUATION IN STUDY IV

All available hospital records of the probands and their family members were obtained from hospitals listed by the study patients at recruitment and analysed in detail. The echocardiography criteria stated in 4.1 for the diagnosis of DCM were used. Electrocardiograms, anthropometrics, clinical echocardiography data, age at DCM

diagnosis, age at death, detection of atrial fibrillation, angiography, pacemaker implantation, resuscitation or appropriate ICD events, and transplantation were recorded.

Familial (dilated) cardiomyopathy was defined as a confirmed family history of any cardiomyopathy, including hypertrophic, dilated or arrhythmogenic right ventricular, or two or more family members with atrial fibrillation before age 40, or rhythm/conduction disturbances necessitating pacemakers in the family.

4.3 GENETIC ANALYSES IN STUDIES I-IV

4.3.1 STUDIES I-III

The genetic analyses were mainly performed in the Diabetes and Heart Disease Research Unit at the University of Eastern Finland. Peripheral blood leukocytes were used for DNA extraction. Amplification was performed using polymerase chain reaction (PCR). Sequencing of the coding regions of the *LMNA* gene was done using ABIPRISM 310 or 3100 Genetic analyzer (PE Applied Biosystems, Foster City, CA, USA).

The genetic analyses concerning desmosomal proteins took place at the University of Helsinki. Protein-coding exons and exon-intron junctions of plakophilin-2b (*PKP2b*) were targeted. Amplification was performed using PCR. Sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). Additionally the previously known Finnish ARVC-related variants were sought using either direct sequencing or restriction enzyme assays. (96, 97)

The five *LMNA* mutations and the mutation carriers further phenotyped in studies II and III were identified in two previous studies. (110, 111)

4.3.2 STUDY IV

A targeted sequencing method, OsSeq was used. (122) The gene panel used, Pan Cardiomyopathy Panel, Blueprint Genetics v. 1.0, consisted of 51 DCM-related and 50 candidate genes implicated in other cardiomyopathies. Peripheral blood samples were used for DNA extraction. DNA capture and sequencing were performed using the MiSeq sequencer.

The raw data was pre-processed with Trimmomatic in paired-end mode. (160) The Burrows-Wheeler Aligner (BWA) (161) was used for mapping the reads to the human genome reference sequence (hg19). The Genome Analysis Toolkit (GATK) (162) version 3.1-1 was used for genotyping. Variant annotation was done with Ensembl's Variant Effects Prediction (VPE) tool. (163) *In silico* prediction of missense variants was performed using the dBNSFP database. (164) Candidate variants obtained were compared to the ExAC (Exome Aggregation Consortium) database containing the exome data of over 60 000

individuals including the Finnish SiSu database. Variants were classified by a group of clinicians and geneticists into five categories: pathogenic, likely pathogenic, variant of unknown significance (VUS), likely benign, or benign. Both likely pathogenic and pathogenic variants were considered diagnostic.

4.4 IMMUNOHISTOCHEMISTRY IN STUDY I

Myocardial samples of two patients in study I carrying the *LMNA* variant c.710T>C, p.(Phe237Ser) were assayed immunohistochemically for plakoglobin, a desmosomal protein. The immunohistochemical analyses were performed in the Neuromuscular Research Unit of the University of Tampere. Myocardial sample sections were stained with plakoglobin antibody (Sigma-Aldrich, St Louis, MO, USA) in dilution 1:10000 using the official protocol of the BenchMark immuno-stainer. For visualization a detection kit (UltraView Universal DAB detection kit, Ventana Medical Systems Inc, Tucson, AZ, USA) for primary antibodies was used.

4.5 ELECTRON MICROSCOPY IN STUDY I

Electron microscopy was performed on an endomyocardial specimen of a patient in study I carrying the *LMNA* variant c.710T>C, p.(Phe237Ser). The electron microscopy sample preparation took place as follows: 2.5% glutaraldehyde in 0.1M phosphate buffer was used at pH 7.4 and at room temperature for 2 hours. Postfixation was performed in 1% osmium tetroxide for an hour, followed by dehydration in ethanol and embedding in LX 112. Toluidine blue was used for semithin sections, and uranyl acetate and lead citrate for thin sections. A Jeol 1400 electron microscope was used to visualize the specimen.

4.6 PARAMETRIC LINKAGE ANALYSIS IN STUDY I

Parametric linkage analysis to assess co-segregation of the *LMNA* mutation c.1380G>C, p.(Glu460Asp) and cardiac involvement was performed on a pedigree in Study I (see the pedigree in Figure 1 of the original publication). An affected-only model was used on Merlin software (165) designed for pedigree analysis. The disease and marker allele frequencies were set at 1:10,000. Individuals with atrioventricular blocks, arrhythmias, or DCM were considered affected.

4.7 CARDIOPULMONARY EXERCISE TESTING IN STUDY II

An electrically braked bicycle ergometer was used in the work conducted exercise tests, and ECG was monitored continuously. Blood pressure was measured manually before exercise, at each exercise level, and 4 to 6 minutes after exercise. Non-invasive arterial oxygen monitoring with two pulse oximeter sensors was used (one in ear lope and another in left middle finger). For women a 40 W and for men a 50 W initial workload and increments every 3 minutes were used to achieve a maximal exercise level. Reference values using corresponding steps were used to compare the results.(166) To ensure the maximality of the test a subjective level 17-19/20 on the Borg scale and a respiratory quotient > 1,0 from breath gas analysis was required if subjective symptoms did not interrupt the test before this. For the respiratory gas-exchange monitoring a tightly attached face mask was used and the breathing gases were collected and analysed breath-by-breath by the equipment described in brief in in Table 3. The key parameters measured during spiroergometry are listed in Table 4. For the gas exchange parameters 30 seconds mean values were used in calculations. Ventilatory anaerobic threshold was measured at the point when the slope change of carbon dioxide production exceeds oxygen consumption, ventilation over oxygen consumption (VE/VO2) increases in comparison to ventilation over CO2 production (VE/VCO2), and when there is an increase of partial pressure of oxygen (PetO2) compared to carbon dioxide (PetCO2) in expiratory air. (70)

Ergometer	Electrically braked bicycle ergometer (Ergoselect ERG Ergometer; Marquette Hellige, Marquette Medical Systems, Germany),
ECG monitoring	Continuous ECG monitoring and recording (Mason-Likar and CardioSoft version V6.5, GE Medical systems, Milwaukee, WI, USA),
Gas exchange analysers	Vmax Encore, Sensormedics, Yorba Linda, CA, USA, Face mask (Rudolph series 7910, Hans Rudolph, Kansas City, MI, USA),
Blood pressure measurement	Manual blood pressure measurement (Erka, Germany)
Pulse oximeters	Datex-Ohmeda 3900 and Datex-Ohmeda 3800 (Datex-Ohmeda, Louisville, CO, USA)
Initial workload and workload increment at 3-minute intervals	40W for females, 50 W for males
Test completion	Borg 17-19/20, and RQ > 1.0

Table 3. *The equipment used in the spiroergometric testing in study II, and the exercise protocols in brief.*

Spiroergometry parameter	Units	Definition
Maximal heart rate	1/min or % of predicted (205-0,5 * age)	
Maximal working capacity/3min (Wmax/3min)	W or % of predicted (166)	The mean workload during the last 3 minutes of exercise
(Maximal) oxygen uptake (V̇O2)	l/min, ml/min/kg, or % of predicted (166)	
(Maximal) carbon dioxide production (V̇CO2)	l/min	
Oxygen pulse (V̇O2/HR)	ml	Ratio of oxygen consumption and heart rate
Anaerobic threshold (AT)	ml/min	Highest V̇O2 level during which energy production is aerobic
AT % of estimated maximal V̇O2	%	
Breathing reserve	%	((MVV-VE)/MVV) x100) where MVV (maximal voluntary ventilation)=38xFEV1 and VE=maximal measured minute ventilation
Ventilatory equivalent for V̇O2 (V̇E/V̇O2)		Ratio of minute ventilation and oxygen consumption
Ventilatory equivalent for V̇CO2 (V̇E/V̇CO2)		Ratio of minute ventilation and carbon dioxide production
Respiratory quotient (RQ)		V̇CO2/ V̇O2
Fraction of end-tidal CO2 (FetCO2)	%	Fraction of CO2 at the end of expiration
Mechanical efficiency (Wmax/VO2max)	%	
V̇E/V̇CO2 slope		Slope of ventilation over CO2 production
Dead-space ventilation/tidal volume (VD/VT)		

Table 4. *The key spiroergometry parameters.*

4.7.1 SCHEDULE AND DROPOUT

The *LMNA* mutation carriers underwent cardiopulmonary exercise testing, or spiroergometry, annually between 2005-2010 one to six times depending on the time of recruitment, changes in clinical condition (including two ICD implantations, two heart

transplantations and three cases where the clinical condition otherwise progressed) and other conditions which prevented the study (including a bone fracture in one study patient and pregnancy in another). The study attendance numbers and the reasons for dropout are given in Table 5.

	Baseline	Control visit 1	Control visit 2	Control visit 3	Control visit 4	Control visit 5
Symptomatic carriers	14	12	10	5	7	4
Asymptomatic carriers	12	11	11	10	10	6

Table 5. *Study attendance in study II. Reasons for dropout in the symptomatic group: heart transplants (n=2), disease progression (n=3), ICD (n=2), late enrolment (n=3). Reasons for dropout in the asymptomatic group: bone fracture (n=1), pregnancy (n=1), or late enrolment (n=4).*

4.8 ECG ANALYSIS AND SEPTAL REMODELLING IN STUDY III

Study III contained the analysis of the ECG characteristics of the cohort of 27 *LMNA* mutation carriers, 20 healthy controls and 78 controls with dilated cardiomyopathy. One standard 12-lead ECG recording at 50mm/sec speed was used. The ECG assessment was performed by an experienced investigator (K.N.) blinded to the clinical data. The criteria used in ECG analyses are given in Table 6.

1st degree atrioventricular block	PR interval >200ms (167)
P terminal force	P wave in lead V1 ≥ -0.4 (168)
Flat P wave	P wave amplitude <1 mm in lead II (169)
Broad P wave	P wave ≥120ms in lead II (169)
Left ventricular hypertrophy (LVH)	Sokolow-Lyon (170) or the Cornell voltage duration product criteria (QRS-duration (ms) x (RaVL (mm) + SV3 (mm) (+6mm for women)) ≥2440) (171, 172)
Criteria for ST segment depression	≥0.5mm if the pattern was horizontal or descending, and ≥1mm if ascending in ≥2 adjacent leads measured at the J point + 60ms (173)
T wave inversion	≥1 mm in ≥ 2 adjacent leads, except for leads aVR and V1 (174)
QRS fragmentation	Das criteria (in ≥2 adjacent leads) (175)
Septal fragmentation	Presence of QRS fragmentation in ≥2 septal leads (V1-V3)
Non-specific intraventricular conduction block	QRS ≥120ms not fulfilling criteria for right or left bundle branch block
Septal remodelling	At least one of the following in V1-V3: 1) pathological Q waves in ≥2 parallel leads, or 2) QRS fragmentation in ≥2 parallel leads, 3) poor R wave progression (R wave <3mm) in leads V1-V3 accompanied by QRS fragmentation, or disorderly distributed R wave amplitudes, either RV2>RV3 or RV1>RV2

Table 6. *The criteria used in the ECG analyses in study III.*

4.9 STATISTICAL ANALYSES

4.9.1 STUDY II

Statistical analyses in Study II were done using SPSS version 20.0 (SPSS Inc, Chicago, IL, USA). For the statistical analyses the study group was divided in two, symptomatic and asymptomatic *LMNA* mutation carriers, depending on phenotype. Covariance analysis was used to compare the spiroergometry results of the symptomatic and asymptomatic mutation carriers to healthy controls. For the spiroergometry parameters concerning oxygen uptake, heart rate or working capacity age, gender, weight, height and the use of

beta-blockers were included as covariates. For the spiroergometry parameters measuring ventilation and echocardiography parameters the covariates used were age, gender, weight and height. In the few instances where the parameters were not normally distributed the comparisons of the mutation carriers to the healthy controls giving statistically significant results were confirmed using the non-parametric Mann-Whitney U test. Paired t-test was used to compare the results of the follow-up visits to the baseline visit in both the symptomatic and asymptomatic mutation carrier group. Bivariate correlation tests were performed between LVEF in echocardiography and some gas exchange variables. Pearson correlation was used as the parameters were normally distributed.

4.9.2 STUDY III

SPSS version 22.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analyses. Shapiro-Wilk test for normality was used to assess the normality of continuous variables. Student's t test was used to analyse continuous variables. Mann Whitney U test was used if the variables were not normally distributed. The Chi-square test or the Fisher exact test was used for categorical variables when appropriate. In the ECG analyses the p-values obtained were multiplied by two to account for the number of paired-wise comparisons. For survival analysis Kaplan-Meier was used. Due to the size of the group studied and the small number of events observed compound parameters of different events were used to compare outcomes between *LMNA* mutation carriers and DCM controls.

4.9.3 STUDY IV

Statistical analyses were performed using R statistical software. Continuous data was expressed as mean ± SD when normally distributed and median ± interquartile range when not. Normality of the data was assessed using the Shapiro-Wilk test. Continuous variables were compared using independent samples t test combined with Levene's test to assess the equality of variances if the data were parametric and Mann Whitney U test if the data were non-parametric. Differences were considered statistically significant when $p<0.05$ (two-sided p-value was used). Categorical data was expressed as frequencies. Comparisons of frequencies were done with either the Chi-square test or the Fisher exact test as appropriate.

4.10 ETHICAL CONSIDERATIONS

All the studies I-IV were approved by the Ethical Committee of the University of Helsinki (Decision number for studies I-III: Dnro 322/E5/03, decision number for study IV: Dnro 307/13/03/01/11) and were in compliance with the Helsinki Declaration. All the study

patients have signed informed consent to participate in the study. To justify this project the aim was to provide clinically relevant information to the patients and their family members.

5 RESULTS

5.1 STUDY I

Phenotypes associated with four *LMNA* variants with special focus on variant c.710T>C p.(Phe237Ser) causing an ARVC-like phenotype

5.1.1 *LMNA* MUTATIONS

From the 135 cardiomyopathy patients studied four *LMNA* variants considered disease-causing, c.497G>C p.(Arg166Pro), c.710T>C p.(Phe237Ser), c.1442dupA p.(Tyr481*), and c.1380G>C p.(Glu460Asp), were found yielding a prevalence of 3.0% in this cohort. None of the variants were present in the 186 healthy controls of Finnish origin. The Arg166Pro *LMNA* variant has been previously described (176) and the other three were novel. None of the four variants are present in the reference population database ExAC.

5.1.2 C.1380G>C, P.(GLU460ASP)

The proband harbouring the c.1380G>C, p.(Glu460Asp) variant first presented at age 48 with atrioventricular block, which was complete during night time. After pacemaker implantation his condition remained stable, apart from intermittent atrial flutter/fibrillation, which later became chronic. However, some 2 and a half years later he developed New York Heart Association (NYHA) class III symptoms compatible with heart failure. In echocardiography LVEDD was 58mm and an LVEF 20%. Beta-blocker, ACE-inhibitor, digoxin, furosemide, and warfarin medications were started, and he responded well to medication. His latest echocardiography findings at age 64 showed a similar LVEDD and an LVEF of 42%.

All in all, the c.1380G>C, p.(Glu460Asp) variant was found in 13 family members, the proband included, nine or ten of whom had clinical cardiac findings compatible with cardiolaminopathy. Three mutation carriers, aged 17-33 at the time of clinical assessment were completely asymptomatic with normal ECG and echocardiography findings. One 34-year-old female had normal standard ECG and echocardiography findings, but some nocturnal unconducted P waves in 24-hour Holter. Three family members (aged 23, 44 and 53) had only first-degree atrioventricular block in ECG with possible extrasystoles as a clinical manifestation compatible with laminopathy. Four mutation carriers had third

degree atrioventricular block necessitating a pacemaker, and three of them also atrial fibrillation. One 54-year-old mutation carrier had atrial fibrillation.

The *in silico* prediction tool PolyPhen-2 gave a benign prediction of the pathogenicity of the variant. Using a parametric affected-only model on Merlin software and classifying family members with atrioventricular blocks, arrhythmias or dilated cardiomyopathy as affected, a logarithm of odds (LOD) score of 2.96 was obtained. The mutation was not present in the 150 healthy Finnish controls of a previous study. (110)

5.1.3 C.497G>C, P.(ARG166PRO)

The proband carrying the previously described c.497G>C, p.(Arg166Pro) *LMNA* mutation (176) first presented with atrial fibrillation at age 50. After cardioversion she had first to third degree atrioventricular block, non-sustained ventricular tachycardia and short sinus pauses, and some months later she received a pacemaker. At age 53 she had a likely cardiogenic stroke during an episode of paroxysmal atrial fibrillation. She developed a progressive phenotype of dilated cardiomyopathy over the course of several years leading to heart transplantation at age 61. Compatible with laminopathy her left ventricle was only moderately dilated with an LVEDD of 54mm, corresponding to 29mm/m^2, shortly prior to transplantation. Additionally, she suffers from a mild progressive distal sensorimotor primary axonal polyneuropathy.

The proband's mother received a pacemaker due to total block at age 57. According to hospital records, three aunts or uncles from her mother's side had cardiac abnormalities. There was no available material for genetic testing. The proband's daughter did not carry the c.497G>C, p.(Arg166Pro) mutation and was healthy in clinical assessment including electrocardiography and echocardiography at age 35.

Poly-Phen-2 predicted the c.497G>C, p.(Arg166Pro) variant to be possibly damaging with a score of 0.950 (sensitivity 0.79; specificity 0.95).

5.1.4 C.1442 DUP A P.(TYR481*)

The proband carrying the c.1442 dupA p.(Tyr481*) mutation first presented with atrial fibrillation at age 41. 10 years later she received a pacemaker due to bradycardia, which was shortly upgraded to an ICD due to non-sustained ventricular tachycardia. For several years she had a fairly conserved systolic function and only mildly dilated left ventricle, and at the time of recruitment to the study she did not fulfil the echocardiography criteria for dilated cardiomyopathy. However, her cardiolaminopathy followed a progressive course. At age 59 she had some episodes of ventricular tachycardia and ICD therapy, and her pacemaker was updated to CRT-D. Some four years later she started having frequent episodes of ventricular tachycardia, and developed heart failure. Recently, she had an LVEDD of 61mm and an LVEF of 30% in echocardiography, and is currently under evaluation for a heart transplant.

5.1.5 C. 710T>C, P.(PHE237SER)

The novel c.710T>C, p.(Phe237Ser) mutation was present in six family members and none of the studied 186 Finnish controls. Three mutation carriers had the severe phenotype affecting in particular the right side of the heart. The three asymptomatic mutation carriers were all under 30 years old. There were additionally two obligatory mutation carriers with the phenotype.

The proband first presented with mild chest pain and dyspnoea at age 43. In echocardiography he had a dilated hypokinetic right ventricle, a dilated right atrium, a moderately dilated left ventricle with an LVEF of 40%, and a severe tricuspid insufficiency. His paroxysmal atrial fibrillation soon became chronic. In coronary angiography he had no signs of coronary artery disease. It was first interpreted that the tricuspid insufficiency was the main reason for the right ventricular failure, and thus, a mechanical tricuspid valve was installed. However, despite the successful valve replacement, the symptoms remained. Two years after the initial presentation the heart symptoms progressed, and the left ventricular pump function worsened (LVEF was down to 35%). There were still no signs of coronary artery disease in coronary angiography. Due to a still deteriorating clinical condition he received a heart transplant at age 47. There was fibrotic replacement in the histology of the explanted heart especially in the right atrium.

The proband's brother presented with atrial fibrillation and heart failure at age 41. He had hypokinesia, a mild mitral insufficiency and a significant tricuspid insufficiency in echocardiography. After a cardioversion he went to asystole, and after that received a pacemaker. In three years the tricuspid insufficiency had progressed to severe stage, and all the chambers of the heart were dilated. There were no signs of coronary artery disease in coronary angiography. At age 44 he also received a heart transplant.

The proband's cousin presented with heart disease at age 44. She had chronic atrial fibrillation, and echocardiography showed right ventricular dilatation and failure with a normal left ventricular diameter. An endomyocardial biopsy revealed nuclear blebbing; a common finding in *LMNA* mutated cells, and unspecific degenerative changes. She developed symptomatic bradycardia 18 months after the initial symptoms, and received a pacemaker. The right ventricular failure progressed, but due to follicular lymphoma she was not a suitable candidate for a heart transplant. She suffered a cardiogenic stroke at age 47, and died of heart failure six months later.

The proband's mother and aunt, both obligatory carriers of the c.710T>C, p.(Phe237Ser) mutation, had similar phenotypes. The proband's mother had elevated blood pressure before the age of 30, received a pacemaker at 43 because of sick sinus syndrome and atrial fibrillation. Six months after pacemaker implantation she had a clinically diagnosed transient ischemic attack. At 48 she underwent surgery for a ruptured intracranial aneurysm. She was diagnosed with heart failure at age 53. Echocardiography showed a significantly dilated right side of the heart, a tricuspid insufficiency and a combined aortic valve defect. She had liver cirrhosis due to the right-sided heart failure, and additionally developed renal failure. She died at age 56.

The proband's aunt presented with sick sinus syndrome and atrioventricular block at age 52, and received a pacemaker. She had a mild combined aortic valve defect, mitral insufficiency and a massive tricuspid insufficiency in echocardiography. Mitral and

tricuspid valvuloplasties were performed when she was 58, but she died of heart failure only four months after surgery. The proband has two children who are both carriers of the c.710T>C, p.(Phe237Ser) mutation, but at ages 24 and 28 showed no signs of heart disease. The proband's nephew is also a mutation carriers, and at age 22 showed non-specific intraventricular conduction defect in ECG, and a slightly reduced performance in spiroergometry, but no abnormalities in echocardiography.

Due to the family's ARVC-like phenotype myocardial samples of the proband and his brother were analysed using immunohistochemistry for plakoglobin. The staining pattern was normal.

Additionally, for the same reason, genetic analyses on the proband's brother and cousin were performed. None of the previously described Finnish ARVC-linked desmosomal mutations (*PKP2* c.176A>T, p.(Gln59Leu), *PKP2* c.184C>A, p.(Gln62Lys), *PKP2* c.1839C>G, p.(Asn613Lys), *DSP* c.4117A>G, p.(Thr1373Ala) and *DSG2* c.3059_3062delAGAG, p(Glu1020Alafs*18) were found. (96, 97) Neither did the sequencing of the protein-coding regions and exon-intron junctions of the plakophilin 2b reveal any mutations.

5.2 STUDY II

Spiroergometry follow-up results of 26 *LMNA* mutation carriers

Due to the heterogeneity of the phenotype in the study group of 26 *LMNA* mutation carriers the study group was divided into symptomatic and asymptomatic *LMNA* mutation carriers. The symptomatic group comprised 14 mutation carriers with at least one of the following clinical manifestations: atrial fibrillation, sustained ventricular tachycardia, pacemakers or ICDs, or dilated cardiomyopathy. The remaining 12 mutation carriers lacked clinically relevant manifestations of their *LMNA* mutation.

Overall the symptomatic mutation carriers showed lower oxygen uptake (statistical significance at baseline visit), a non-significantly lower anaerobic threshold and signs of an increased ventilatory response during exercise compared to the control group marked by higher ventilatory equivalents ($\dot{V}EO_2$ and $\dot{V}ECO_2$), lower fraction of end-tidal CO_2 ($FetCO_2$) and higher $\dot{V}E/\dot{V}CO_2$ slope values. In echocardiography the symptomatic mutation carriers had a lower LVEF than the control group, the difference being statistically significant at the baseline and control visits 1 and 3.

The overall spiroergometry performance and LVEF in echocardiography of the asymptomatic carriers compared to the control group was quite similar. However $\dot{V}E/\dot{V}CO_2$ slope of the asymptomatic mutation carriers was higher (statistically significant difference from baseline to control visit 2) and $FetCO_2$ was lower (statistically significant difference from control visit 1 to 4) than in the control group.

Figure 3 presents the $FetCO_2$ and $\dot{V}E/\dot{V}CO_2$ slope values of all the *LMNA* mutation carriers and the healthy controls.

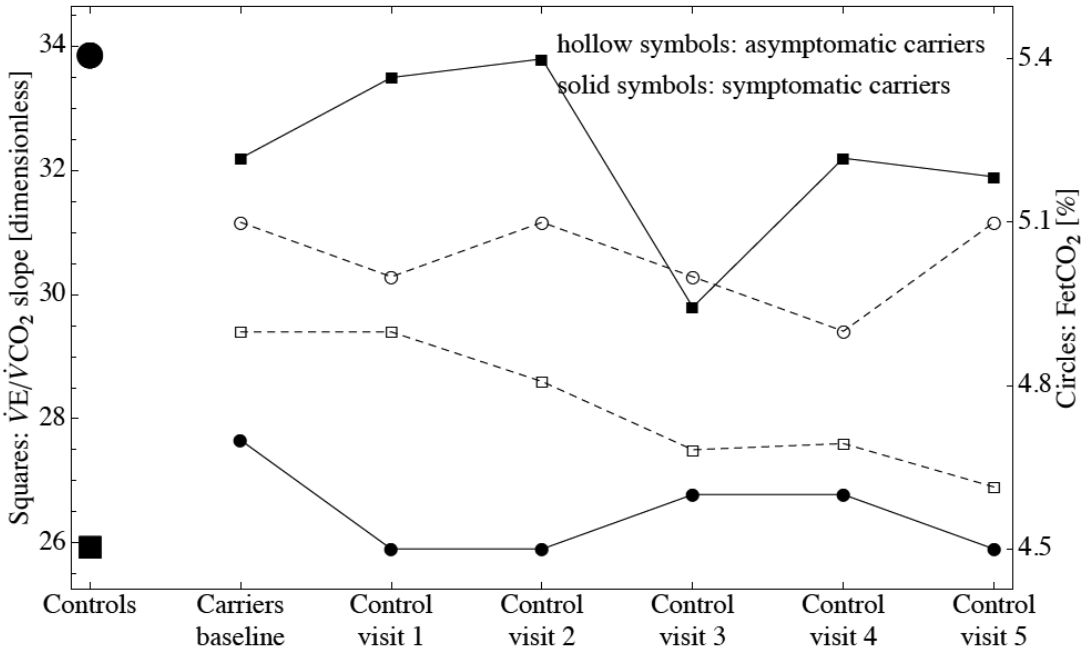


Figure 3 The FetCO2 (circles) and V̇E/V̇CO2 slope (squares) values of all *LMNA* mutation carriers. Hollow symbols represent the asymptomatic carriers and solid symbols represent the symptomatic carriers. The healthy controls (depicted as larger solid symbols) were the same for both asymptomatic and symptomatic carriers.

5.3 STUDY III

Disease progression and ECG characteristics of 27 *LMNA* mutation carriers

5.3.1 GENERAL

Among the 27 *LMNA* mutation carriers 12 fulfilled the criteria set for DCM (*LMNA*-DCM subgroup). The *LMNA* mutation carriers were younger at the end of the follow-up than the DCM controls: the mean age at last follow-up or major endpoint was 48 and 59 y, respectively ($p<0.001$). There was more atrial fibrillation in the *LMNA*-DCM subgroup than in the DCM controls (91.7% vs. 50.0%, $p=0.007$), but no difference between all the *LMNA* mutation carriers (55.6%) and the DCM controls. *LMNA* mutation carriers also had a first recorded episode of atrial fibrillation at a younger age than the DCM controls (46.9 vs. 56.9, $p=0.003$); the finding was essentially the same in the *LMNA*-DCM subgroup.

Similarly, there were more implanted pacemakers in the *LMNA*-DCM subgroup than in the DCM controls (83.3% vs. 47.7%, p=0.020), but again no statistically significant difference between all the *LMNA* mutation carriers (59.3%) and the DCM controls. There was no difference in the number of ICDs between all the *LMNA*-mutation carriers (33.3%) or DCM controls (33.3%). The prevalence was a bit higher in the *LMNA*-DCM subgroup (50.0%), but not statistically significantly. Thrombosis was as common among all the *LMNA*-mutation carriers (14.8%) than among the DCM controls (15.4%). Again the number was statistically non-significantly higher among the *LMNA*-DCM subgroup (33.3%). Figure 4 shows the endpoint prevalences in all the *LMNA* mutation carriers, the *LMNA*-DCM subgroup, and the DCM controls. The number of major endpoints (deaths, resuscitations, appropriate ICD events, or heart transplants) was lower among all the *LMNA* mutation carriers (25.9%) than the DCM-controls (48.7%). However in Kaplan-Meier analysis there was no difference in event-free survival of these two groups when the definition of major endpoint was the same as above; the median age estimate was 62.8 (CI: 53.3-72.3) y for *LMNA* mutation carriers and 68.0 (CI: 64.3-71.7) y for DCM controls (the figure presented in the original article). When first incidence of atrial fibrillation, pacemaker implantations and thrombosis were included additionally as events *LMNA* mutation carriers had a lower event-free survival age than the DCM controls (median age estimates 47.0, CI: 37.4-56.6 for *LMNA* mutation carriers and 56.9, CI: 53.7-60.1 for DCM controls) (Figure 5). NSVT was common among all the *LMNA* mutation carriers (77.8%). By age 50 all *LMNA* mutation carriers had a clinically relevant manifestation.

In Figure 6 the incidence ages of *LMNA* mutation manifestations in the entire *LMNA* mutation carrier group in study III are given.

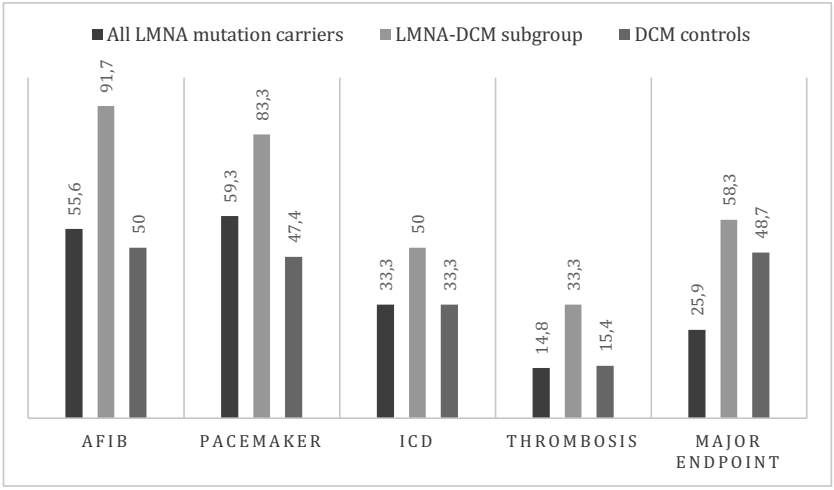


Figure 4 Prevalence of atrial fibrillation (AFib), pacemakers, ICDs (implantable cardioverter-defibrillators), thrombosis, and major endpoints including death, heart transplantation, resuscitation, or appropriate ICD event in all *LMNA* mutation carriers, those *LMNA* mutation carriers with DCM and the DCM controls.

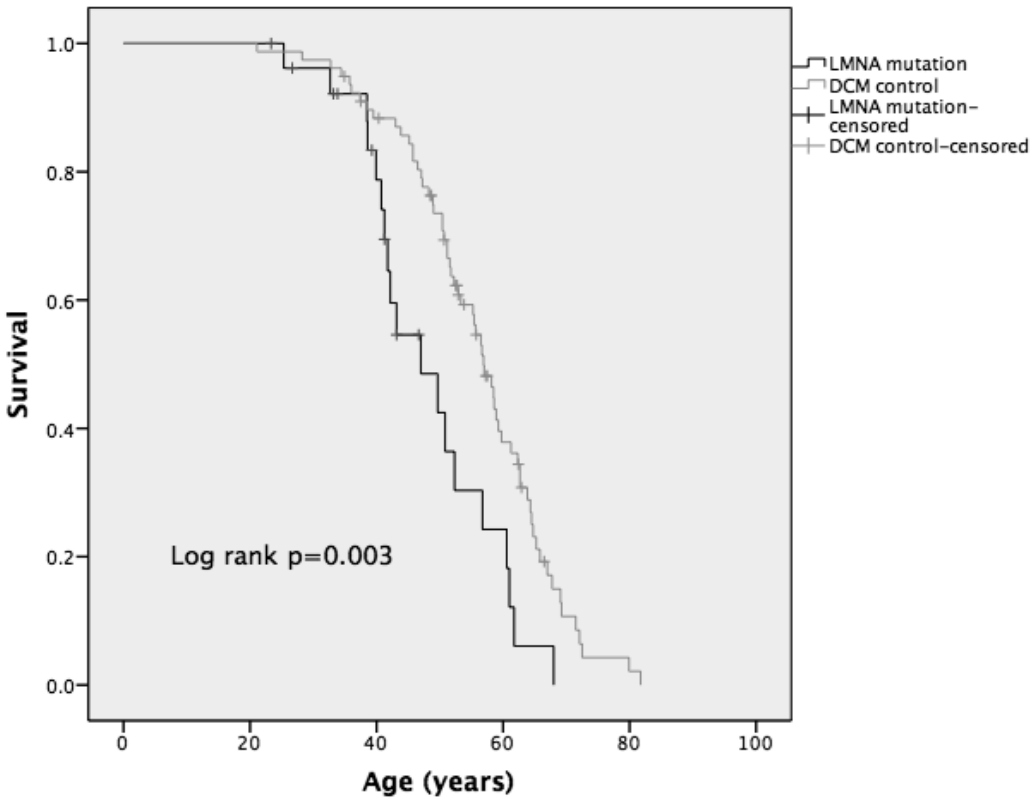


Figure 5 Kaplan-Meier analysis of event-free survival of *LMNA* mutation carriers and DCM controls when death, heart transplantation, resuscitation, appropriate ICD event, atrial fibrillation, pacemaker implantation, and thrombosis were included as events. Median age estimate 47.0 years (CI: 37.4-56.6) for *LMNA* mutation carriers and 56.9 years (CI: 53.7-60.1) for DCM controls, p=0.003.

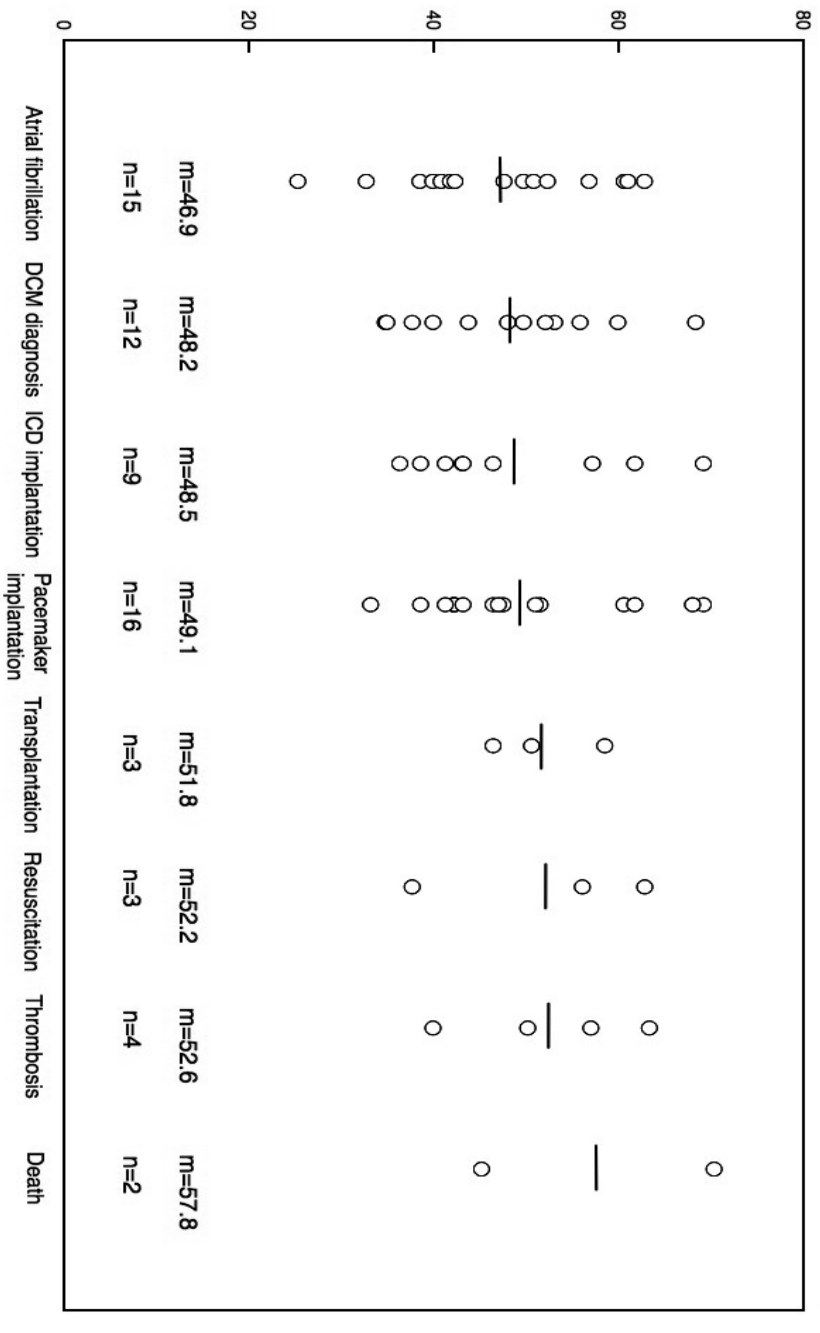


Figure 6 The incidence ages of *LMNA* mutation manifestations in the entire *LMNA* mutation carrier group in study III.

5.3.2 GENDER-SPECIFIC DIFFERENCES

Figure 7 shows clinical endpoint prevalences in male and female *LMNA* mutation carriers and DCM controls, and Figure 8 the clinical endpoint incidence ages in male and female *LMNA* mutation carriers and DCM controls. Male *LMNA* mutation carriers had their first recorded episode of atrial fibrillation (40.9 vs. 55.4 y, p<0.001), pacemaker implantation (41.8 vs. 55.8, p<0.001) and ICD implantation (41.5 vs. 51.2, p=0.007) earlier than male DCM controls. Female *LMNA* mutation carriers differed from the female DCM controls only in the number of major endpoints occurred (14.3% vs. 50.0%, p=0.03).

Looking at the *LMNA* mutation carriers there was a general tendency for each measured event or endpoint to take place at least a decade earlier in males than in females. The incidence ages for the different endpoints were: 40.5 and 50.3 y, non-significant, for NSVT, 40.9 and 53.7 y, p=0.016 for atrial fibrillation, 41.8 and 58.5 y, p=0.001, for pacemaker implantation, 41.5 and 62.7 y, p<0.001, for ICD implantation, 42.2 and 54.1 y, p=0.042, for fulfilling the echocardiography criteria for DCM, 48.5 and 56.8 y, non-significant, for likely cardiogenic thrombosis, and 47.7 and 56.3 y, non-significant, for age at major endpoint for males and females, respectively.

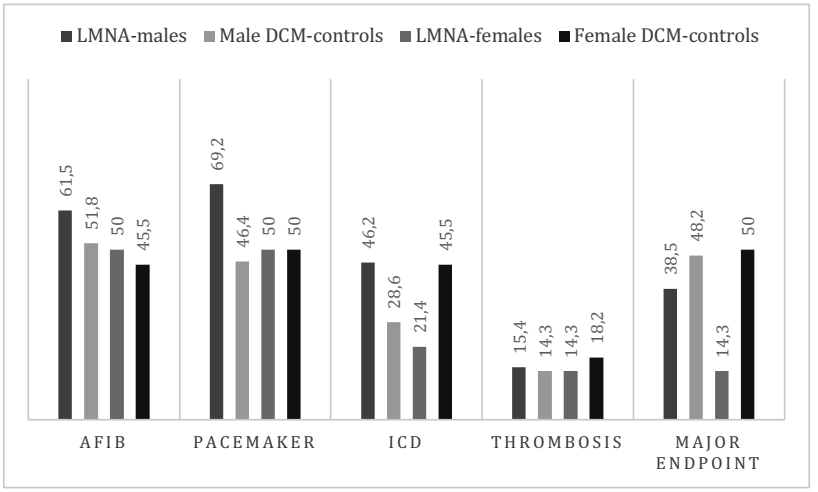


Figure 7 The endpoint prevalences in male and female *LMNA* mutation carriers and DCM controls.

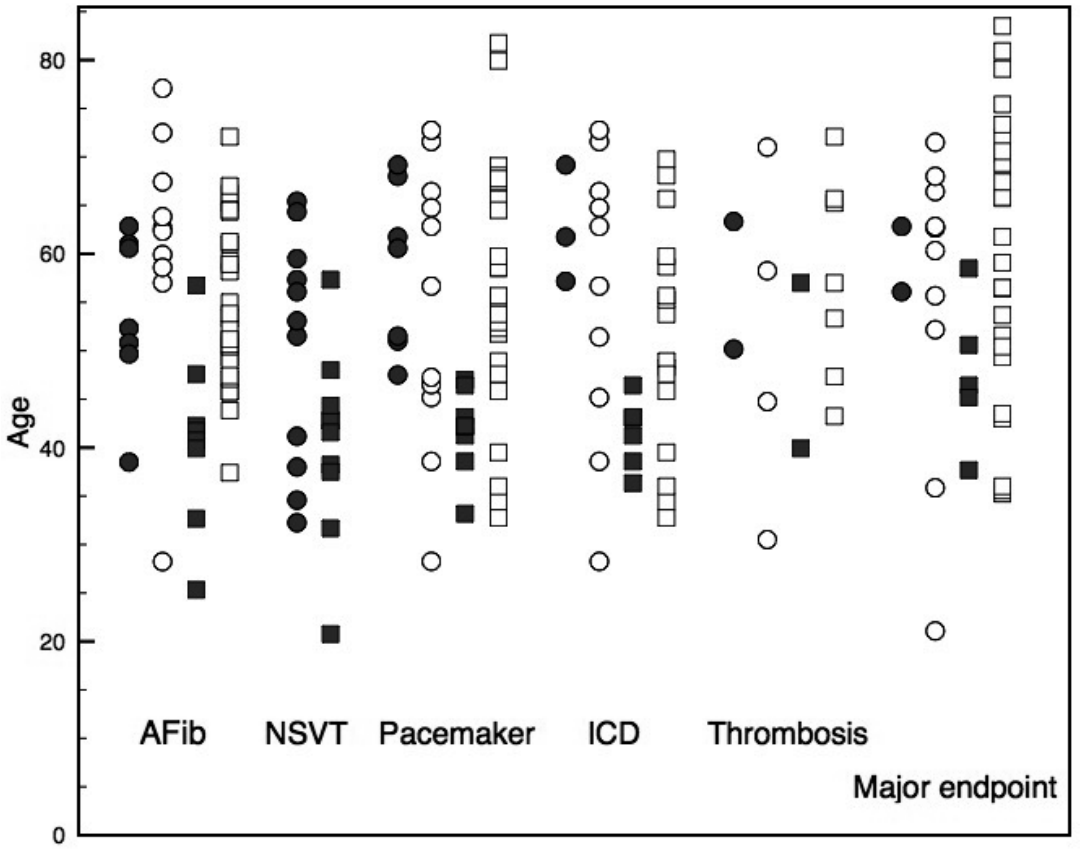


Figure 8 First incidences of atrial fibrillation (AFib), NSVT (non-sustained ventricular tachycardia), pacemaker implantation, ICD implantation, thrombosis and major endpoint (death, heart transplantation, resuscitation, or appropriate ICD event) in female *LMNA* mutation carriers (filled circle), female DCM controls (white circle), male *LMNA* mutation carriers (filled square) and male DCM controls (white square).

5.3.3 ECG FINDINGS AND SEPTAL REMODELLING IN ECG

The previously known ECG abnormalities typical to laminopathy were also seen in this study. Namely, flat P wave and AV blocks were significantly more frequently present in *LMNA* mutation carriers than the DCM controls. Neither was present in any of the 20 healthy controls used as an additional control group for the ECG analyses. Additionally septal remodelling, for the definition see 4.6.7, was present in 81.5% of *LMNA* mutation carriers, only 20.5% of DCM controls and none of the healthy controls. Figure 9 shows the prevalence of the abovementioned ECG abnormalities in the three groups. Current or previous AV block was used because the presence of an AV block could not be analysed in a fairly large proportion of the *LMNA* mutation carriers or in the DCM controls due to atrial fibrillation and ventricular pacemakers. Table 7 shows the sensitivities, specificities, and

positive and negative predictive values for ECG septal remodelling in classifying *LMNA* mutation carriers from DCM controls, healthy controls or all the controls.

	***LMNA* vs. DCM control**	***LMNA* vs. healthy control**	***LMNA* vs. DCM or healthy control**
Sensitivity	81.5	81.5	81.5
Specificity	79.5	100	83.7
PPV	57.9	100	57.9
NPV	92.5	80	94.3

Table 7. *Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for septal remodelling in distinguishing LMNA mutation carriers from DCM control, healthy controls, and either healthy controls or DCM controls.*

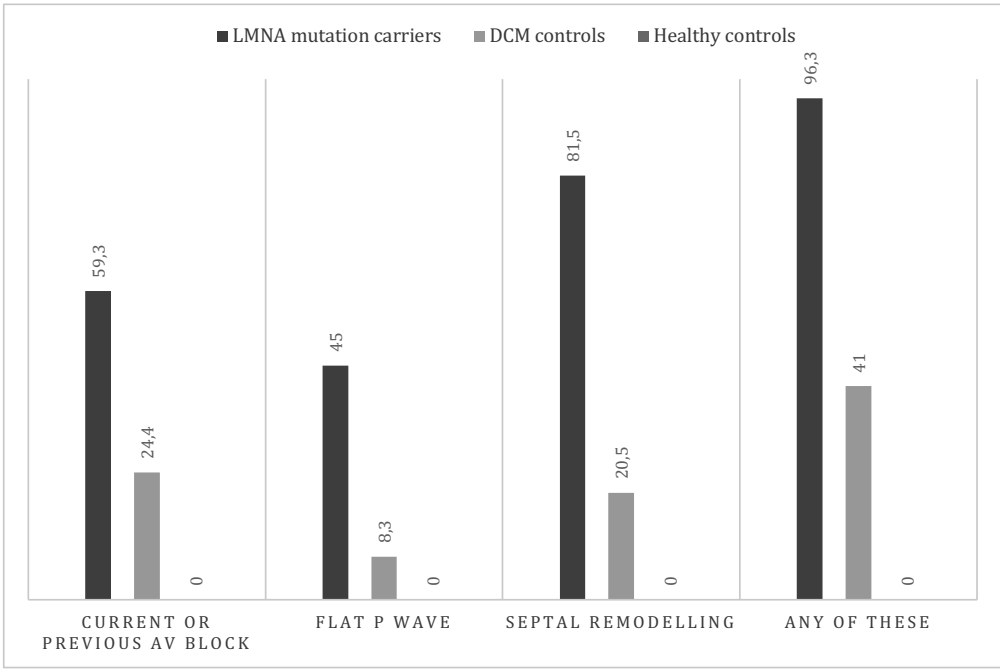


Figure 9 Prevalence of AV block (at the time of the ECG recording or previously), flat P wave, septal remodelling, and any of these in *LMNA* mutation carriers, healthy controls, and DCM controls.

5.4 STUDY IV

Finn-DCM

5.4.1 GENERAL

Sixty-three patients (43.4%) had familial DCM and 82 (56.6%) sporadic DCM. In the familial DCM group 69.8% (n=44) and in the sporadic group 76.8% (n=63) were males. Males had a larger LVEDD (72 vs. 64mm, p<0.001) and a lower LVEF (23 vs. 28%, p=0.003) than females. There were no gender-specific significant differences concerning the frequencies of atrial fibrillation, arrhythmias, resuscitation, or implanted pacemakers. However, men were younger than women at the time of resuscitation or appropriate ICD event (47.6 vs. 65.4, p=0.001). There were no differences in frequencies of atrial fibrillation, pacemakers, heart transplants, resuscitations, histological samples (=endomyocardial biopsy or a histology report of the explanted heart), or angiography in the familial vs. non-familial groups.

When the pathogenic and likely pathogenic variables were considered disease-causing the diagnostic yield was 35.2% (n=51) for the entire group. The diagnostic yield was significantly higher for the familial than the sporadic cases (47.6%, n=33 vs. 25.6%, n=21, p=0.004).

5.4.2 TITIN (*TTN*)

Truncating titin mutations explained 17.2% in the entire study population, 20.6% among the familial DCM patients and 14.6% among those with sporadic DCM. Segregation analysis was possible in five families. The *TTN*-mutation co-segregated with the disease in all five cases.

Altogether there were 64 unique rare *TTN* variants in the cohort present in 71 probands (48%). 38 of these variants were missense. 21 *TTN* variants were truncating; 11 non-sense, 9 frameshift, and one consensus splice-site variant. Additionally, one truncating variant was classified as a VUS due to its commonness and the fact that it affects only one transcript.

62% (13/21) of the truncating *TTN* mutations were located in the A band region of the sarcomere, and 95% (20/21) of them affected all transcripts of the gene.

5.4.3 OTHERS

LMNA mutations were present in 12 (8.3%) and DSP mutations in 8 (5.5%) probands. Six of the probands with *DSP* mutations had the same novel c.6310delA p.(Thr2104Glnfs*12)

variant whereas three of the *LMNA* mutation carriers had the Finnish founder mutation c.427T>C, p.(Ser143Pro). Additional causative variants found were two *RBM20* variants, one dystrophin (*DMD*) variant and one telethonin (*TCAP*) variant.

5.4.4 CLINICAL FINDINGS AND GENOTYPE-PHENOTYPE CORRELATIONS

Table 8 shows clinical characteristics of the probands carrying *TTNtvs* compared to the others probands. No statistically significant differences were seen. Co-segregation was possible to analyse in five families (13 individuals) carrying *TTNtvs*. The penetrance was full by age 70 as seen in Figure 10.

LMNA mutation carriers had a statistically significantly smaller LVEDD and LVEDD index than *TTN* mutation carriers. They also had more pacemakers than *TTN* mutation carriers and had been resuscitated or had experienced an appropriate ICD shock more often than *TTN* mutation carriers. Atrial fibrillation and pacemakers were more common in *LMNA* mutation carriers than *TTN* or *DSP* mutation carriers.

	***TTN* (n=25)**	**Others (n=120)**
Age at diagnosis	43.4	46.5
LVEDD	70.1	70.7
LVEF	23.1	23.5
Pacemaker	0	8.3
CRT	4	7.5
CRT-D	8	15.8
ICD	20	17.5
Transplantation	28	30
Resuscitation	12	20.8

Table 8. *Clinical characteristics of probands carrying TTNtvs compared to others probands*

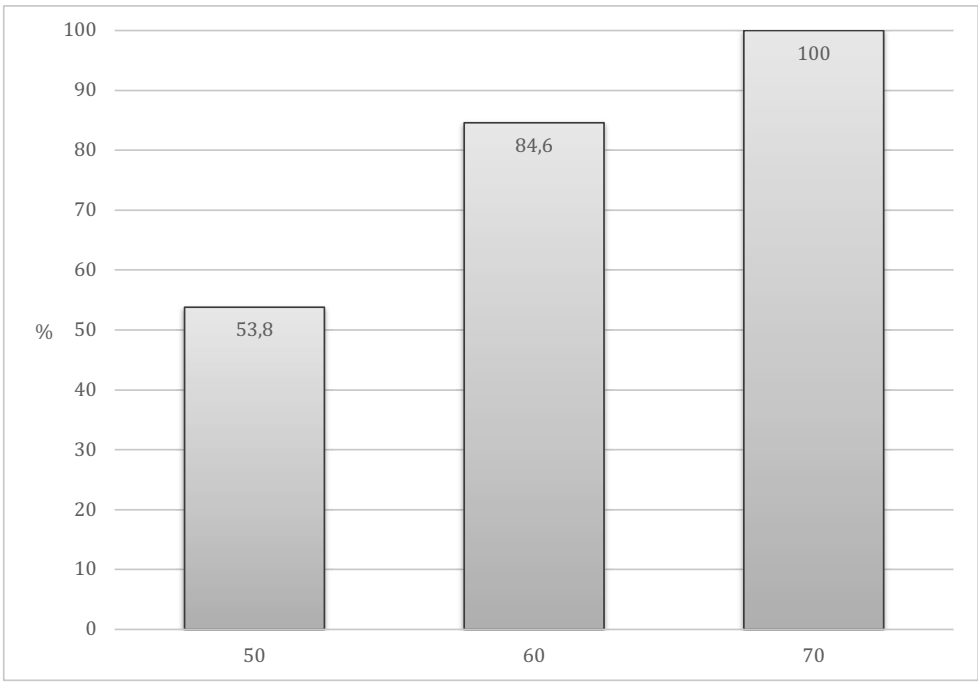


Figure 10 Penetrance of cardiomyopathy manifestations in 13 *TTNtv* carriers by ages 50, 60, and 70.

6 DISCUSSION

6.1 LAMINOPATHY

6.1.1 RIGHT VENTRICLE PREDOMINANT LAMINOPATHY

Cardiomyopathy caused by *LMNA* mutations is well characterized, the most typical manifestations being atrioventricular conduction disturbances, atrial and ventricular arrhythmias, and dilated cardiomyopathy often with somewhat modest left ventricular dilatation. (22, 139) Study I describes an atypical cardiomyopathy phenotype present in five carriers of a novel c.710T>C, p.(Phe237Ser) *LMNA* variant. The phenotype consists of severe cardiomyopathy mainly in the right side of the heart leading to heart transplantation in two carriers and death in three. Three carriers did not yet show the phenotype characteristics in early adulthood, which is compatible with the age-dependent presentation of dilated cardiomyopathy and cardiolaminopathy. (22, 103) The mutation carriers did not fulfil the ARVC criteria, (87) however they did not undergo systematic assessment to exclude or diagnose ARCV, for instance 24 hour ECG data was available from only one mutation carrier and signal averaged ECG from none. Subsequent to the publication of study I the c.710T>C, p.(Phe237Ser) variant was found in another Finnish proband with a similar right-predominant cardiomyopathy phenotype diagnosed initially at age 44. The patient's father had died of heart failure before age 50. (T. Heliö, personal communication)

LMNA mutations have also been found in 4% (4/108 patients) of the patients in an ARVC cohort reported by Quarta et al. Two mutations, c.214C>T p.(Arg72Cys), and c.1145G>T p.(Gly382Val), were novel, and two, c.568C>T p.(Arg190Trp), and c.1930C>T p.(Arg644Cys), were previously described. (92) 81 of the patients had a definite and 27 borderline diagnosis according to the 2010 ARVC criteria. The c.568C>T p.(Arg190Trp) mutation has been reported multiple times in DCM patients including a Finnish study of DCM patients having undergone heart transplantation. (110, 139, 177, 178) It is also one of the mutations present in our studies II and III. The c.1930C>T p.(Arg644Cys), mutation has been reported multiple times before, in association with varying laminopathy phenotypes including DCM, ARVC, muscular dystrophy, and atypical progeria. (179-183) In a study by Mercuri et al. the proband and his mother had a similar phenotype of muscular dystrophy and cardiomyopathy, but only the proband was a mutation carrier suggesting another cause for the familial disease. (179) In a further study it was found that the proband also carried a desmin mutation and his explanted heart showed an abundance of desmin accumulation. Additionally the proband's healthy father was found to be a

carrier of the c.1930C>T p.(Arg644Cys) *LMNA* mutation. This evidence put together suggests that the pathogenic mutation in this case was in fact the desmin variant as opposed to the c.1930C>T p.(Arg644Cys) variant. In a study by Csoka et al. the proband carrying The c.1930C>T p.(Arg644Cys) variant suffered from "atypical progeria" described as short stature, thinned skin, generalized wasting and survival to a relatively old age. No segregation-analysis was done, but in immunofluorescence microscopy of the mutant fibroblast cell line mild nuclear irregularity was seen, less so than in the other mutant *LMNA* cell lines, but more than in a control cell line. (180) Rankin et al. presented nine patients carrying the same *LMNA* variant with varying manifestations compatible with laminopathy, such as lipodystrophy, neuropathy, DCM, and muscular dystrophy. However, the variant did not show co-segregation with a laminopathy phenotype in any of the studied families. (184) In a study of SCD victims the c.1930C>T p.(Arg644Cys) variant was found in one victim, but family history was unavailable, thus again co-segregation was not shown. (183) Furthermore, the c.1930C>T p.(Arg644Cys) variant is present in ExAC with an allele frequency of 0.0016 for non-Finnish Europeans suggesting that the c.1930C>T p.(Arg644Cys) is more likely a VUS. (131) The other three *LMNA* variants reported in ARVC patients in the Quarta et al. study are not found in ExAC. In 2015 Forleo et al. reported an *LMNA* variant c.418_438dup, p.(Leu140Ala146dup) in a large Italian family with a varying phenotype of ARVC, DCM, conduction disturbances, arrhythmia and SCD co-segregating with the *LMNA* variant. (185)

Our study is compatible with the other recent studies cited here, presenting a cardiolaminopathy affecting the right side of the heart. While Quarta et al. presented probands fulfilling the ARVC criteria, Forleo et al. presented a family with both DCM and ARVC phenotypes. (92, 185) The other three mutations presented in study I represent the more typical form of cardiolaminopathy.

6.1.2 INEFFICIENT EXERCISE VENTILATION IN *LMNA* MUTATION CARRIERS

In study II individuals carrying DCM-causing *LMNA*-mutations were studied repeatedly using spiroergometry. The study group was heterogenic ranging from asymptomatic mutation carriers to individuals with dilated cardiomyopathy. With increasing genetic testing of cardiomyopathy patients and their family members the number of asymptomatic individuals known to carry cardiomyopathy-causing mutations also increases. The clinical follow-up protocols of these individuals vary, and there are no detailed guidelines for this purpose.

To address the issue of asymptomatic individuals in particular the study group was divided in symptomatic and asymptomatic mutation carriers. The symptomatic mutation carrier group consisted of patients with clinically significant arrhythmias, pacemakers or with dilated cardiomyopathy. Consistent with this they showed a lower maximal working capacity, maximal oxygen uptake and $FetCO_2$, and an increased slope of ventilation/carbon dioxide exhaled, or VE/VCO_2 slope, namely, changes seen typically in heart failure patients. (71, 77, 83) The mechanisms underlying inefficient ventilation in heart failure patients are considered multifactorial including a lowered anaerobic

threshold, altered breathing patterns, ventilation-perfusion mismatching caused by reduced perfusion in areas with good ventilation, increased sensitivity of chemoreceptors to metabolic changes and abnormalities in the ergoreflex. (76, 186, 187)

The overall performance of the asymptomatic mutation-carriers was comparable to the control group. However, also the asymptomatic mutation carriers had a higher VE/VCO2 slope level and a lower FetCO2 level than the controls suggesting inefficient ventilation during incremental exercise. (76, 85) Due to the generally good performance in spiroergometry of the asymptomatic mutation carriers, the mechanisms underlying the abnormalities seen are not obvious. Possible mechanisms might include changes in chemosensitivity or ventilation-perfusion coupling. (187) These results suggest that inefficient ventilation during exercise might be a sign of evolving cardiomyopathy in *LMNA* mutation carriers who are considered still unaffected by the mutation by other means of clinical assessment.

6.1.3 EFFECT OF GENDER ON LAMINOPATHY PHENOTYPE

There was a consistent difference in the ages of onset of clinical manifestations in men and women in study III, namely men presented with the various clinical cardiomyopathy or laminopathy manifestations at a younger age than women. In the landmark Olmsted county study from 1989 both the incidence and prevalence of idiopathic DCM was threefold in men compared to women. (16) Other studies have also found a difference, albeit a smaller one, in the prevalence of DCM between men and women. (41, 188, 189) Furthermore women are far less likely to undergo heart transplantation than men; between 2005 and 2010 women comprised 23% of heart transplant recipients. (190) Also the outcome in heart failure is more favorable in women as shown by a follow-up study of the Framingham Heart Study reporting 5-year survival rates of 25% for men and 38% for women after congestive heart failure diagnosis. (191)

Given these well-established gender-specific differences in disease prevalence and outcome in the wider heart failure population it is not surprising that similar differences should be seen also in *LMNA* mutation carriers. In a study by VanRijsingen et al. gender-specific differences were also reported; male *LMNA* mutation carriers were more likely to have LVEF≤45% than women. Men also had a higher prevalence of end-stage heart failure and malignant arrhythmias and a higher mortality than women. However, the prevalence of AV block, atrial tachyarrhythmia, and NSVT did not differ between the sexes. (192)

The mechanisms underlying these clinical differences between genders are likely multifactorial. Possible biological mechanisms include the effects of sex hormones on cardiomyocytes. Estradiol acts as a cardioprotective agent by preventing cardiomyocyte apoptosis, cardiac hypertrophy and fibrosis. (193) A homozygous *LMNA* c.665A>C, p.(His222Pro) knock-in mouse-model has also been studied giving some mechanistic evidence of the effects of androgens on the development of the cardiomyopathy phenotype. (194) However, the applicability of these results to humans and cardiomyopathy inherited in an autosomal dominant pattern can be questioned. Behavioral patterns can also play a role; in a large American study women were 50% more likely to follow national

recommendations of smoking abstinence, physical activity and fruit and vegetable consumption. (195) Men also consume more alcohol than women globally and in Finland. (196, 197) Although it is still debated whether excessive alcohol consumption alone can cause the dilated cardiomyopathy phenotype or if a genetic vulnerability is needed, it is well accepted that there is a link between excessive alcohol use and the dilated cardiomyopathy phenotype. (198)

6.1.4 NON-SUSTAINED VENTRICULAR TACHYCARDIA

The study patients in study III were under rigorous follow-up. Possibly due to this we found an unprecedentedly high prevalence of NSVT among *LMNA* mutation carriers. Sudden death is quite common in *LMNA* mutation carriers and it has been speculated that lethal tachyarrhythmia is the likeliest mechanism as sudden death was as common among those with pacemakers as those without in a meta-analysis of *LMNA* mutation carriers. (22) This does not mean, however, that the risk for malignant ventricular arrhythmia is the same for all *LMNA* mutation carriers. Another study tackling malignant ventricular arrhythmia in *LMNA* mutation carriers reported that reduced ejection fraction, male gender, non-missense mutations, and NSVT were independent risk factors, and malignant ventricular arrhythmias occurred in those with at least two risk factors. (68) The same study reported NSVT in 37% of *LMNA* mutation carriers. Our markedly higher number of NSVT suggests that with repeated monitoring most *LMNA* mutation carriers have at least one of these risk factors.

6.1.5 SEPTAL REMODELLING

Study III included 27 individuals 24 of whom were also study patients in study II. Of the 12 individuals defined as asymptomatic in study II 10 (83%) had septal remodeling in study III. Likewise, of the 12 individuals defined as symptomatic in study II 10 (83%) had septal remodeling in study III. This is to say that septal remodeling in ECG was very common among all *LMNA* mutation carriers including those appearing asymptomatic in clinical assessment. Given that also late gadolinium enhancement (LGE) seen in CMR known to detect cardiac scarring appears to localize in the septum (199, 200), it seems feasible that septal remodeling is a sensitive indicator of scarring, and furthermore, that *LMNA* mutation carriers appear to have myocardial scarring in the septum. In a study presenting a large kindred carrying a cardiomyopathy-causing *LMNA* mutation Raman and colleagues also showed LGE localizing in the basal septum. Furthermore, scarring was seen in the hearts of deceased relatives in autopsy. (200)

The European Society of Cardiology Working Group on Myocardial and Pericardial Diseases recommends continuous clinical follow-up of asymptomatic carriers of cardiomyopathy-causing mutations. (103) With limited resources and an increasing number of asymptomatic mutation carriers this is a burdensome task for the health-care system. In the case of asymptomatic *LMNA*-mutation carriers the standard ECG might be

a useful tool for allocating resources. For instance those with yet normal ECG could be monitored less vigorously and those showing signs of cardiolaminopathy, such as septal remodeling in ECG, could undergo more frequent assessment. However, to assess the specificity and clinical usefulness of ECG septal remodeling, studies including patients with other cardiac diseases, such as ischemic and inflammatory heart disease, should be performed.

6.2 FINN-DCM

In Finn-DCM a targeted sequencing panel using OS-Seq technology and covering 101 genes associated with cardiomyopathy was used to study 145 Finnish DCM patients. Although the variant classification criteria for pathogenicity were strict, more than 1 in three of all DCM patients (35%) and nearly half of those with familial disease (48%) received a genetic diagnosis showing the utility of such an approach in the study of genetic DCM. Pugh et al. using a smaller panel of up to 46 genes, and similar criteria for pathogenicity found a diagnostic yield of 27-37% with the lower number containing only pathogenic or likely pathogenic variants and the higher additionally those variants of unknown significance whose assessment favored pathogenicity. (101) Haas et al. studied a large cohort of 639 DCM patients with a panel containing 84 genes. They reported a higher diagnostic yield of 73% with 38% of the patients carrying two or more mutations. The variant classification system they used, however, was very different from ours, or the one used by Pugh at al. As a basis for classification they considered variants listed as cardiomyopathy or channelopathy variants in Human Gene Mutation Database (HGMD) pathogenic. (106) This can be criticized, however, as HGMD contains variants previously considered pathogenic but with the emergence of reference databases now known to be likely polymorphisms. (201) As an additional check-up Haas et al. excluded variants present in ESP (Exome Sequencing Project), containing the genomes of 6500 individuals, with an allele frequency greater than 1%. (106) Suitable cutoff values for allele frequencies when assessing pathogenicity are not straightforward to define, but significantly lower values, for instance 0,04%, have been proposed for dilated cardiomyopathy. (202) The possibility remains, of course, that some of the variants considered possibly pathogenic using looser criteria for pathogenicity are in fact modifying variants. An interesting study using exome sequencing to study an Italian family with severe DCM found an *LMNA* c.656A>C, p.(Lys219Thr) variant, previously reported to cause DCM (181), in all affected patients and additionally a *TTN* missense variant c.14563C>T, p.(Leu4855Phe) in five individuals. (181, 203) Those individuals carrying both the *LMNA* and the *TTN* variant had a more severe clinical phenotype than those *LMNA* variant carriers without the *TTN* variant. Furthermore the histology of the cardiac tissue of the double heterozygotes was more disturbed than the histology of the individuals carrying only the *LMNA* variant.

The novel *DSP* variant c.6310delA p.(Thr2104Glnfs*12) found in six probands who were not apparently related, as confirmed by a genealogy search spanning over several centuries, is present in ExAC in four individuals of European ancestry, two Finnish individuals and two non-Finnish Europeans.

The Finn-DCM study comprised of DCM patients predominantly from the Southern part of Finland. However, as heart transplantations are only performed in Helsinki, patients from all over the country were included. It is likely that the results of Finn-DCM are somewhat representative of the entire country. However, it should be accounted that recruitment took place in a tertiary centre, which likely biases the patient material towards more severe phenotypes. Since the genetic etiology of DCM is varied, local differences between studies similar to Finn-DCM performed elsewhere are bound to exist. The *DSP* variant c.6310delA p.(Thr2104Glnfs*12) is a good example of this; the prevalence of pathogenic or likely pathogenic *DSP* variants was unprecedentedly high in Finn-DCM, 5.5% altogether and 4.1% for the c.6310delA p.(Thr2104Glnfs*12) variant. This is to say the high prevalence was mainly due to one variant, a likely Finnish founder mutation. Previously, Elliot et al. reported a prevalence of 2%, (94) and Pugh et al. a prevalence of 2.4% for pathogenic *DSP* variants in DCM. (101)

6.2.1 *TTN*

Study IV confirmed the significance of truncating titin variants as the most significant known cause for DCM. Of the 21 truncating titin variants considered pathogenic in the study all but one affected all transcripts. This is in line with the observations made in other studies finding that clinically significant variants affect highly expressed parts of the *TTN* gene, most often the constitutively expressed A-band. (101, 108, 109)

The prevalence of *TTNtvs* was 20.6% for probands with familial disease, 14.6% for sporadic cases, and 17.2% for the entire study population. In the first *TTN* report by Herman et al. the prevalence of *TTNtvs* was 25% for familial disease and 18% for sporadic disease. The definition for familial disease they used, family history of DCM in a first degree relative, was tighter than ours. (108) In the study by Haas et al. the prevalence of *TTNtvs* was 19% in familial and 11% in sporadic DCM. The definition for familial disease used was DCM or sudden cardiac death before age 35 in the family. (106) Roberts et al. found the prevalence of *TTNtvs* to be 20% among end-stage and 13% among unselected DCM patients. (109) And finally, Pugh et al. reported a prevalence of 14% for *TTNtvs* in a referral genetics laboratory population. (101) The numbers reported in the first *TTN* study are higher than the ensuing ones including ours, which can be explained by the looser variant classification criteria used in the Herman et al. study. The initial *TTN* report also raised questions about the relevance of *TTNtvs* in the general population and the distinction between clinically relevant and irrelevant *TTNtvs*. Following studies have come to the conclusion that causative *TTNtvs* are localized in highly expressed exons whereas variants residing in areas exhibiting abundant alternative splicing are less likely to cause cardiomyopathy. (109, 149) However, analyzing variants of *TTN* is still far from simple. A recent study presented a computational model for the assessment of pathogenicity of *TTNtvs* taking in account the disruption of an internal *TTN* promoter, named cronos, and

the localization of the variant in the most C terminal part of the gene, in addition to the level of expression, or PSI (proportion spliced in). (204) If variant analysis of *TTNtvs* is somewhat complex, the assessment of *TTN* missense variants is even more so. In study IV *TTN* missense variants were considered at most variants of unknown significance, which is the same approach others have used. (101, 108) Since study IV a *TTN* c.14563C>T, p.(Leu4855Phe) variant has been reported as a possible disease-modifying agent making cardiolaminopathy more severe as described above. (203) A recent study using whole genome sequencing and linkage analysis also reported a *TTN* missense variant c.533C>A, p.(Ala178Asp) as the only plausible causative variant for a combined phenotype of LVNC and DCM with an autosomal dominant inheritance pattern in a family with nine affected individuals in three generations. Additionally to co-segregation with disease functional evidence including reduced binding of the mutant titin to *TCAP* was seen. (205) The largest study of *TTN* missense variants in DCM to date found missense variants to be very common and although preliminary assessment suggested pathogenicity, co-segregation with disease was often not seen. They reported, however, four families with *TTN* missense mutations they considered causative showing co-segregation with disease. They concluded that *TTN* missense variants should not be considered disease-causing without careful assessment including co-segregation with disease. (206)

6.2.2 GENOTYPE-PHENOTYPE CORRELATIONS

Comparison of clinical characteristics of the probands carrying *TTNtvs* compared to the other probands showed no statistically significant or clinically relevant differences. Thus, *TTNtv* carriers do not seem to form a phenotypically distinct group differing from other DCM patients like *LMNA* mutation carriers do. Similarly, in the first large *TTN* paper by Herman et al. there were no distinct clinical characteristics seen. (108)

In line with previous studies *LMNA* mutation carriers in study IV had a distinct phenotype characterized by a somewhat small left ventricular size, atrial fibrillation, pacemakers and a high incidence of resuscitation or appropriate ICD shock. (15, 22, 139)

Statistically significant differences were seen comparing the phenotype of the carriers of causative *TTNtvs* compared to the carriers of *LMNA* mutations regarding left ventricular size, pacemaker implantations, atrial fibrillation, resuscitation or appropriate ICD shock. Similar differences in left ventricular diameter, atrial fibrillation, presence of pacemakers, and having undergone treatment for malignant ventricular arrhythmia could be seen when comparing *LMNA* mutation carriers to *DSP* mutation carriers and other mutation carriers, but due to smaller group sizes only some differences reached statistical significance.

6.3 STRENGTHS AND LIMITATIONS

Studies I-III aimed to deepen the understanding of cardiomyopathy caused by *LMNA* mutations. The main finding in study I was the description of an unusual cardiolaminopathy phenotype affecting particularly the right side of the heart. The phenotype resembled arrhythmogenic right ventricular cardiomyopathy but did not fulfil the ARVC criteria in retrospect. However, ARVC was not extensively sought in the study patients, and for example, signal-averaged ECG was not available from any of the study patients, and 24 hour ECG was available from only a single patient. It is therefore possible that a more extensive workup for the diagnosis of ARVC might have changed this interpretation.

Studies II-III were based on rigorous follow-up of a group of *LMNA* mutation carriers. In study II a weakness concerning the control group was that they were studied only once. Another limitation was dropout in the study patient group, which limited the statistical analysis in the assessment of disease progression. Spiroergometry is a reproducible method to measure cardiorespiratory fitness, (69) although some variation between tests of even the same individuals at short intervals has been reported. (207) Spiroergometry has also been proven useful in estimating prognosis in heart failure. (77, 82, 83) Recently, the ability of spiroergometry as a prognostic tool in the idiopathic DCM population has also been shown. (84) While the possible variation in the reproducibility in the spiroergometry method is also a limitation in this study it is unlikely to overestimate the main findings.

The repeated examinations were a strength of both studies II and III and lead to the finding in study III of an unprecedentedly high prevalence of NSVT in *LMNA* mutation carriers. An obvious weakness of study III was the number of participants limiting the number of events during the follow-up. Another limitation was the less frequent follow-up of the DCM patients used as controls. A strength of the control group was that they were all genotyped using the Pan Cardiomyopathy test panel of study IV. Therefore it is certain that there were no *LMNA* mutation carriers in the DCM control group. In study III ECG septal remodelling was introduced as a readily available tool for distinguishing *LMNA* mutation carriers from other cardiomyopathy patients. To establish the clinical usefulness of ECG septal remodelling it should be studied in other groups of cardiac patients as other conditions affecting the septum might present with the same ECG pattern.

The study group in study IV was fairly large and well phenotyped. The technology used allowed for the sequencing of all genes previously associated with cardiomyopathies. Even with strict criteria for pathogenic variants the diagnostic yield was high. However, many patients even with known familial disease were still left without genetic diagnosis. As in other studies using large gene panels based on next generation sequencing methods many variants of unknown significance were found also in this study. Some of them will likely be reassessed later as pathogenic variants with more segregation data, which will increase the diagnostic yield.

All the index patients of this project were recruited in a tertiary centre. This should be taken in account when assessing whether the results apply to the broader DCM population, as the study patients comprise more severe cases, such as heart transplant recipients. A common issue in the study of genetic disease is the availability of segregation data. In this project family segregation data was available to analyse in many cases. However, at times the availability of family members to phenotype and genotype limited the possibility of discovering new causative variants. An aim of this thesis was to find clinically relevant genotype-phenotype correlations, and indeed some were found. A problem with this specific aim is the number of genes associated to DCM emphasizing the need of significantly larger sample sizes, international studies, and sharing of data, to identify more genotype-phenotype correlations.

7 CONCLUSIONS

Dilated cardiomyopathy is a substantial cause of morbidity and mortality. The possible genetic etiology is still often not considered, although, as study IV shows, using currently available genetic diagnostics up to a half of patients with familial disease and one in four of those without a family history of DCM receive a genetic diagnosis. Study IV also re-confirmed the role of truncating titin mutations as the most important genetic defect causing DCM. Additionally study IV illustrated the usefulness of targeted panels when studying the genetic etiology of DCM. With the use of large panels rare variants are found in practically all individuals. The only logical approach to variant classification in this setting is the use of strict criteria for pathogenicity. The logic behind the classification criteria has not changed from the times when single genes were sequenced individually. Co-segregation of the variant in question with disease remains the single most important evidence of pathogenicity requiring thorough clinical phenotyping of genetic defects. Thus, the phenotyping of cardiomyopathy-causing genetic defects is as important as ever, although sometimes this appears to be forgotten due to the excitement over technological advancements made in the genotyping front.

A consequence for both strict criteria used in variant classification and the increased testing itself is a rising number of DCM patients known to carry variants of unknown significance. An important clinical concept concerning variants of unknown significance is that they should be considered temporary. With further information gathered from family members, other laboratories etc. a VUS should, in time, become a known pathological or benign variant. A significant recent improvement to variant classification is the publication of population reference databases, the largest of which is gnomAD. Many variants previously considered disease-causing have been proven common variants after the population databases became available, increasing the accuracy of variant classification. However, many populations are still underrepresented in population databases, which remains an obvious limitation to their usefulness.

This thesis also broadened the known clinical spectrum of cardiolaminopathy, cardiac disease caused by *LMNA* mutations. In Study I a severe right predominant phenotype with overlapping characteristics of DCM and ARVC was presented in one family. Study II described the spiroergometry results of 26 *LMNA* mutation carriers. The mutation carriers with a cardiolaminopathy phenotype showed results typical to heart failure patients. The yet healthy mutation carriers, on the other hand, had an overall performance similar to the healthy controls, but also showed milder signs of inefficient exercise ventilation suggesting that increased ventilation during exercise might be an early sign of symptomatic cardiolaminopathy. Study III described clinical follow-up data from 27 *LMNA* mutation carriers. *LMNA* mutation carriers presented with atrial fibrillation at a younger age than DCM patients, however no difference in event-free survival concerning major event was seen between *LMNA* mutation carriers and DCM controls. Male mutation carriers presented with clinical signs of cardiolaminopathy significantly earlier than female

mutation carriers. In Study III a new ECG concept, ECG septal remodeling, typical to *LMNA* mutation carriers was also introduced. The high prevalence of ECG septal remodeling in *LMNA* mutation carriers suggests that localized septal fibrosis is common in this group.

Overall this thesis added to the knowledge of genetic DCM, and gave insight into the clinical features of cardiolaminopathty.

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