Inherited Cardiomyopathies

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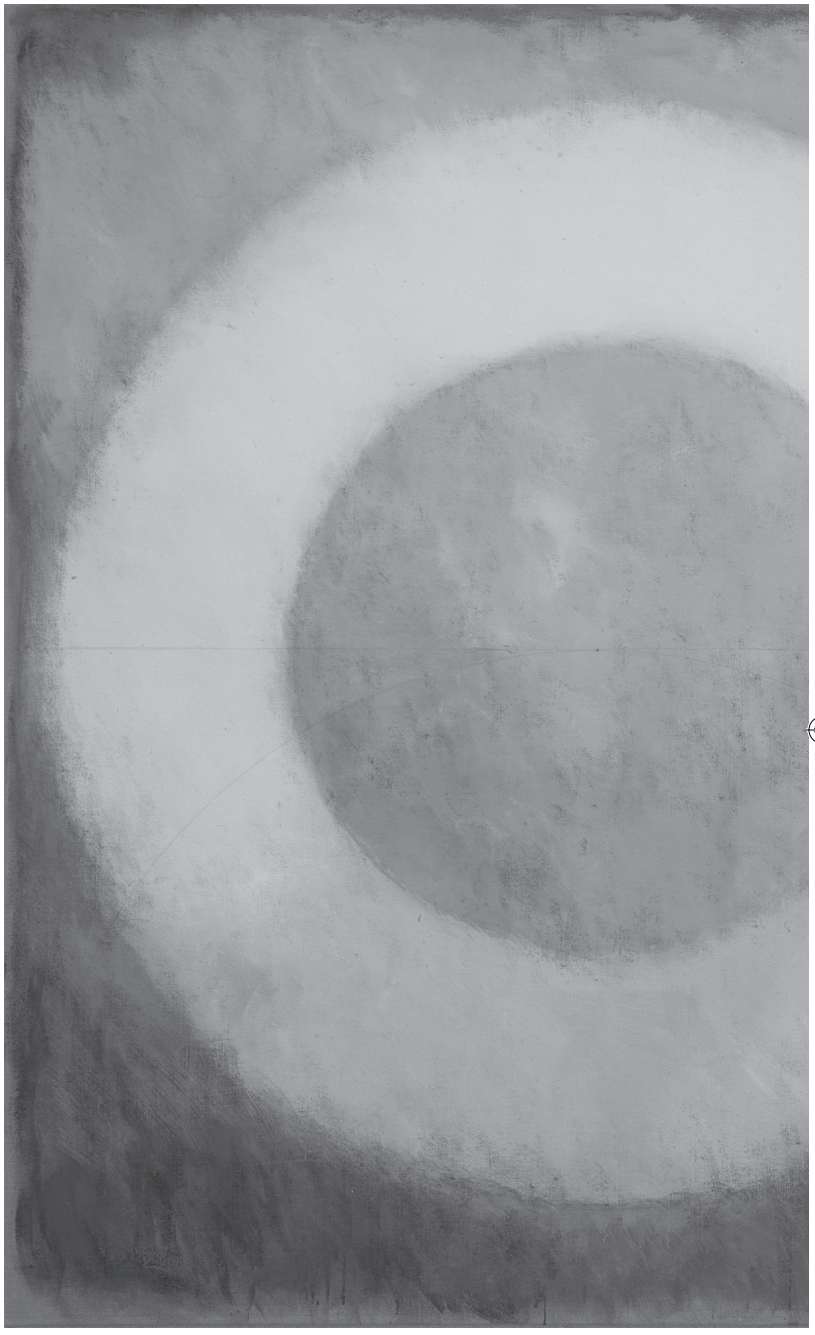
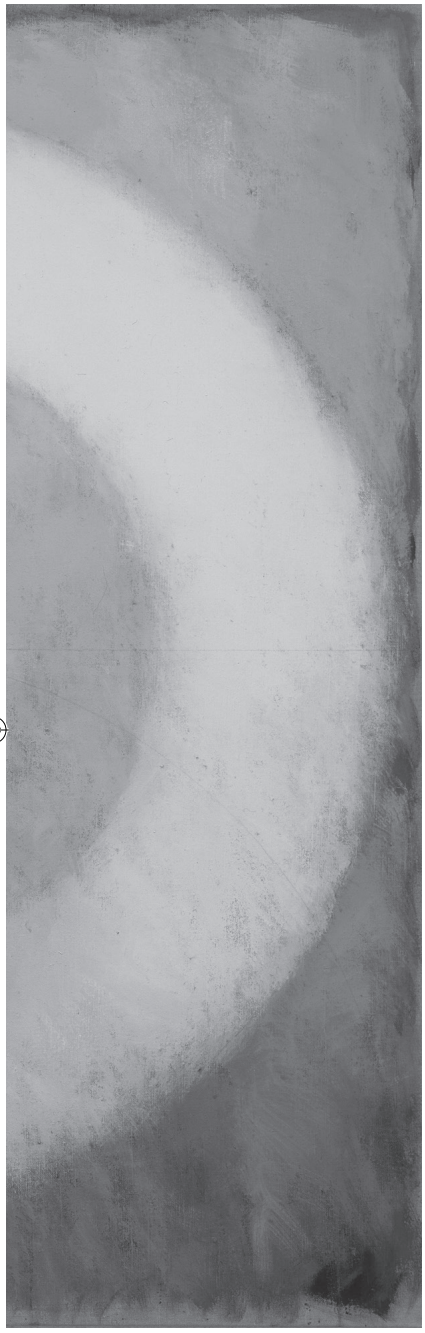
Part I

Inherited Cardiomyopathies





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Genetic analysis in 418 index patients with idiopathic dilated cardiomyopathy: overview of 10 years' experience

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ABSTRACT

Aims With more than 40 dilated cardiomyopathy (DCM)-related genes known, genetic analysis of patients with idiopathic DCM is costly and time-consuming. We describe the yield from genetic analysis in DCM patients in a large Dutch cohort.

Methods and results We collected cardiological and neurological evaluations, family screenings, and genetic analyses for 418 index patients with idiopathic DCM. We identified 35 (putative) pathogenic mutations in 82 index patients (20%). The type of DCM influenced the yield, with mutations found in 25% of familial DCM cases, compared with 8% of sporadic DCM cases and 62% of cases where DCM was accompanied by neuromuscular disease. A *PLN* founder mutation (43 cases) and *LMNA* mutations (19 cases, 16 different mutations) were most prevalent and often demonstrated a specific phenotype. Other mutations were found in: *MYH7*, *DES*, *TNNT2*, *DMD*, *TPM1*, *DMPK*, *SCN5A*, *SGCB* (homozygous), and *TNNI3*. After a median follow-up of 40 months, the combined outcome of death from any cause, heart transplantation, or malignant ventricular arrhythmias in patients with a mutation was worse than in those without an identified mutation (hazard ratio 2.0, 95% confidence interval 1.4–3.0). This seems to be mainly attributable to a high prevalence of malignant ventricular arrhythmias and end-stage heart failure in *LMNA* and *PLN* mutation carriers.

Conclusion The yield of identified mutations in DCM index patients with clinical clues, such as associated neuromuscular disease or familial occurrence, is higher compared with those without these clues. For sporadic DCM, specific clinical characteristics may be used to select cases for DNA analysis.

INTRODUCTION

Idiopathic dilated cardiomyopathy (DCM) is characterized by dilatation and impaired contraction of the left ventricle or both ventricles, in the absence of underlying causes such as CAD, valve disease, congenital heart disease, or pericardial disease.[1] Most patients present with symptoms of heart failure or arrhythmias, or even sudden cardiac death.[2,3] An extensive family history (pedigree covering three or four generations), in combination with cardiological screening of first-degree relatives, results in a diagnosis of a familial form of DCM in up to 35% of cases.[4] Causative mutations have been described in > 40 genes encoding proteins that play a role in the formation and function of the cytoskeleton and (its linkage to) the sarcomere and nucleus.[5,6] The majority of these genes only account for a minority of cases, and many mutations remain unique to one family. Until recently, a genetic cause was found in less than one-third of cases.[4,7] Only a few genotype–phenotype associations are known.[8]

Identifying the causative mutation in individuals with DCM may help to confirm a diagnosis, which can be useful in borderline cases. More importantly, identifying the causative mutation facilitates genetic cascade screening in relatives, which can help to identify other individuals at risk. This enables a timely diagnosis to be made, with the possibility of preventing complications and reducing morbidity and mortality. It also implies that relatives who do not carry the mutation can be excused from regular monitoring.[9] Identifying specific mutations may sometimes guide clinical management, since for some specific mutations, the clinical course is now becoming better established.[10] For instance, *LMNA* mutations are associated with a high risk of sudden cardiac death, even before the occurrence of overt DCM, so that an implantable cardioverter defibrillator (ICD) is recommended based on a genotype-specific algorithm.[11,12]

In a recent expert consensus statement, it was stated that genetic analysis is recommended for patients with DCM and cardiac conduction disease or a family history of premature sudden cardiac death, but there were no specific recommendations on genetic analysis in sporadic cases vs. familial DCM.[8,13]

Our aim here is to describe the results of the 10-year effort to analyse DCM-related genes and to report on the yield of genetic analysis and genotype–phenotype associations. For this purpose, we collected and analysed the clinical and genetic data of a cohort of 418 unrelated index patients with DCM.

METHODS

Subjects and clinical evaluation

Data were collected from all consecutive index patients (first patient in their family) diagnosed with DCM (≥16 years old) admitted to one of the cardiogenetics clinics of four Dutch university hospitals (Amsterdam, Groningen, Utrecht, and Leiden) in whom genetic analysis was performed after a genetic counselling procedure between 2000 and 2011. We excluded cases with suspicion of a syndromal diagnosis or mitochondrial cause based on extracardiac features.

All index patients and their first-degree relatives who consented to cardiological and genetic evaluation underwent cardiological evaluation using the criteria proposed by Mestroni *et al.*[1] Available data on medical history, 12-lead ECG, echocardiography, Holter monitoring, and exercise testing were collected at the time of diagnosis of DCM. Follow-up data on death, heart transplantation, and malignant ventricular arrhythmias were recorded. DCM was diagnosed when a patient had both a reduced systolic function of the left ventricle (LVEF <0.45) and dilation of the left ventricle (LV end-diastolic dimension >117% of the predicted value corrected for body surface area and age) on echocardiography, and only after other identifiable causes such as severe hypertension, CAD, and systemic diseases had been excluded. Cardiac conduction disease and atrial tachyarrhythmias were specifically documented. Atrioventricular (AV) block was classified as first, second, or third degree. First-degree AV block was defined by a PR interval ≥0.20 s. Atrial tachyarrhythmias were classified as paroxysmal (>30 s), persistent, or permanent AF, or atrial flutter. Complete left and right bundle-branch blocks (LBBB and RBBB) were defined by QRS duration ≥120 ms and specific LBBB or RBBB pattern.[14]

Index patients were classified as: (i) familial DCM; (ii) sporadic DCM; or (iii) DCM associated with neuromuscular disease. In patients with a primary cardiological phenotype, the initial neuromuscular examination was restricted to taking a patient and family case history of neuromuscular complaints, while in a subset of cases a physical neurological examination and/or determination of creatine kinase (CK) level were also performed. Associated neuromuscular disease was defined as objective muscular weakness or dystrophy in either the index patient or in (a) close relative(s). Data on family history (of at least three generations) were obtained through interviews with patients and relatives, from medical reports, and from cardiological screening of first-degree relatives. Sudden cardiac death (SCD) was noted when it occurred in first-, second-, or third-degree relatives in two age groups: ≤45 years and ≤60 years. Families with confirmed disease in the index patient and with confirmed or probable disease in at least one first-, second-, or third-degree relative were classified as having familial DCM.

DNA analysis

Genomic DNA was extracted from blood samples obtained from all index patients and affected relatives. Genetic analysis was designed to cover DCM-related genes using different genetic techniques over time (i.e. denaturing high-performance liquid chromatography, denaturing gradient gel electrophoresis, conformation-sensitive capillary electrophoresis, and direct sequencing) to screen the protein-coding region of all exons, as well as the adjacent intronic regions essential for splicing. Details of these analyses are available upon request. To detect large deletions or duplications of one or more exons of *LMNA*, we used the multiplex ligation-dependent probe amplification test (MLPA kit-P048-B, MRC-Holland, Amsterdam, The Netherlands).[15]

DNA analysis of several DCM-related genes was performed in the index patients. When a mutation

was found, affected relatives were screened for carriership to study co-segregation. Our strategy over the past 10 years was to screen genes consecutively, based upon the knowledge available at that moment. In the course of time, this strategy was adjusted because new genes were identified or the yield of screening for a gene was too low according to the literature or from our own experience.

The following criteria were used to classify variations/mutations. We use a list of mutation-specific features based on *in silico* analysis using the mutation interpretation software AlaMut (version 1.5). A score is given depending on the outcome of a prediction test for each feature (i.e. Grantham distance). Then, depending on the total score and the availability of the variant in at least 300 ethnically matched control alleles (data obtained from the literature and/or the internet, e.g. http://evs.gs.washington.edu/EVS, or from our own control alleles), we classified them as: (putative) pathogenic, not pathogenic, or as a variant of unknown clinical significance (VUS: VUS1, unlikely to be pathogenic; VUS2, uncertain; VUS3, likely to be pathogenic). Family information (co-segregation), phenotypic features, and/or functional analysis are needed to classify a variant as (putative) pathogenic. For this, we use strict criteria (*Supplementary data*). In this manuscript, the term 'mutation' was exclusively used for (putative) pathogenic mutations and consistently not for variants with unknown clinical significance (VUS).

Statistical analysis

Clinical characteristics at the moment of DCM diagnosis and family history were compared between subgroups, based on the presence of neuromuscular disease and a family history of DCM, and on the presence of a mutation. Data were compared using the Student's unpaired *t*-test for continuous variables, and the χ^2 test for categorical variables expressed as proportions. The Cox proportional hazard model was used for the event-free survival analyses for time from DCM diagnosis to event, by comparing the presence or absence of a mutation. Three events were used: death from any cause, heart transplantation, or malignant ventricular arrhythmias. Malignant ventricular arrhythmias were defined as cardiopulmonary resuscitation or appropriate ICD treatment. The data were analysed in two separate analyses: death from any cause alone, and a combination of the three events. For the combined outcome, survival time was the time to the first event. Data were corrected for age at DCM diagnosis. Index patients with double-heterozygous (putative) pathogenic mutations or VUS3s were excluded from these analyses. Statistical analyses were performed using PASW software.[16] A *P*-value of <0.05 was considered statistically significant.

RESULTS

Subjects and mutations

We collected 418 index patients with DCM: mean age at onset 46 ± 13 years, 56% male, 224 with familial DCM, 173 with sporadic DCM, and 21 with DCM associated with neuromuscular disease, the latter of which was familial in 62% of these cases (*Table 1*).

The tested genes are shown in *Figure 1*. Per index patient we analysed 1–14 genes (mean 6.1 ± 3.2). We identified 35 different mutations in 82 of 418 (20%) index patients. Seven of the 35 (20%) mutations were novel. Mutations were identified in 25% (55/221) of cases with familial DCM, 8% (14/169) of cases with sporadic DCM, and 62% (13/21) of cases with DCM and neuromuscular disease (*Table 1*; note that patients with VUS3 are excluded from these numbers). The 35 mutations and the variants classified as VUS3 ($n = 8$) are listed in *Tables 2* and *3*, respectively. Mutation types were: 18 missense (1 homozygous), 5 intragenic insertion/deletion, 5 splice site, 2 deletion/duplication of a whole exon, 2 nonsense, 1 trinucleotide repeat expansion, and 2 complex genetic status. The cases with a complex genetic status included: 1 double-mutant allele (2 missense mutations) and 1 double-heterozygous (in-frame deletion plus nonsense). In addition, one case had an in-frame deletion plus a missense VUS3.

The most prevalent mutated gene in our Dutch cohort was *PLN*, due to the founder mutation p.Arg14del (43/314 = 14% of tested index patients) (*Table 1*, *Figure 1*). The clinical characteristics of carriers of this specific mutation have recently been published.[17] *LMNA* was the second most prevalent mutated gene in our cohort (19/384 = 5% of tested index patients), especially in the subgroup with AV block (11/48 = 23% of tested index patients with AV block). *MYH7* was the third in this row (7/294 = 2% of tested index patients). Although the total yield of mutation analysis was lower in all other genes, the detection rate was high in genes that are related to a specific clinical expression because they were only tested on the basis of a specific indication (i.e. 100% in *DMPK*, 100% in *SGCB*, 13% in *DMD*) (*Figure 1*).

Clinical expression

The characteristics at the moment of DCM diagnosis are given in *Table 4*. An LVEF <35% was less common in index patients with a mutation compared with those without an identified mutation (65% vs. 46%, $P < 0.05$). AV block, atrial tachyarrhythmias, neuromuscular complaints, a positive family history for DCM, and a family history for neuromuscular disease were present significantly more often in index patients with a mutation compared with those without an identified mutation. When focusing on the subgroup of index patients with an *LMNA* mutation and comparing these with index patients without an *LMNA* mutation, we recognized similar differences. Also, complete RBBB and LBBB were less frequently noted in index patients with an *LMNA* mutation. In index patients with a *PLN* mutation, LVEF <35% was present less frequently, whereas SCD in relatives ≤45 years old was recorded more frequently.

After a median follow-up of 40 months (interquartile range: 13–90 months), survival in index patients with a mutation and in those without a known mutation was comparable, with a hazard

Table 1: Yield of genetic analysis in 418 index patients with dilated cardiomyopathy

	Neuromuscular –		Neuromuscular +	Total
	FDCM (n = 224)	SDCM (n = 173)	(n = 21)	(n = 418)
Male gender	123/224 (55%)	102/173 (59%)	8/21 (38%)	233/418 (56%)
Age at onset, years	47 ± 12	47 ± 13	42 ± 13	46 ± 13
DCM characteristics at diagnosis				
NYHA ≥III	70/221 (32%)	47/168 (28%)	8/20 (40%)	125/409 (31%)
LVEF <35%	137/224 (61%)	112/173 (65%)	8/21 (38%)[a]	257/418 (61%)
Complete RBBB/LBBB	46/222 (21%)	50/169 (30%)[a]	3/20 (15%)	99/411 (24%)
AV block	21/217 (10%)	21/168 (13%)	9/19 (47%)[a]	51/404 (13%)
Atrial tachyarrhythmias	45/223 (20%)	26/172 (15%)	7/21 (33%)	78/416 (19%)
Familial				
Sudden death ≤45 years	55/224 (25%)	41/173 (24%)	7/21 (33%)	103/418 (25%)
Sudden death ≤60 years	87/224 (39%)	65/173 (38%)	10/21 (48%)	162/418 (39%)
DCM	224/224 (100%)	–	13/21 (62%)	237/418 (57%)
Mutation	55/221 (25%)[a,b]	14/169 (8%)[a,b]	13/21 (62%)[a]	82/411 (20%)[b]
PLN	33[c]	10	0	43
LMNA	9	2	8	19
MYH7	6	1	0	7
DES	1	0	2	3
TNNT2	3	0	0	3
DMD	0	1	1	2
TPM1	2	0	0	2
DMPK	0	0	1	1
SCN5A	1[c]	0	0	1
SGCB	0	0	1	1
TNNI3	1	0	0	1

All variables are given as number present/total number (%), except for age, which is given as mean ± SD.
AV= atrioventricular; DCM=dilated cardiomyopathy; FDCM=familial DCM; RBBB= right bundle branch block; SDCM=sporadic DCM.
[a]Reflects a *P*-value <0.05 comparing subgroups of individuals with familial DCM, sporadic DCM, and DCM associated with neuromuscular disease.
[b]Exclusion of VUS3 (variants of unknown clinical significance type 3).
[c]Found in the same index patient.

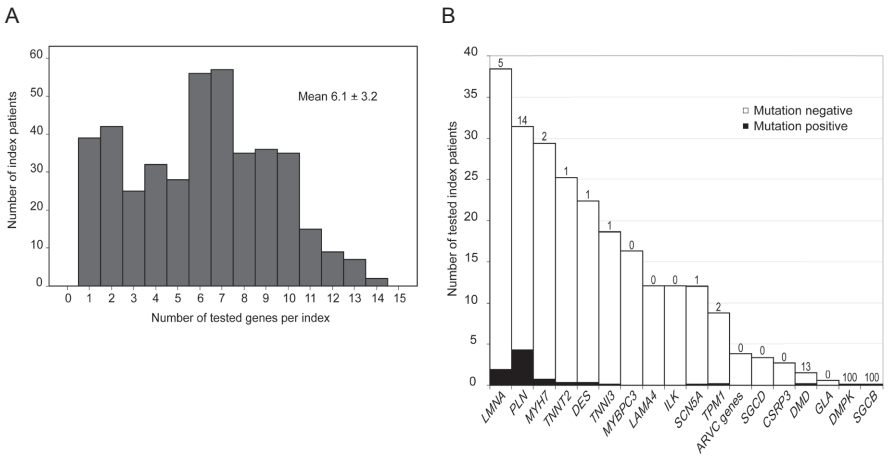
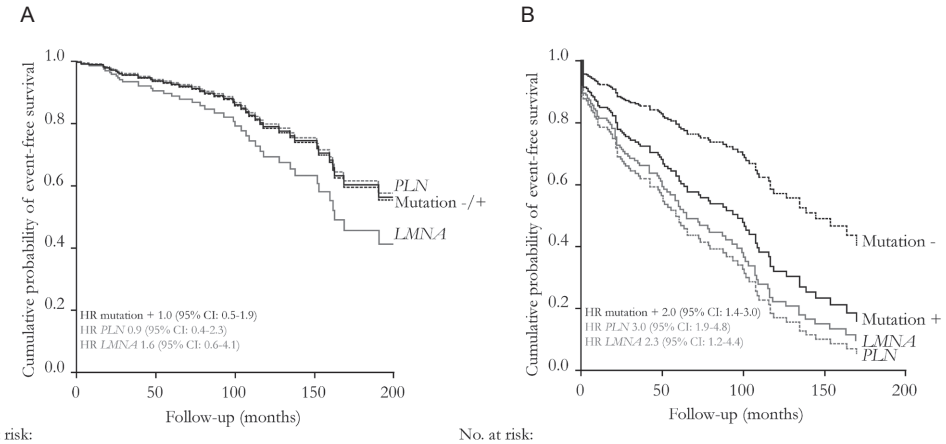


Figure 1: Number of analysed genes per index patient (*A*) and the number of tested index patients per gene, with mutation yield per gene (*B*). Genes that were analysed in at least five index patients and genes with mutations are depicted. Five arrhythmogenic right ventricular cardiomyopathy (ARVC)-related genes are shown together in one bar (*PKP2*, *DSP*, *DSC2*, *DSG2*, and *JUP*).



No. at risk:					
Mutation -	329	280	230	180	130
Mutation +	80	53	32	13	7
PLN	41	25	13	5	3
LMNA	19	12	7	4	3

No. at risk:					
Mutation -	329	111	40	18	4
Mutation +	80	37	18	2	1
PLN	41	15	6	0	0
LMNA	195	8	2	1	1

Figure 2: Event-free survival curves for index patients with a mutation or without an identified mutation, and the subgroups of index patients with a *PLN* or *LMNA* mutation, corrected for age at diagnosis of dilated cardiomyopathy. (*A*) Outcome of 'death from any cause'. (*B*) Combined outcome of 'death from any cause, heart transplantation, or malignant ventricular arrhythmias'. CI=confidence interval; HR=hazard ratio.

ratio (HR) of 1.0 [95% confidence interval (CI) 0.5–1.9]. In addition, we detected no difference in survival between the subgroups of index patients with a *PLN* or *LMNA* mutation compared with those without either a *PLN* or *LMNA* mutation (HR 0.9, 95% CI 0.4–2.4, and HR 1.6, 95% CI 0.6–4.1, respectively). However, when combining the outcome parameters 'death from any cause, heart transplantation, or malignant ventricular arrhythmias', the outcome in index patients with a mutation was worse (HR 2.0, 95% CI 1.4–3.0). In the subgroups of index patients with a *PLN* or a *LMNA* mutation, the outcome was worse than for index patients without either a *PLN* or a *LMNA* mutation (HR 3.0, 95% CI 1.9–4.8, and HR 2.3, 95% CI 1.2–4.4, respectively) (*Figure 2* and *Supplementary data, Table S1*).

DISCUSSION

Dilated cardiomyopathy is genetically highly heterogeneous, with >40 causally related genes, which in most cases are mutated in only a minority of cases.[6] We investigated one of the largest well-defined DCM cohorts (418 index patients) to evaluate the yield of genetic testing and to search for clinical clues that could increase the likelihood of certain mutations. We identified a mutation in 62% of index patients with DCM and neuromuscular disease, in 25% of familial DCM cases, and in only 8% of sporadic DCM cases. Compared with the studies of Hershberger *et al.*, who identified a putative genetic cause in one-third of the DCM patients after sequencing 14 genes per patient, we identified a putative genetic cause in one-fifth (20%) of our total group of DCM patients, with a mean number of six tested genes per patient.[18–20] Another study, with a similar study design to ours, identified a genetic cause in 17% of their DCM patients.[7] Although the yield of genetic testing in these studies seems comparable with our yield, the yield in our cohort is highly determined by the occurrence of the *PLN* founder mutation, p.Arg14del. Gaining insight into genotype–phenotype associations is important because, in several cases, it is possible to perform a more targeted DNA analysis based on specific clinical features. In this study, *LMNA* mutations were associated with atrial tachyarrhythmias, AV block, neuromuscular complaints, a family history of DCM, and a family history of neuromuscular disease, which corresponds to the literature on *LMNA*-associated phenotypes.[12,21,22] The *PLN* founder mutation, p.Arg14del, is associated with an arrhythmogenic cardiomyopathy with left and/or right cardiac involvement, frequently associated with a low voltage on ECG.[17] We were not able to perform gene-specific analyses on other genes in our study because of the small numbers of patients with mutations in the same gene.

We did not observe any differences between index patients with a mutation and those without an identified mutation for mortality ('death from any cause'), but a combined endpoint ('death from any cause, heart transplantation, or malignant ventricular arrhythmias') was seen significantly more frequently in index patients with a mutation. This is due to high prevalence of both malignant ventricular arrhythmias and end-stage heart failure of *LMNA* and *PLN* mutation carriers.[17,22,23] The presence of associated neuromuscular disease in the patient or in relatives is an important

Table 2: Overview of 35 identified mutations in 418 index patients with dilated cardiomyopathy

Gene	Nucleotide change	Amino acid change	No. of index patients	Reference
PLN	40_42del[b,b]	Arg14del	43	17, 27[a]
LMNA	del exon 1		1	15[a]
	73C > T	Arg25Cys	1	28[a]
	250G > A	Glu84Lys	1	29[a]
	568C > T	Arg190Trp	1	30
	624_626del	Lys208del	2	28[a]
	777T > A	Tyr259X	1	28[a]
	936 + 1delG		1	29[a]
	936 + 2T > G		1	–
	1001G > A	Ser334Asn	2	–
	1045C > T	Arg349Trp	1	28[a]
	1130G > A	Arg377His	1	28[a]
	1130G > T	Arg377Leu	2	28[a]
	1157 + 1G > A		1	31
	1380 + 1G > A		1	28[a]
	1512–1513insAG		1	28[a]
	1608 + 4A > G		1	–
MYH7	1357C > T	Arg453Cys	1	32
	1633G > A and 2863G > A[b]	Asp545Asn and Asp955Asn	2	33
	1976T > C	Met659Thr	1	–
	2710C > T	Arg904Cys	1	34[a]
	3113T > C	Leu1038Pro	1	35
	5754C > G	Asn1918Lys	1	36
DES	38C > T	Ser13Phe	2	37, 38[a]
	1360C > T	Arg454Trp	1	39[a]
TNNT2	282_283delAG		1	–
	634C > T	Arg205Trp	1	40
	650_652delAGA	Lys217del	1	40, 41[a]
DMD	Duplication exon 12		1	42
	3516G > A	Trp1172X	1	43
TPM1	602C > T	Thr201Met	2	–
DMPK	CTGn repeat expansion		1	44
SGCB	341C > T homozygous	Ser114Phe	1	45
SCN5A	3318dupC[b]	Glu1107ArgfsX24	1	–
TNNI3	555C > G	Asn185Lys	1	46

Nomenclature according to HGVS (Human Genome Variation Society) using the reference sequences: PLN (NM_002667.3), LMNA (NM_170707.2), MYH7 (NM_000257.2), DES (NM_001927.3), TNNT2 (NM_000364.2), DMD (NM_004006.2), TPM1 (NM_001018005.1), DMPK (NM_004409.2), SGCB (NM_000232.4), SCN5A (NM_198056.2), and TNNI3 (NM_000363.4).
[a]Reference includes our index patient.
[b]Complex genetic status: one double-mutant allele MYH7 and two double-heterozygous [PLN founder mutation plus SCN5A nonsense mutation; and PLN founder mutation plus MYH7 VUS3 (variant of unknown clinical significance type 3)].

Table 3: Overview of eight identified variants of unknown clinical significance type 3 in 418 index patients with dilated cardiomyopathy

Gene	Nucleotide change	Amino acid change	No. of index patients	Reference
LMNA	71C > T	Thr24Ile	1	–
	992G > A	Arg331Gln	1	47
	1930C > T	Arg644Cys	1	47, 48
MYH7	1699C > T[a]	Arg567Cys	1	–
	2890G > C	Val964Leu	1	20
	2945T > C		1	49
EMD	454C > T	Arg152Cys	1	–
MYBPC3	2618C > A	Pro873His	1	50

Nomenclature according to HGVS (Human Genome Variation Society) using the reference sequences: *LMNA* (NM_170707.2), *MYH7* (NM_000257.2), *EMD* (NM_000117.2), and *MYBPC3* (NM_000256.3).
[a]The patient also carried the *PLN* p.Arg14del founder mutation.

feature for selecting genes for targeted gene analysis. We identified mutations in *LMNA* and *DES* irrespective of the specific clinical neurological diagnosis (i.e. limb girdle muscular dystrophy, Markesberry myopathy, etc.). *DMD* and *DMPK* are other genes involved in DCM associated with neuromuscular disease.

We specifically analysed the phenotypes and family history of the index patients with sporadic DCM and an identified mutation (8%, $n = 14$), with the aim of seeing if it is justified to reserve DNA analysis for specific sporadic DCM cases, i.e. only for those with clinical characteristics that raise suspicion for certain genetic defects (such as AV block, neuromuscular involvement, X-linked inheritance, low voltage on ECG, or right cardiac involvement). In most of these index patients (12/14), retrospectively we could see features pointing towards specific clinical expression (i.e. features known to be associated with *LMNA* mutations or with the *PLN* founder mutation, and an increased CK level in a *DMD* mutation carrier). We could not identify any specific clues in two index patients with sporadic DCM, who proved to have a *MYH7* and *LMNA* mutation, respectively.

We were extremely careful in classifying the (putative) pathogenic mutations and VUSs. This is

Table 4: Characteristics at time of diagnosis of dilated cardiomyopathy in index patients with dilated cardiomyopathy

	Mutation – (n = 329)[a]	Mutation + (n = 82)	*PLN* + (n = 41)[b]	*LMNA* + (n = 19)
Characteristics				
Male gender	186/329 (57%)	42/82 (51%)	18/41 (44%)	12/19 (63%)
Age at onset, years	47 ± 12	44 ± 14	48 ± 12	43 ± 15
NYHA ≥III	98/321 (31%)	23/81 (28%)	10/40 (25%)	5/19 (26%)
LVEF <35%	213/329 (65%)	38/82 (46%)[c]	19/41 (46%)[c]	7/19 (37%)[c]
Complete RBBB/LBBB	81/327 (25%)	17/78 (22%)	10/40 (25%)	1/18 (6%)[c]
AV block	32/320 (10%)	18/78 (23%)[c]	4/40 (10%)	11/17 (65%)[c]
Atrial tachyarrhythmias	52/328 (16%)	25/82 (30%)[c]	8/41 (20%)	12/19 (63%)[c]
Neuromuscular	7/329 (2%)	10/82 (12%)[c]	0/41 (0%)	7/19 (37%)[c]
Familial				
Sudden death ≤45 years	77/329 (23%)	25/82 (30%)	16/41 (39%)c	5/19 (26%)
Sudden death ≤60 years	126/329 (38%)	33/82 (40%)	18/41 (44%)	7/19 (37%)
DCM	168/329 (51%)	66/82 (80%)[c]	31/41 (76%)[c]	17/19 (89%)[c]
Neuromuscular	3/329 (1%)	10/82 (12%)[c]	0/41 (0%)	6/19 (32%)[c]

All variables are given as number present/total number (%), except for age, which is given as mean ± SD.
AV=atrioventricular; DCM=dilated cardiomyopathy; RBBB=right bundle branch block.
[a] Exclusion of variants of unknown clinical significance type 3.
[b] Double-heterozygous mutations excluded.
[c] Reflects a P-value < 0.05 comparing individuals with and without a (*PLN/LMNA*) mutation.

a challenge in the genetics of DCM, as the majority of mutations are unique or only identified in a few cases or families, and functional data are often lacking.[24] Reports like this, on identified mutations, may assist other professionals in judging mutations/VUSs in daily practice.
A limitation of our study is that it underestimates the prevalence of disease-causing mutations in DCM. The number of index patients screened for each gene was not the same due to the ongoing recruitment of families over a period of 10 years and greater insight into the prevalence of mutations in specific genes, which led to stopping screening for certain genes. Only one double-heterozygous carrier of mutations was identified in our study, a phenomenon that has been reported to be present in at least a few per cent of cases with cardiomyopathy.[18,25] However, we have no suitable data to discuss this because we often refrained from further DNA analysis once a mutation had been identified. We have screened relatively large numbers of genes per index patient (mean 6.1 ± 3.2), but we have not yet evaluated *TTN* in our cohort. Recently, mutations in this gene have been reported in a substantial proportion of DCM cases (25% of familial cases).[26] The *PLN* founder mutation, p.Arg14del, is relatively frequent in our cohort, but it is not known how much this mutation occurs outside the Netherlands. There

might have been a referral bias towards patients with a more severe clinical course and/or DCM accompanied by cardiac conduction disease. In the next few years, we foresee a significant increase in knowledge about the genetics of DCM because the use of next-generation sequencing developed for DCM has now entered clinical practice. This will provide high-throughput, rapid, and affordable molecular analysis for DCM, leading to many new data on the genetics of DCM.

Considerations

A correct clinical classification, knowledge of gene-specific clinical symptoms, and expertise on interpreting genetic results and classifying mutations and VUSs is pivotal. We recommend genetic testing in all cases with familial DCM and in cases of DCM with neuromuscular disease. However, the indication for genetic testing should not be restricted to familial cases, since sporadic cases can also harbour DCM-related mutations. These can be *de novo* mutations, or the family history may be negative due to an incomplete evaluation of family members, variable expression, or age-related/age-reduced penetrance. Because the yield of genetic screening in sporadic DCM is substantially lower than in familial DCM, genetic analyses can be targeted by noting specific clinical characteristics, such as gene-specific characteristics or sudden cardiac death in relatives. This is important particularly when genetic or financial recourses are scarce. Next-generation sequencing will provide rapid molecular analysis for DCM. However, in cases where gene-specific characteristics are present, it might be justified to start with low-cost testing of a single gene based on the phenotype.

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SUPPLEMENTARY DATA

Classification of variations/mutations

To classify the sequence variants we have used two scoring lists. Scoring list 1 was used for missense and in-frame variants. Scoring list 2 was used for non-sense and frame-shift variants. For each variant we have analysed the outcome of many mutation specific features. If a feature could not be calculated, we selected "not possible". This score was not taken into account for the final classification. With these scoring lists the variants were classified in 5 different classes: not pathogenic, variant of unknown clinical significance 1, 2, 3 (VUS1, unlikely to be pathogenic; VUS2, uncertain; VUS3, likely to be pathogenic), (putative) pathogenic.

Scoring list 1 (see below) consists of two parts. In the first part mutation specific features are calculated and scored based on *in silico* analysis using the mutation interpretation software AlaMut (version 1.5, parts 1-6 and 9) and protein alignments as offered by AlaMut and/or home made (part 6 and 7). All these features are basically based on conservation and the alteration in biophysical characteristics of the amino acid substitution. The score given to each feature is based on years of experience in interpreting sequence variants in autosomal dominant cardiac conditions in DNA diagnostics. Based on the presence and the frequency of the variant in a (preferably ethnically matched) control population a score is given (part 8 and *table S1*). For substitutions the exome variant server (http://evs.gs.washington.edu/EVS/) can be very useful. If a variant (i.e. insertions or deletions) was not available in the exome variant server, internet (databases, publications) or information from our own laboratory (number of index cases, control population) was used. A score is given based on the frequency and number of control alleles analysed see *table S1* for the conversion. In part 9 a score is given based on splice site prediction obtained by several splice site prediction software's available in AlaMut. *Table S2* is used for the conversion. The scores obtained from the "in silico" part (1-9) are added and used to determine a sub-classification. Based on this information the highest score a variant can get is a VUS3. Family information (co-segregation), phenotypic features and/or functional analysis are needed to classify a variant as (putative) pathogenic (part 10 and 11).

Scoring list 2 (see below) consists also of two parts. The first part is based on splice predictions, general characteristics of the mutation type of variant and frequency in a control population (parts 1 till 3). *Table S1* and *S2* are used to determine the score with respect to frequency of the variant in controls and splice site prediction, respectively. The scores obtained from the first part (1-3) are added and used to determine a sub-classification. Based on this information the highest score a variant can get is a VUS3. Family information (co-segregation), phenotypic features and/or functional analysis are needed to classify a variant as (putative) pathogenic (part 4 and 5).

Scoring list 1

1. PolyPhen:

a. HumDiv

Probably damaging	=> score 1
Possibly damaging	=> score 0.5
Benign	=> score 0

not possible or **Score:……..**

b. HumVar

Probably damaging	=> score 1
Possibly damaging	=> score 0.5
Benign	=> score 0

not possible or **Score: …..**

2. SIFT

0.00-0.05: intolerant	=> score 1
>0.05: tolerant	=> score 0

not possible or **Score: …..**

3. Grantham dist (0-215)

Large distance (>140)	=> score 2
Moderate distance (≤140)	=> score 1
Benign (≤70)	=> score 0

not possible or **Score: …..**

4. Align-GVGD

Class C65 most likely	=> score 1.25
Class C55	=> score 1
Class C45	=> score 0.75
Class C35	=> score 0.5
Class C15/25	=> score 0.25
Class C0	=> score 0

not possible or **Score: …..**

5. Blosum 62

≥ -2	=> score 1
-1	=> score 0.5
≥0	=> score 0

not possible or **Score: …..**

6. Conservation between species using protein aligments
(use at least human, 3 other mammals and 3 lower animals like bird, frog, fly, fish)

All mammals and almost all lower animals	=> score 1
All mammals and a few lower animal	=> score 0.75
Almost all mammals and no lower	=> score 0.5
Other	=> score 0

not possible or **Score: …..**

7. Conservation between isoforms (different genes) using protein aligments

75-100% conserved	=> score 0.5
35-74% conserved	=> score 0.25
0-34% conserved	=> score 0

not possible or **Score: …..**

8. Frequency in control population

a. Ethnical background of the patient matches the control population:

Yes
Unknown
No

b. Exome variant server (http://evs.gs.washington.edu/EVS/):

# variant alleles	# total alleles		freq in %
1…….	…….	EA (European American) alleles	…….%
2…….	…….	AA (African American) alleles	…….%
total:…….	…….		……..%

c. Own laboratory:
freq. of the variant in index cases:
100*(…….. variant in index / ……. total number of index cases) =…….%

freq. of the variant in controls:
100*(…….. variant in controls / ……. total number of controls) =…….%

d. Other sources (databases, literature): …….
…….

Determine the score based on table S1 **not possible** or **Score: …..**

9. In silico analysis of splicing
(Splice prediction using AlaMut, see table 2 for grouping)

Very likely (group 4)	=> score 2
Probably (group 3)	=> score 1
Possibly (group 2)	=> score 0.5
Not likely (group 1)	=> score 0

not possible or **Score: …..**

Total score for 1 till 9: …….
Maximum score possible*: ……..
("not possible" is excluded)

10. Family information/Phenotype?

The information can also come from other families or literature.

Summaries below all available information and evidence (e.g. literature references).

……..…

…….

Very likely pathogenic **=> score 4**

(de novo mutation or ≥6 affected family members with the mutation and no affected without the mutation*)

Probably co-segregation **=> score 3**

(5 affected family members with the mutation and no affected without the mutation*)

Possible co-segregation **=> score 2**

(3-4 affected family members with the mutation and no affected without the mutation*)

Co-segregation unclear **=> score 1**

(2 affected family members with the mutation and no affected without the mutation*)

Only index **=> score 0**

No co-segregation **=> score 0**

(affected family member without mutation)

Score: …..

*This does not count when the phenotype is likely due to a non genetic cause like hypertension in cardiac hypertrophy or when it is likely that more than a single mutation explains the phenotype in a severely affected patient).

11. Functional analysis

Experimentally:

Is the variant functionally tested in vitro, in culture or in an animal model? If so judge based on the method used and the experimental data how convincing the conclusion is. This is important because functional assays are often not well validated.

Summaries below all available information and evidence (e.g. literature references):

……

……

Convincingly functionally aberrant => score 3

Possibly functionally aberrant => score 1

Unclear or not functionally aberrant => score 0

not possible or **Score: …..**

Sub-classification based on parts 1-9:
Calculate % score:
100* (total score for 1 till 9/ maximum score possible for 1 till 9) = …. %

% score ≥ 70%	➔ VUS3
45% ≤ % score < 70%	➔ VUS2
25% ≤ % score < 45%	➔ VUS1
% score < 25%	➔ not pathogenic*

*missense mutations which get a sub-classification "not pathogenic" but were less than two times observed in a large (>10000) population of control alleles will be upgraded to a VUS1

Final classification (including part 10 and 11):
Family information (co-segregation), phenotypic features and/or functional analysis are needed to classify a variant as (putative) pathogenic.

1. A combined score of 2 or 3 for part 10 and 11 will upgrade the score from the sub-classification one level.
2. A maximum score for part 11 (functional analysis) and a score 0 for part 10 (Family information) upgrades every sub-class to a VUS3 (a functional test on its own is not enough to give a variant the classification pathogenic).
3. A combined score of 4 for part 10 and 11 and none of the parts have a maximum score will upgrade the score from the sub-classification to a VUS3.
4. When in part 10 a maximum score is obtained the sub-classification is upgraded to a pathogenic mutation.
5. A combined score of 5 or 6 for part 10 and 11 and part 10 has not the highest score will upgrade the score from the sub-classification to a pathogenic mutation.

Score part 10 (family information): …….
Score part 11 (functional analysis): …….
Total score parts 10 & 11: …….

Conclusion:

1. **Not pathogenic (neutral variant or weak modifier)**
2. **VUS1 (unlikely pathogenic)**
3. **VUS2 (unclear)**
4. **VUS3 (likely pathogenic)**
5. **Pathogenic (putative)**

Comment:
If the scoring list is not in agreement with other information not included in this list indicate this below and correct the conclusion accordingly.
…….
…….

Scoring list 2

1. Non-sense variant or predicted effect on splicing (table S2)

c.x-1 or -2 (acceptor) or c.y+1 or +2 (donor) and clear reduction of splice site Predicated values in AlaMut (group 5) => Score 4

Stop or frameshift mutation* => Score 4

New or altered splice site predicted (see table S2)

Probably functional (group 4)	=> score 2
Possibly functional (group 3)	=> score 1
Unlikely (group 2)	=> score 0.5
Not very likely (group 1)	=> score 0

not possible or **Score:……..**

* When the stop or frameshift mutation is in the last 2 exons this may result in a stable protein and if the N-terminal part of the protein is not well conserved it remains uncertain whether the variant will be pathogenic.

2. Does the mutation type fit with the disease?

(Think about gain or loss of function, dominant negative, haplo-insufficiency etc)

Analysis of "real" control alleles are not necessary in case of stop, frame-shift or clear splice site mutations (c.x-1 or -2 or c.y+1 or +2) and this type of mutation fits with the disease.

Type of variant fits with the disease	=> score 5
Type of variant not described before in disease	=> score 1
Unlikely disease causing	=> score 0.5
(Not) pathogenic,	
because:…..……..	
……	

not possible or **Score: …..**

3. Frequency in control population

a. Ethnical background of the patient matches the control population:

Yes
Unknown
No

b. Exome variant server (http://evs.gs.washington.edu/EVS/):

# variant alleles	# total alleles		freq in %
1. …….	…….	EA (European American) alleles	…….%
2. …….	…….	AA (African American) alleles	…….%
total:…….	…….		……..%

c. Own laboratory:

freq. of the variant in index cases:

100*(…….. variant in index / ……. total number of index cases) =…….%

freq. of the variant in controls:
100*(…….. variant in controls / ……. total number of controls) =…….%

Other sources (databases, literature ….):……
…….

Determine the score based on table S1 **not possible** or **Score: …..**

4. Family information/Phenotype?
The information can also come from other families or literature.
Summaries below all available information and evidence (e.g. literature references):
……..…
……

Very likely pathogenic **=> score 4**
(de novo mutation or ≥6 affected family members with the mutation and no affected without the mutation*)
Probably co-segregation **=> score 3**
(5 affected family members with the mutation and no affected without the mutation*)
Possible co-segregation **=> score 2**
(3-4 affected family members with the mutation and no affected without the mutation*)
Co-segregation unclear **=> score 1**
(2 affected family members with the mutation and no affected without the mutation*)
Only index **=> score 0**
No co-segregation **=> score 0**
(Affected family member without mutation)

Score: …..

*This does not count when the phenotype is likely due to a non genetic cause like hypertension in hypertrophy or when it is likely that more than a single mutation explains the phenotype in a severely affected person).

5. Functional analysis
Experimentally:
Is the variant functionally tested in vitro, in culture or in an animal model? If so judge based on the method used and the experimental data how convincing the conclusion is. This is important because functional assays are often not well validated.
Summary of all available information and evidence (e.g. literature references):
……..…
…….

Convincingly functionally aberrant => score 3
Possibly functionally aberrant => score 1
Unclear or not functionally aberrant => score 0

not possible or **Score: …..**

Sub-classification based on parts 1-3:
Calculate % score:
100* (total score for 1 till 3/ Maximum score possible for 1 till 3) = …. %

% score ≥ 70%	➔ VUS3
45% ≤ % score < 70%	➔ VUS2
25% ≤ % score < 45%	➔ VUS1
% score < 25%	➔ not pathogenic*

Final classification (including part 4 and 5):
Family information (co-segregation), phenotypic features and/or functional analysis are needed to classify a variant as (putative) pathogenic.

1. A combined score of 2 or 3 for part 4 and 5 will upgrade the score from the sub-classification one level.
2. A maximum score for part 5 and a score 0 for part 4 upgrades every sub-class to a VUS3 (a functional test on its own is not enough to give a variant the classification pathogenic).
3. A combined score of 4 for part 4 and 5, and none of the parts have a maximum score, will upgrade the score from the sub-classification to a VUS3.
4. When in part 4 a maximum score is obtained the sub-classification is upgraded to a pathogenic mutation.
5. A combined score of 5 or 6 for parts 4 and 5, and part 4 has not the highest score, will upgrade the score from the sub-classification to a pathogenic mutation.

Score part 4 (family information):	…….
Score part 5 (functional analysis):	…….
Total score parts 4 & 5:	..……

Conclusion:
1. **Not pathogenic (neutral variant or weak modifier)**
2. **VUS1 (unlikely pathogenic)**
3. **VUS2 (unclear)**
4. **VUS3 (likely pathogenic)**
5. **Pathogenic (putative)**

Comment:
If the scoring list is not in agreement with other information not included in this list indicate this below and correct the conclusion accordingly.
……
……

Table S1: Score table for the frequency of the variant in control alleles

≤1500 control alleles analysed:			
# variant observed	**# alleles analysed**	**Action**	**Score**
1x in index	<200 index alleles	Analyse >300 “real” control alleles	
1x in index	1x in >200 index alleles and/or not in >300 “real” control alleles (unknown or same ethnical background)		1.5
1x in index + SNP database (not genotyped)	1x in <500 index alleles	Analyse >500 “real” control alleles	
1x in index + SNP database (not genotyped)	1x in >500 index alleles or not in >500 “real” control alleles		1.0
1x in index + SNP database (not genotyped)	1x in >500 index alleles or ≥ 1x in >500 “real” control alleles		0
1x in index + SNP database (genotyped)	≥ 2x in >200 “real” control alleles (freq ≥ 1%)		Not pathogenic*
1x in index + SNP database (genotyped)	≤ 2x in >500 “real” control alleles (freq < 1%)		0
≥2x in index		Analyse >500 “real” control alleles	
≥2x in index	Not in >500 “real” control alleles (unknown or same ethnical background)		1.5
≥2x in index	Not in >500 “real” control alleles (different ethnical background)		0
≥2x in index	≥1 in >500 “real” control alleles		0.5
≥2x in index	1x SNP database (not genotyped) and not in >500 “real” control alleles		1
>1500 control alleles analysed:			
Variant allele frequency (%)		**Match in ethnical background**	
Not present		Yes	2
Not present		Unknown	2
Not present		No	1
0<freq≤0.02		Not important	1.5
0.02<freq≤0.05		Not important	1
0.05<freq≤0.1		Not important	0.5
>0.1		Not important	0
In >40 control alleles		Not important	Not pathogenic*

*variant should not be known as a founder mutation (at least 200 index alleles should have been analysed
Real control alleles indicate DNA from healthy people or patients that suffer from a non cardiac disease.

Table S2: Classification of potential splice site mutations using splice site predictions in AlaMut

	# programs with (aberrant) values	Difference between the potential splice site and genuine splice site in %*	Classification
Aberrant values of the genuine splice donor or acceptor site			
	≥2 (2 or more)	All: 70≤%diff≤100%	Group 1
	1	<70%	Group 2
	≥2 (2 or more)	At least one program: 40≤%diff≤70%; Others 70≤%diff≤100%	Group 2
	C	One program: ≤40%; Others 40<%diff≤100%	Group 3
	≥2 (2 or more)	At least two programs: ≤40%	Group 4
c.x-1 or -2 (acceptor) or c.y+1 or +2 (donor)			Group 5
New donor site or different value of predicted donor site that is not used in the wt gene			
Downstream site	≤5 (5 or less)	%diff <20%	Group 1
	≤5 (5 or less)	At least one program: 80%≤%diff≤100%	Group 2
	≤5 (5 or less)	At least one program: %diff≥100%	Group 3
Upstream (in the exon)	≤5 (5 or less)	%diff <50%	Group 1
Upstream (in the exon)	1	%diff<90%	Group 2
Upstream (in the exon)	≥2 (2 or more)	At least one program: 50%≤%diff≤60%; Others <60%	Group 3
Upstream (in the exon)	1	%diff≥90%	Group 3
Upstream (in the exon)	≥2 (2 or more)	At least one program: 60%<%diff≤90%; Others <60%	Group 3
Upstream (in the exon)	≥2 (2 or more)	At least one program: %diff>90%; Others >20%	Group 4
New acceptor site or different value of predicted acceptor site that is not used in the wt gene			
Downstream (in the exon)	≤5 (5 or less)	%diff <80%	Group 1
	≤5 (5 or less)	One program: 80%≤%diff≤100%; Others: %diff <80%	Group 2
	≤5 (5 or less)	One program: >100% Others: %diff <100%	Group 3
Upstream site	≤5 (5 or less)	%diff <60%	Group 1
Upstream site	≥2 (2 or more)	At least one program: 50%≤%diff≤60%; Others <60%	Group 2
Upstream site	1	%diff≥90%	Group 3
Upstream site	≥2 (2 or more)	At least one program: 60%<%diff≤90%; Others <60%	Group 3
Upstream site	≥2 (2 or more)	At least one program: %diff>90%; Others >20%	Group 4

% chosen arbitrarily

Table S3: Overview of the frequencies of separate endpoints in DCM index patients with and without a putative pathogenic mutation

Endpoints	Mutation – (n=329)**	Mutation + (n=80)***	*PLN* + (n=41)***	*LMNA* + (n=19)
Death from any cause	29/329 (9%)	14/80 (18%)*	6/41 (15%)	6/19 (32%)*
Age of death	57 (44-64)	59 (33-65)	64 (59-72)	51 (30-62)
HTX	6/329 (2%)	14/80 (18%)*	8/41 (20%)*	4/19 (21%)*
Age of HTX	57 (39-61)	49 (39-60)	56 (45-62)	49 (42-58)
MVA	37/319 (12%)	21/79 (27%)*	14/40 (35%)*	4/19 (21%)*
Age of MVA	48 (39-57)	44 (32-58)	45 (37-58)	37 (31-51)

The endpoints death from any cause, HTX and MVA are mentioned as number present/total number (%). Ages of the endpoints are mentioned as median (interquartile range).
HTX= heart transplantation
* Reflects a p-value < 0.05 comparing individuals with and without a (*PLN/LMNA*) mutation; ** Exclusion of VUS3; *** Double-heterozygous mutations excluded