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The clinical outcome of *LMNA* missense mutations appears to be associated with the amount of mutated protein in the nuclear envelope

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Abstract

Aims: Lamin A/C mutations are generally believed to be associated with a severe prognosis. It was the aim to investigate the disease expression in three affected families carrying different *LMNA* missense mutations. Furthermore, the potential molecular disease mechanisms of the mutations were investigated in fibroblasts obtained from mutation carriers.

Methods and Results: A *LMNA*-p.Arg216Cys missense mutation was identified in a large family with 36 mutation carriers. The disease expression was unusual with a late onset and a favourable prognosis. Two smaller families with a severe disease expression were shown to carry a *LMNA*-p.Arg471Cys and *LMNA*-p.Arg471His mutation, respectively. *LMNA* gene and protein expression was investigated in eight different mutation carriers by Western blotting, immunohistochemistry, quantitative reverse transcriptase PCR, and protein mass spectrometry. The results showed that all mutation carriers incorporated mutated lamin protein into the nuclear envelope. Interestingly, the ratio of mutated- to wild type- protein was only 30:70 in *LMNA*-p.Arg216Cys carriers with a favourable prognosis while *LMNA*-p.Arg471Cys and *LMNA*-p.Arg471His carriers with a more severe outcome expressed significantly more of the mutated protein by a ratio of 50:50.

Conclusion: The clinical findings indicated that some *LMNA* mutations may be associated with a favourable prognosis and a low risk of sudden death. Protein expression studies suggested that a severe outcome was associated with the expression of high amounts of mutated protein. These findings may prove to be helpful in counselling and risk assessment of *LMNA* families.

Keywords: Dilated cardiomyopathy; *LMNA*; lamin; heart failure; sudden death, cardiac conduction disease; dominant-negative effect

Introduction

Mutations in the gene for lamin A and lamin C (*LMNA*) are associated with the development of cardiac conduction disease (CCD), atrial and ventricular arrhythmias, muscular dystrophy, and dilated cardiomyopathy (DCM).[1, 2] Recently, a European multicentre study of 269 *LMNA*-mutation carriers suggested that the presence of two or more risk-factors including an episode of non-sustained ventricular tachycardia (VT), a left ventricular ejection fraction (LVEF) < 45%, male sex, and non-missense mutations were associated with a high risk of malignant ventricular arrhythmias (VA) and sudden cardiac death (SCD). [3, 4] These observations were recently confirmed in a study of 122 *LMNA*-mutation carriers in the US.[5] However, novel data suggest that some *LMNA* mutations may be associated with a more benign outcome.[6]

Lamin A and lamin C proteins constitute the main structural proteins of the inner nuclear envelope. Both isoforms are transcribed from the *LMNA* gene through alternative splicing of messenger RNA (mRNA).[7] They are expressed in almost all differentiated tissues including the myocardium and fibroblasts. In addition to their structural and supportive function of the nucleus, these proteins are believed to influence regulation of gene expression through an interaction with transcription factors, DNA, and chromatin.[7] It has been suggested that haploinsuffiency is the disease mechanism in patients carrying truncating *LMNA* mutations, while *LMNA* missense mutations have been proposed to act through a dominant negative pathway.[8-10] A recent study of a missense mutation suggested that mutant lamin protein may accumulate and form intra-nuclear aggregates and thereby exhibit a dominant negative effect.[11]

In the current study, we report the disease expression associated with a *LMNA*-p.Arg216Cys variant in a large family with 36 mutation carriers. The phenotype was remarkable since most of

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the affected mutation carriers had a late onset of disease manifestations and a favourable prognosis. Furthermore, we describe two additional *LMNA* families with a total of 17 mutation carriers, which were characterised by an early onset and a severe disease expression of DCM. To elucidate if the highly variable disease expression observed in the three families was associated with different molecular disease mechanisms we performed gene and protein expression studies in fibroblasts obtained from *LMNA* mutation carriers.

Methods

Subjects and clinical investigations

The study included three unrelated index patients who carried a disease associated *LMNA* mutation and their relatives who accepted an offer of clinical investigation including 12-lead ECG-recording, two-dimensional Doppler echocardiography, and 48-hour Holter-monitoring. DCM in index-patients was defined as LV end-diastolic volumes or -diameters >2 standard deviations (SD) from normal and LVEF < 0.45.[12] VA were defined as sustained VT lasting >30 s, non-sustained VT as ≥3 ventricular ectopics with a frequency of > 120 beats/minute, or as ventricular fibrillation. Relatives were diagnosed in accordance with recent recommendations.[13] Coronary artery disease was excluded by coronary angiography in patients older than 40 years. The study was carried out in accordance with the Declaration of Helsinki. The local ethics committee approved the study and informed consent was obtained from all participants (protocol no. VEK S-20140073).

Genetic diagnosis

Index-patients underwent genetic testing in 84 cardiac specific genes as previously reported and relatives at risk were offered predictive testing for the specific variant identified in the index patient.[14] Genetic investigations of deceased individuals were made with DNA extracted from formalin-fixed paraffin embedded tissue.

Cell cultures

Fibroblast cell lines were established from skin biopsies of eight individuals with *LMNA* mutations and three healthy controls as previously described.[9]

Quantitative reverse transcriptase PCR (qRT-PCR)

cDNA was synthesized from RNA extracted from cultured fibroblasts as previously described.[9] For qRT-PCR analysis, custom designed Taqman gene expression assays covering both *LMNA(Hs00153462_m1)* and *TUBA4A* (Hs01081795_g1) transcripts were used. Step One Plus (Life technologies) was used to analyse the samples as triple determinations. *LMNA* gene expression levels were normalised to α-tubulin levels.

Western blotting (WB)

The cytoskeletal protein fractions were extracted from patient and control fibroblasts visualised by WB as previously described.[9] Primary antibodies used were: anti-lamin A/C (N-terminal epitope, Sigma-Aldrich, #HPA006660) and anti-α-tubulin (Sigma-Aldrich, #T6074), followed by incubation with secondary antibodies (goat-anti-mouse- or goat-anti-rabbit-HRP, DAKO) in one hour. ECL-Plus (Thermo Scientific) was used for protein detection. ImageQuant LAS 4000 (GE Healthcare) was used for visualisation.

Targeted mass spectrometry analysis of lamin proteins

Targeted mass spectrometry analysis of lamin proteins was performed as previously described.[15] Twenty-five µg of the extracted proteins from the isolated cytoskeletal fraction from each sample was separated by SDS-PAGE. The gel pieces containing the proteins of interest, lamin A and lamin C, migrating between 100 and 40 kDa in-gel were digested by trypsin. A mix of heavy labelled peptides covering the mutation sites and other parts of lamin A and lamin C (Supplementary Table S2) was added to each peptide sample, followed by selected reaction monitoring (SRM) using nano-liquid chromatography coupled with tandem mass spectrometry (TSQ Vantage, Thermo Scientific, see Supplement S2 for details).[15] Skyline software was used to assure correct peptide identifications and to calculate the ratios between the light (endogenous) and the heavy (internal standard) peptides.[16] The peptide mass data were validated according to retention time and fragmentation pattern. For quantitation, the ratio of the area under the curve of endogenous peptide to heavy labelled peptide was calculated.

Histology and Immunohistochemistry of cardiac tissue and fibroblasts

Myocardial tissue was available from one carrier for each *LMNA* mutation. Samples from non-*LMNA* DCM patients served as controls (n=4). Four µm thick formalin-fixed paraffin-embedded sections of myocardial tissue were plated on glass coverslips and were stained with either hematoxylin and eosin, or with mouse anti-lamin A/C antibody (#4C11, Cell Signalling technology) followed by incubation with anti-IgG mouse Alexa-Fluor-488-conjugated antibody (Invitrogen). The slides were imaged using a confocal laser scanning microscope (Leica TCS SP8). Immunocytochemistry staining of cultured fibroblasts was performed with similar antibodies as previously described.[9]

Statistics

Normally distributed data are expressed as means ± SD or otherwise as medians with interquartile ranges. Statistical analyses were performed using Student's t-test with significance level of 0.05.

Results

Clinical investigations and genetic diagnosis

For a complete overview of clinical features in mutation carriers, see Table S1 in the Data Supplement.

Family A

The proband of family A, (IV-9), presented at the age of 59 with sustained VT (Fig. 2A, Table S1). Following cardioversion ECG monitoring showed sinus node dysfunction and advanced second degree AVB. Echocardiography showed a dilated LV with a LVEF of 40%. An ICD was implanted and he has subsequently received appropriate ICD-therapy multiple times due to sustained VT. Genetic investigations identified a novel *LMNA*-p.Arg216Cys/c.464C>T variant which was considered to be disease associated since it segregated with the condition in the family and was absent in more than 60.000 ExAC exomes.[17]

Clinical cascade screening identified 12 affected individuals (mean age 68 years, range: 59-87) who all carried the mutation (Fig. 1+2A and Table 1+S1). All had CCD with advanced AVB of which six had been diagnosed before this investigation. Family investigations identified five patients with total AVB of which one, (III-8), declined the offer of pacemaker implantation two years before he died at the age of 89 years. All but one, (IV-6), had chronic or paroxysmal SVA while eight fulfilled diagnostic criteria of familial DCM with an average LVEF of 40%. Only one relative, (IV-4), received appropriate ICD-therapy during follow-up, while another, (IV-27), received a cardiac transplant at the age of 50 due to end-stage heart failure. This patient

however had extensive cardiac sarcoidosis confirmed by histological examination of the explanted heart, which was believed to be the main cause of her condition.

A total of 17 healthy mutation carriers (mean age 49 years, range: 23-68, Table S1) were identified. Five of these individuals developed advanced AVB with preserved LV function and no significant VA during an average follow-up period of 5.2 years at a mean age of 60 years (range: 55-69). One younger mutation carrier, (V-9), had 10% monomorphic premature ventricular contractions (PVCs) originating from the right ventricular outflow tract and short episodes of non-sustained VT of similar morphology on Holter monitoring. She had mild LV dysfunction (LVEF 0.50) without structural abnormalities or late gadolinium enhancement on cardiac magnetic resonance imaging. The patient underwent successful ablation for the arrhythmic substrate and no VA have been detected during five years of follow-up. In addition, her LV function normalised without medication. It is unclear if the disease expression of this patient was related to the genotype or if she represented a phenocopy with a different cause of her PVCs.

A total of six deceased obligate mutation carriers were identified with little clinical information available, (Fig. 2A, Table S1). One of these individuals (II-2) died of cancer at the age of 54, while the remaining individuals died at an average age of 77 years, which indicated a near normal life expectancy despite their genotype.

In summary, during this investigation 20 of 36 (46%) mutation carriers were affected or developed disease manifestations that required device implantation at an average age of 61.7 years. Although all affected individuals had or developed advanced AVB requiring pacemaker

implantation only two patients (5%) including the proband had significant VA. Only nine of 36 (25%) mutation carriers fulfilled diagnostic criteria of DCM.

Family B

The proband, (III-3), was diagnosed with DCM (LVEF 0.35) at the age of 54 and has remained stable on heart failure therapy for the past 6 years (Fig. 1+2B, Table 1+S1). Her son, (IV-6), died suddenly at the age of 18 while at rest and autopsy revealed an enlarged heart consistent with a diagnosis of DCM. The brother, (IV-5), of the deceased was diagnosed with DCM (LVEF 0.40) at the age of 25 and was treated with a prophylactic ICD from which he has received appropriate anti-tachycardia pacing once during twelve years of follow-up. Family investigations identified three additional surviving relatives with DCM of which one received a cardiac transplant at the age of 59, (III-5). Four mutation carriers had died prior to this investigation from CHF at an average age of 61 years.

Genetic investigations identified a novel *LMNA*-p.Arg471His/c.1412G>A mutation. The mutation co-segregated with the disease in the family. Genetic cascade screening identified three healthy gene carriers with an average age of 38 years (one individual lost to follow-up). The p.Arg471His mutation was considered pathogenic since it co-segregated with the DCM-phenotype in this family, was absent in the ExAC control exomes[17], and has been reported previously in a DCM-patient.[18]

Family C

The proband was diagnosed at the age of 41 with atrial fibrillation (Fig. 1+2C, Table 1+S1). He later developed DCM with a LVEF of 45% and received a dual-chamber pacemaker at the age of 45 years due to total AVB. Four years later he died suddenly at rest. Post-mortem examination of the heart was consistent with DCM. His sister developed DCM with an LVEF of 25% at the age of 38 years and has had episodes of nsVT and sVT.

Genetic investigations identified a *LMNA*-p.Arg471Cys/ c.1411C>T missense mutation carried by both affected individuals. The same mutation has been identified in three additional unrelated probands with DCM at our institution (unpublished data). The p.Arg471Cys variant was considered to be a likely pathogenic variant since it was located in the same codon as the recognised disease causing mutation identified in family B, (p.Arg471His), and the frequency among ExAC control alleles was very low (3/117.104).[17]

In summary, the disease expression in family B and C appeared to be severe since 65% of mutation carriers had DCM of which seven (47%) had died from either CHF or SCD. Surprisingly, no one had CCD. The average age of onset of disease was 48 years (Table 1).

LMNA gene and lamin A and C protein expression in mutant fibroblast cell lines

LMNA transcript and protein levels in fibroblast of mutation carriers were measured by *qRT-PCR* and WB and were not significantly different from control fibroblasts (Fig. 3A+B). However, the specific expression of lamin C protein was significantly increased in mutation carriers (Fig. 3C). This finding was supported by SRM MS analysis which showed that the expression of a lamin A-specific peptide relative to expression of common lamin A and lamin C peptides from p.Arg471His and p.Arg216Cys fibroblasts was lower compared to peptides from

control fibroblasts (Fig. 3D). In accordance with the result by WB this finding indicated that expression of the lamin C isoform was relatively increased compared to lamin A expression in mutation carriers.

Lamin A and C proteins in the cytoskeletal fraction of cells

Mutant lamin A/C peptides were identified and discriminated from wild type (WT) peptides by use of heavy-isotope labelled mutant peptides in the SRM MS analysis. In p.Arg216Cys fibroblasts SRM analysis was able to detect the endogenous mutant peptide NIYSEEL**C**ETK (red in fig. 4A, variation in bold) with high intensities (Fig. 4A). Similarly, the mutant peptides **H**QNGDDPLLTYR and **C**QNGDDPLLTYR were detectable in p.Arg471His and p.Arg471Cys fibroblast cells, respectively (Fig. 4B+C). As expected, the mutant peptides were not present in controls.

Quantification of the WT peptide corresponding to the mutated p.Arg216Cys peptide demonstrated that the WT peptide accounted for approximately 70% of total lamin A and C protein in p.Arg216Cys fibroblasts (Fig. 4D). In contrast, only 50 % of the lamin protein was derived from the WT allele in the *LMNA*-p.Arg471His- and *LMNA*-p.Arg471Cys carriers indicating that these mutant lamin proteins were incorporated in the cytoskeletal fraction with similar efficiency as the WT protein (Fig. 4D).

Immunohistology

Histological examination did not show any obvious differences between *LMNA* and non-*LMNA* DCM patients (Suppl. Figure S1). Antibody staining with an anti-*LMNA* antibody

demonstrated nuclear staining in all *LMNA*-patients without cytosolic aggregates which indicated nuclear incorporation of mutant lamin protein. Additional immuno-staining of p.Arg471His fibroblasts showed a normal nuclear morphology without intracellular aggregates supporting the expression and incorporation of mutated protein into the cytoskeleton (Sup. Figure S2).

Discussion

Clinical and genetic investigations

Several clinical investigations have shown that *LMNA*-mutations in cardiac conditions are associated with a severe prognosis and that the presence of specific risk factors are associated with an increased risk of VA and sudden death.[3-5] The disease expression in family B and C was in accordance with previous findings and showed that more than half of the patients died from CHF or VA. In contrast, the clinical investigations in family A, which included 36 carriers of the p.Arg261Cys mutation, showed a late onset of disease manifestations and a low frequency of VA. A substantial part of the mutation carriers had a near normal life span of which one patient even survived until the age of 89 years without a pacemaker, despite the presence of a complete AVB. Overall, the disease expression was associated with an apparently mild phenotype and few adverse disease complications which suggested that *LMNA* mutations may also be associated with a favourable prognosis. These findings were consistent with the results of recent Dutch study of a *LMNA*-p.Arg331Gln mutation in 57 mutation carriers, which was also associated with a lower risk of VA, end-stage CHF and SCD than previously anticipated.[6]

So far clinical and genetic data of *LMNA* families have largely been provided by investigations of highly selected families at tertiary referral centres, which may introduce selection bias towards severely affected families.[1, 2, 4, 5, 19, 20] The results of the current study underscore the importance of investigating the entire family and assess the phenotype of all individuals at risk of having inherited the condition to provide a firm basis for individualised risk assessment.

Expression studies

For the first time to our knowledge protein MS was used to perform targeted analyses of wild type and mutant lamin A and C protein expression in heterozygous mutation carriers. The results showed that the mutated peptides were present in the cytoskeletal protein fraction and thereby likely to be incorporated into the nuclear lamin polymer. Furthermore, SRM revealed that the ratio of WT and mutated protein was 50:50 in fibroblasts obtained from mutation carriers of p.Arg471His and p.Arg471Cys in whom the disease expression was associated with a severe outcome. Interestingly, the proportion of mutant protein was only 30% in mutation carriers of p.Arg216Cys which may explain the apparently milder disease expression, although further conformational studies of additional families need to be performed. However, the results of the current study indicated that the proportion of incorporated mutant lamin protein may serve as a prognostic marker of disease which has the potential to improve current algorithms for risk assessment of individual *LMNA* families.

The mutations investigated were all located in the common region of lamin A/C and thereby expected to affect both isoforms equally. However, the experimental results indicated that the protein expression levels of lamin A were unaffected in mutant cells. Surprisingly, the expression of lamin C protein was significantly increased in mutation carriers, leading to a decreased lamin A to lamin C ratio in *LMNA* carriers. This finding was also observed in our previous study of fibroblasts from carriers of a truncating *LMNA*-p.Arg321X mutation.[9] Similarly a decreased lamin A to lamin C ratio at mRNA and protein level has been observed in patients with progeria and Emery-Dreifuss muscular dystrophy.[21, 22] The impact of these findings remain unclear and needs to be investigated further.

Immunohistochemistry of cardiac tissues and p.Arg471His fibroblasts did not show intracellular aggregates of mutant lamin protein (Sup. Fig. 1 and 2). Therefore, it is likely that the combination of a skewed lamin A to C ratio and the incorporation of mutated protein may lead to destabilisation of the nuclear envelope and impair the mechanical support of the nucleus. The results support the hypothesis that *LMNA* missense mutations act by a dominant-negative disease mechanism.[18, 23-25]

Limitations

Skin fibroblasts are easily accessible from mutation carriers compared to cardiac tissue. In addition, these fibroblasts are likely to share the same genetic background as cardiomyocytes of the patients, which may minimise potential bias compared to *in vitro* studies of mutations expressed in artificial cell systems unrelated to the patient. However, studies on protein functions in fibroblasts need confirmation in cardiac tissue in which the electromechanical coupling is a complex interplay between myocytes, fibroblasts, and extracellular matrix.

Conclusion

The clinical investigations of a large family with a novel pathogenic *LMNA* mutation showed that the disease expression of *LMNA* mutations may also be associated with a favourable prognosis in contrast to the findings in most previous reports. Furthermore, the results suggested that the clinical outcome in *LMNA* mutation carriers may be associated with the amount of mutant peptide incorporated into the nuclear envelope.

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Conflicts of interests

None

References

1. Fatkin D, MacRae C, Sasaki T, Wolff MR, Porcu M, Frenneaux M, Atherton J, Vidaillet HJ, Jr., Spudich S, De Girolami U, Seidman JG, Seidman C, Muntoni F, Muehle G, Johnson W and McDonough B. Missense mutations in the rod domain of the lamin A/C gene as causes of dilated cardiomyopathy and conduction-system disease. *N Engl J Med*. 1999;341:1715-24.
2. Bonne G, Barletta MRD, Varnous S, Becane H-M, Hammouda E-H, Merlini L, Muntoni F, Greenberg CR, Gary F, Urtizberea J-A, Duboc D, Fardeau M, Toniolo D and Schwartz K. Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. *Nat Genet*. 1999;21:285-288.
3. Meune C, Van Berlo JH, Anselme F, Bonne G, Pinto YM and Duboc D. Primary Prevention of Sudden Death in Patients with Lamin A/C Gene Mutations. *New England Journal of Medicine*. 2006;354:209-210.
4. van Rijsingen IAW, Arbustini E, Elliott PM, Mogensen J, Hermans-van Ast JF, van der Kooi AJ, van Tintelen JP, van den Berg MP, Pilotto A, Pasotti M, Jenkins S, Rowland C, Aslam U, Wilde AAM, Perrot A, Pankuweit S, Zwinderman AH, Charron P and Pinto YM. Risk Factors for Malignant Ventricular Arrhythmias in Lamin A/C Mutation Carriers: A European Cohort Study. *Journal of the American College of Cardiology*. 2012;59:493-500.
5. Kumar S, Baldinger SH, Gandjbakhch E, Maury P, Sellal JM, Androulakis AF, Waintraub X, Charron P, Rollin A, Richard P, Stevenson WG, Macintyre CJ, Ho CY, Thompson T, Vohra JK, Kalman JM, Zeppenfeld K, Sacher F, Tedrow UB and Lakdawala NK. Long-Term Arrhythmic and Nonarrhythmic Outcomes of Lamin A/C Mutation Carriers. *J Am Coll Cardiol*. 2016;68:2299-2307.
6. Hoorntje ET, Bollen IA, Barge-Schaapveld DQ, van Tienen FH, Te Meerman GJ, Jansweijer JA, van Essen AJ, Volders PG, Constantinescu AA, van den Akker PC, van Spaendonck-Zwarts KY, Oldenburg RA, Marcelis CL, van der Smagt JJ, Hennekam EA, Vink A, Bootsma M, Aten E, Wilde AA, van den Wijngaard A, Broers JL, Jongbloed JD, van der Velden J, van den Berg MP and van Tintelen JP. Lamin A/C-Related Cardiac Disease: Late Onset With a Variable and Mild Phenotype in a Large Cohort of Patients With the Lamin A/C p.(Arg331Gln) Founder Mutation. *Circ Cardiovasc Genet*. 2017;10.
7. Al-Saaidi R and Bross P. Do lamin A and lamin C have unique roles? *Chromosoma*. 2015;124:1-12.
8. Narula N, Favalli V, Tarantino P, Grasso M, Pilotto A, Bellazzi R, Serio A, Gambarin FI, Charron P, Meder B, Pinto Y, Elliott PM, Mogensen J, Bolognesi M, Bollati M and Arbustini E. Quantitative expression of the mutated lamin A/C gene in patients with cardiolaminopathy. *J Am Coll Cardiol*. 2012;60:1916-20.
9. Al-Saaidi R, Rasmussen TB, Palmfeldt J, Nissen PH, Beqqali A, Hansen J, Pinto YM, Boesen T, Mogensen J and Bross P. The LMNA mutation p.Arg321Ter associated with dilated cardiomyopathy leads to reduced expression and a skewed ratio of lamin A and lamin C proteins. *Exp Cell Res*. 2013;319:3010-9.
10. Arimura T, Helbling-Leclerc A, Massart C, Varnous S, Niel F, Lacene E, Fromes Y, Toussaint M, Mura AM, Keller DI, Amthor H, Isnard R, Malissen M, Schwartz K and Bonne G. Mouse model carrying H222P-Lmna mutation develops muscular dystrophy and dilated cardiomyopathy similar to human striated muscle laminopathies. *Hum Mol Genet*. 2005;14:155-69.
11. West G, Gullmets J, Virtanen L, Li SP, Keinanen A, Shimi T, Mauermann M, Helio T, Kaartinen M, Ollila L, Kuusisto J, Eriksson JE, Goldman RD, Herrmann H and Taimen P. Deleterious assembly of the lamin A/C mutant p.S143P causes ER stress in familial dilated cardiomyopathy. *J Cell Sci*. 2016;129:2732-43.

12. Mestroni L, Maisch B, McKenna WJ, Schwartz K, Charron P, Rocco C, Tesson F, Richter A, Wilke A and Komajda M. Guidelines for the study of familial dilated cardiomyopathies. Collaborative Research Group of the European Human and Capital Mobility Project on Familial Dilated Cardiomyopathy. *Eur Heart J*. 1999;20:93-102.
13. Pinto YM, Elliott PM, Arbustini E, Adler Y, Anastasakis A, Bohm M, Duboc D, Gimeno J, de Groote P, Imazio M, Heymans S, Klingel K, Komajda M, Limongelli G, Linhart A, Mogensen J, Moon J, Pieper PG, Seferovic PM, Schueler S, Zamorano JL, Caforio AL and Charron P. Proposal for a revised definition of dilated cardiomyopathy, hypokinetic non-dilated cardiomyopathy, and its implications for clinical practice: a position statement of the ESC working group on myocardial and pericardial diseases. *Eur Heart J*. 2016;37:1850-8.
14. Haas J, Frese KS, Peil B, Kloos W, Keller A, Nietsch R, Feng Z, Muller S, Kayvanpour E, Vogel B, Sedaghat-Hamedani F, Lim WK, Zhao X, Fradkin D, Kohler D, Fischer S, Franke J, Marquart S, Barb I, Li DT, Amr A, Ehlermann P, Mereles D, Weis T, Hassel S, Kremer A, King V, Wirsz E, Isnard R, Komajda M, Serio A, Grasso M, Syrris P, Wicks E, Plagnol V, Lopes L, Gadgaard T, Eiskjaer H, Jorgensen M, Garcia-Giustiniani D, Ortiz-Genga M, Crespo-Leiro MG, Deprez RH, Christiaans I, van Rijsingen IA, Wilde AA, Waldenstrom A, Bolognesi M, Bellazzi R, Morner S, Bermejo JL, Monserrat L, Villard E, Mogensen J, Pinto YM, Charron P, Elliott P, Arbustini E, Katus HA and Meder B. Atlas of the clinical genetics of human dilated cardiomyopathy. *Eur Heart J*. 2014;36:1123-35.
15. Birkler RI, Nochi Z, Gregersen N and Palmfeldt J. Selected reaction monitoring mass spectrometry for relative quantification of proteins involved in cellular life and death processes. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences*. 2016;1035:49-56.
16. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC and MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 2010;26:966-8.
17. Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG and Exome Aggregation C. Analysis of protein-coding genetic variation in 60,706 humans. *Nature*. 2016;536:285-91.
18. Cowan J, Li D, Gonzalez-Quintana J, Morales A and Hershberger RE. Morphological analysis of 13 LMNA variants identified in a cohort of 324 unrelated patients with idiopathic or familial dilated cardiomyopathy. *Circ Cardiovasc Genet*. 2010;3:6-14.
19. Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NS, Abeysinghe S, Krawczak M and Cooper DN. Human Gene Mutation Database (HGMD): 2003 update. *Hum Mutat*. 2003;21:577-81.
20. van Rijsingen IA, Arbustini E, Elliott PM, Mogensen J, Hermans-van Ast JF, van der Kooi AJ, van Tintelen JP, van den Berg MP, Pilotto A, Pasotti M, Jenkins S, Rowland C, Aslam U, Wilde AA, Perrot A, Pankuweit S, Zwinderman AH, Charron P and Pinto YM. Risk factors for malignant ventricular arrhythmias in lamin a/c mutation carriers a European cohort study. *Journal of the American College of Cardiology*. 2012;59:493-500.
21. Reunert J, Wentzell R, Walter M, Jakubiczka S, Zenker M, Brune T, Rust S and Marquardt T. Neonatal progeria: increased ratio of progerin to lamin A leads to progeria of the newborn. *Eur J Hum Genet*. 2012;20:933-7.

22. Niebroj-Dobosz I, Fidzianska A and Hausmanowa-Petrusewicz I. Expression of emerin and lamins in muscle of patients with different forms of Emery-Dreifuss muscular dystrophy. *Acta Myol*. 2003;22:52-7.
23. Lai CC, Yeh YH, Hsieh WP, Kuo CT, Wang WC, Chu CH, Hung CL, Cheng CY, Tsai HY, Lee JL, Tang CY and Hsu LA. Whole-exome sequencing to identify a novel LMNA gene mutation associated with inherited cardiac conduction disease. *PLoS One*. 2013;8:e83322.
24. Arbustini E, Pilotto A, Repetto A, Grasso M, Negri A, Diegoli M, Campana C, Scelsi L, Baldini E, Gavazzi A and Tavazzi L. Autosomal dominant dilated cardiomyopathy with atrioventricular block: a lamin A/C defect-related disease. *Journal of the American College of Cardiology*. 2002;39:981-990.
25. Sylvius N, Bilinska ZT, Veinot JP, Fidzianska A, Bolongo PM, Poon S, McKeown P, Davies RA, Chan KL, Tang AS, Dyack S, Grzybowski J, Ruzyllo W, McBride H and Tesson F. In vivo and in vitro examination of the functional significances of novel lamin gene mutations in heart failure patients. *J Med Genet*. 2005;42:639-47.

Figure legends

Figure 1: Clinical endpoints according to age in *LMNA* mutation carriers. Abbreviations; HTX, heart transplantation; SUD, sudden unexpected death; ICD, implantable cardioverter defibrillator; PM, pacemaker; VT, ventricular tachycardia; SVA, supraventricular arrhythmias; DCM, dilated cardiomyopathy; AVB/LBBB, atrioventricular block or left bundle branch block.

Figure 2: Pedigrees of *LMNA*-families. Square, male; circle, female; filled symbols, affected; grey symbols, possibly affected; crossed symbols, deceased; +, mutation carrier; -, non-mutation carrier; ?, unknown genetic status.

Figure 3: (A) qRT-PCR analysis of *LMNA* transcript levels relative to α–tubulin (TUBA4A) in healthy controls and in *LMNA*-p.Arg471His and *LMNA*-p.Arg216Cys fibroblasts. Error bars denote standard deviation. (B) Western blot analysis. Representative Western blot detecting lamin A and lamin C proteins from the cytoskeletal fraction of patient and control fibroblasts. α-tubulin was used as a loading control. (C) Quantitation of band intensities, from Figure 3A, of lamin A and lamin C relative to α-tubulin. Columns represent the means of signal intensities of lamin A and lamin C relative to α-tubulin. Error bars denote standard deviation; *p <0.05. (D) Lamin A and lamin C ratio quantitation by SRM. Schematic overview of lamin A and lamin C (lamin A/C) common peptides 1-3 (black) and lamin A specific peptide 4 (grey) used for the quantitation of lamin A to lamin C ratio. The graphs represent relative quantitation of lamin A to lamin A/C peptides by SRM-nLC-MS/MS in control and patient fibroblasts. The quantitative robustness was improved by utilization of isotopically labelled peptide standards, for each

quantified peptide, and by normalisation to the reference protein α-tubulin (Supplement S1). Error bars denote standard deviation.

Figure 4: SRM mass spectrometry detection of variation specific peptides of lamin A and lamin C in the cytoskeletal fraction of patient fibroblasts. Endogenous peptide signal depicted in red. The corresponding heavy labelled standard peptides (blue) were detectable in both control and patient samples (A) Representative chromatogram of the mutant peptide (red), NIYSEEL**C**ETK. The peptide was only detected in the p.Arg216Cys patient. (B) Representative chromatogram of the endogenous mutant peptide, **H**QNGDDPLLTYR detected in patient carrying the p.Arg471His mutation and not in control. A small peak representing signal from noise, at a mismatched time point, was detected in control. (C) Chromatogram representing the endogenous mutant peptide (red), **C**QNGDDPLLTYR, detected in the patient with the p. Arg471Cys mutation. (D) Calculations of the relative amounts of wild type lamin A and lamin C in the cytoskeletal fractions of patient and control fibroblasts. The wild type peptides covering the respective mutation sites (WT) only present in lamin A and lamin C from the wild type allele and three reference peptides present in both wild type and mutant lamin A and C sequences (1, 2, and 3) were detected, quantitated by SRM, and used for the calculations of the fractions of wildtype and mutant protein (for details see Supplement S1). Error bars denote standard deviations.

Table 1

Clinical event	*LMNA*-p.Arg216Cys (n=36)		*LMNA*-p.Arg471His/Cys (n=17)	
	n (%)	Mean age (range)	n (%)	Mean age (range)
Unaffected at last follow up	11 (31)	50 (27-60)	4 (24)	32 (19-40)
Conduction disease	17 (47)	66 (50-89)	4 (24)	49 (36-64)
Dilated cardiomyopathy	12 (33)	63 (34-89)	11 (65)	48 (18-64)
Supraventricular arrhythmia	14 (39)	68 (53-89)	3 (18)	54 (40-64)
Ventricular tachycardia	7 (19)	58 (32-69)	3 (18)	40 (25-59)
Sudden unexplained death	2 (6)	65 (61-69)	4 (24)	46 (18-70)
Pacemaker	17 (47)	62 (34-89)	2 (12)	48 (40-56)
Implantable cardioverter defibrillator	11 (31)	59 (34-73)	5 (29)	46 (26-59)
Heart transplantation	1 (3)	58	1 (6)	59
All-cause death	11 (33)	73 (54-91)	8 (47)	53 (18-70)

Legend: Clinical events according to age in *LMNA* mutation carriers. Clinical data from index patients and three first-degree relatives have been included in a previous paper.[4]

Supplementary online material

Supplementary Figure S1: Nuclear morphology of cardiac tissues of patients and control. Immunofluorescence microscopy analysis of control and patient cardiac tissue. The immunofluorescent image showing lamin A/C (green) and alfa- actinin2 (red) detected with mouse anti-lamin A/C antibody and α-actinin-2, respectively. Myocardial tissues were stained with hematoxylin eosin.

Supplementary Figure S2: Immunofluorescence microscopy analysis of control and patient fibroblasts. The immunofluorescent image showing lamin A/C (green) and cell nuclei (blue) detected with mouse anti-lamin A/C antibody and DAPI, respectively.

Supplementary Table S1

ID/ sex	Presentation	Age*	CCD[2]	DCM[3]	SVA[4]	VA[5]	Device[6]	Comment
Family A: *LMNA*-p.Arg216Cys								
II-2/M		-/54						Died of cancer
II-5/F		-/84						Unknown cause of death
III-1/F		-/60						Unknown cause of death
III-3/F	CHF	89/91	AVB+LBBB(89)	57/45%(89)	AFLU(89)		VVI-PM(89)	Death cause unknown
III-4/F		-/83						Died of cancer
III-6/F		-/69						Sudden death
III-7/F	Syncope	62/74	AVB+LBBB(62)		AFIB(73)		VVI-PM(62)	Stroke(74)
III-8/M	CHF	87/89	AVB+LBBB(87)	65/50%(87)	AFLU(87)			CHF(89), declined PM
III-11/F		-/54						Died of multiple sclerosis
IV-1/M	Bradycardia	59/75	AVB(59)	54/45%(67)	AFIB(59)	nsVT(69)	DDD-PM(59)	Died of cancer, IHD
IV-3/M	Postoperative AVB	67/70	AVB(67)	60/40%(67)	AFIB(67)	20% PVCs, nsVT(67)	DDD-PM(67), CRT-D(67)	Mitral valve surgery RFA-PVCs twice
IV-4/M	Svt	61/65	AVB(61)	50/49%(61)	AFIB(61)	13% PVCs, sVT(61)	DDD-ICD(61)	RFA-VT trice. CMR with septal and RV LGE
IV-6/F	FS	73/78	AVB(73)				DDD-ICD(73)	
IV-9/M	sVT	59/65	AVB(59)	57/40%(59)		11%PVCs, multiple sVT(65)	DDD-ICD(59), CRT-D(65)	RFA-VT twice
IV-10/F	FS	57/64	PRP(57), AVB+LBBB(59)		AFIB(61)		DDD-ICD(59)	Mild limb-girdle muscular dystrophy
IV-12/F	FS	73/76	PRP+AVB(73)		AFLU(73)		DDD-PM(73)	RFA-AFLU(73)
IV-14/F	FS	70/75	AVB(70)		AFIB(70)		DDD-PM(70)	
IV-15/M	FS	69/73	AVB(69)		AFLU(69)		DDD-ICD(69)	RFA-AFLU(69)
IV-16/M	AVB	61/72	AVB(61) LBBB(69)	73/10%(65) 51/50%(70)	AFLU(65) AFIB(66)		DDD-PM(61) CRT-D(65)	IHD, PCI, RFA-AFLA(65)
IV-24/F	CHF	59/66	LBBB(59) AVB(64)	68/30%(59)	AFIB(59)		DDD-PM(59) CRT-P(62)	Alcohol abuse, IHD, died of CHF
IV-27/F	CHF	50/58	AVB(50) LBBB(56)	72/20%(56)	AFIB(53)	nsVT(56)	DDD-PM(50) CRT-P(56)	Cardiac sarcoidosis, HTX(58), Died of graft failure
IV-29/M	FS	59/64	SND+PRP(59) AVB(60)	59/60%(62)	AFIB(61)		DDD-ICD(60)	
IV-30/M	FS	58/60				PVCs(60)		
IV-32/M	FS	55/57						
IV-33/F	FS	56/58						
IV-37/F	FS	54/60	SND+PRP(54)			PVCs(55)	DDD-ICD(57)	

			AVB(57)			nsVT(57)		
IV-38/F	FS	50/55	SND+PRP(50)			PVCs(55)		
V-9/F	FS	31/38		59/50%(34)		PVCs(32)	DDD-ICD(34)	RFA-PVCs from RVOT(34). No
				49/60%(38)		nsVT(32)		VT on FU
V-12/F	FS	23/27						
V-16/M	FS	50/54						Stroke(53)
V-17/F	FS	49/49						
V-18/F	FS	48/48						
V-25/M	FS	48/51		56/45%(50)			VVI-ICD(50)	Normalisation of LVEF on FU
V-27/F	FS	43/47						
V-29/F	FS	46/50						
V-30/M	FS	41/45						
Mean age at endpoint (range)			64.4 (50-89)	63.0/(34-89)	67.6(53-89)	57.8(32-69)	61.7(34-89)	
Family B: *LMNA*-p.Arg471His								
I-1/M	SUD	46						No autopsy, no gene test
II-1/F	CHF	57						No clinical data
II-2/F	CHF	57						No clinical data
II-3/F	CHF	60						No clinical data
III-3/F	FS	54/61	PRP(61)	57/40%(54)			VVI-ICD(54)	Stroke
III-5/M	CHF	53/67	LBBB(59)	78/20%(59)	AFIB(58)	PVCs(56)	CRT-D(56)	HTX(59)
III-6/M	CHF	64/70	LBBB(64)	68/35%(64)	AFIB(64)	PVCs(68)	Declined ICD	SUD(70)
III-7/F	FS	59/59		54/50%(59)		nsVT(59)	VVI-ICD(59)	Lost to FU (foreign citizen)
IV-5/M	FS	25/37	LBBB(36)	57/35%(25)		PVCs(25)	VVI-ICD(26)	Stroke
						nsVT(25)		
IV-6/M	SCD	18						Autopsy: DCM
IV-10/M	FS	22/36						
IV-11/M	FS	34/40						
IV-13/M	FS	32						Lost to FU (foreign citizen)
Family C: *LMNA*-p.Arg471Cys								
I-1/M		-/55						Died from lung cancer, no gene
								test
II-1/M	Stroke/AFIB/br	40/49	AVB(40)	63/45%(40)	AFIB(40)		DDD-PM(40)	Stroke(40), SCD(49), autopsy:
	adycardia				AFLU(42)			DCM
II-2/F	FS	36/45	SND(45)	64/25%(41)		PVCs(36)	VVI-ICD(37)	Stroke(35)
						nsVT(36)		
						sVT (46)		
III-3/F	FS	15/19						

Table legend: Clinical characteristics of *LMNA* mutation carriers. Data from index patients and three first-degree relatives have been included in a previous paper.[4] Value numbers in parenthesis indicate age. [1]Age at initial evaluation and at death, HTx, or last follow-up; [2]Cardiac conduction

disease including sinusnode dysfunction (SND), PR-interval prolongation (PRP; PR-interval >200 ms), atrioventricular block (AVB; advanced AVB type 2 or 3rd degree AVB), left bundle branch block (LBBB); [3]Dilated cardiomyopathy defined as left ventricular dysfunction with ejection fraction < 0.50, or left ventricular end-diastolic dimensions >112% of the predicted value by the Henry formula (16); [4]Supraventricular arrhythmias including atrial fibrillation (AFIB) or atrial flutter (AFLU); [5]Ventricular arrhythmias including premature ventricular contractions (PVCs; >1000/24h), ventricular tachycardia (≥3 consecutive PVCs at a heart rate above 120/min); [6]Dual-chamber pacemaker (DDD-PM), implantable cardioverter defibrillator (VVI-ICD/DDD-ICD), or biventricular pacemaker or ICD (CRT-P/CRT-D). Abbreviations: AFIB, atrial fibrillation; AFLU, atrial flutter; CHF, chronic heart failure; FU, follow up; FS, family screening; HTx, cardiac transplantation; DCM, dilated cardiomyopathy; F, female; LVEF, left ventricular ejection fraction; IHD, ischemic heart disease; M, male; RFA, radiofrequency ablation; SCD, sudden cardiac death; SUD, sudden unexpected death; sVT, sustained ventricular tachycardia.

Supplementary Table S2

Peptide	Sequence	Precursor (*m/z*) values	Transitions (*m/z*) values
Endogenous: Lamin A/C common peptides	LKDLEALLNSK	622.37	1002.55, 887.52, 774.44, 645.39, 565.82
	IDSLSAQLSQLQK	715.90	1002.56, 915.53, 844.49, 603.35, 516.31
	MQQQLDEYQELLDIK	947.47	1378.71, 1265.63, 1150.60, 1021.56, 817.92
Heavy: Lamin A/C common peptides	*LKDLEALLNS[K+4]*	626.37	1010.56, 895.53, 782.45, 653.41, 569.83
	IDSLSAQLSQLQ[K+4]	719.90	1010.57, 923.54, 852.50, 611.36, 524.33
	MQQQLDEYQELLDI[K+4]	951.47	1386.72, 1273.64, 1158.61, 1029.57, 821.92
Endogenous: Lamin A specific peptide	SVGGSGGGSFGDNLVTR	783.88	1179.58, 921.48, 774.41, 602.36, 690.83
Heavy: Lamin A specific peptide	*SVGGSGGGSFGDNLVT[R+5]*	788.88	1189.58, 931.49, 784.42, 612.37, 695.83
Endogenous: Lamin A/C WT peptides	NIYSEELR	512.26	796.38, 633.32, 546.29, 417.25, 288.20
	QNGDDPLLTYR	646.32	877.48, 762.45, 552.31, 439.23, 338.18
Heavy: Lamin A/C WT peptides	NIYSEEL[R+5]	517.26	806.39, 643.33, 556.30, 427.25, 298.21
	QNGDDPLLTY[R+5]	651.32	887.49, 772.46, 562.32, 449.24, 348.19
Endogenous: Lamin A/C mutant peptides	HQNGDDPLLTYR	714.85	1291.63, 1163.57, 1049.53, 762.45
	CQNGDDPLLTYR	726.33	1163.57, 1049.53, 992.50, 877.48, 762.45
	NIYSEELCETK	693.32	1271.58, 1158.50, 995.44, 908.40, 779.36
Heavy: Lamin A/C mutant peptides	*HQNGDDPLLTY[R+5]*	719.85	1301.64, 1173.58, 1059.53, 772.46
	CQNGDDPLLTY[R+5]	731.34	1173.58, 1059.53, 1002.51, 887.49, 772.46
	NIYSEELCET[K+4]	697.32	1279.60, 1166.51, 1003.45, 916.42, 787.37
Endogenous reference peptides: tubulin beta-5 chain (*TBB5*)	ISVYYNEATGGK	651.32	1188.55, 1101.52, 1002.45, 839.39,676.33
	ALTVPELTQQVFDAK	830.45	1275.66, 936.48, 480.25, 687.87, 638.33
Heavy reference peptides: tubulin beta-5 chain (*TBB5*)	*ISVYYNEATGG[K+4]*	655.33	1196.57, 1109.54, 1010.47, 847.40, 684.34
	ALTVPELTQQVFDA[K+4]	834.46	1283.67, 944.49, 488.26, 691.87, 642.34

Table legend: Endogenous and heavy labelled peptides used in SRM analysis