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Genetics and Genetic Testing of Dilated Cardiomyopathy: a New Perspective

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Abstract

The completion of the Human Genome Project was a landmark achievement, but as clinical genetic testing becomes more mainstream, the extent of remarkable genetic variation is increasingly being appreciated. Newer DNA sequencing technology can now complete the sequencing of an entire human genome several times over in a matter of days, but this will undoubtedly add new challenges to the difficulty of distinguishing true pathogenic variants from benign variants in diagnostic genetics and in the research setting. The recent discovery of the role of titin gene (*TTN*) mutations in dilated cardiomyopathy (DCM) will make genetic testing in this disease more efficient. Furthermore, better understanding of genotype-phenotype associations will assist clinicians in identifying early stages of disease and providing more appropriate treatments. This high level of complexity requires an expert genetic team to offer counseling and to manage, deliver, and follow-up over time the results of genetic testing, which is particularly important for screening of family members potentially at risk. In DCM, genetic testing may be useful for the identification of non-carriers and asymptomatic carriers, as well as for prevention strategies, sport recommendations, and defibrillator implantation. It can also guide reproductive decision-making including utilization of pre-implantation genetic diagnostic strategies.

Introduction

Dilated cardiomyopathy (DCM) is the most common form of heart muscle disease and a leading cause of cardiac transplantation (Taylor *et al*., 2007). For many years, the cause of the disease has been considered unknown, possibly autoimmune, or due to a viral myocarditis, in some cases running in families and considered to be of genetic origin only in rare instances. However, the role of genetic factors has gained more importance over the last two decades, with the progressive discovery of a higher frequency of familial cases than previously anticipated (up to 30–50%), a large number genes (over 40) and gene mutations associated with the disease phenotype, and a clear evidence that even the so called "sporadic" cases may harbor a genetic defect (Hershberger *et al*., 2009). In particular in the last few years, impressive technological advances, large multicenter studies, and novel insights into the phenotype have significantly expanded our understanding of the origin of this common disorder and the opportunity for molecular diagnosis (Mestroni and Taylor, 2011).

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Genes and DCM: a New Role for Titin Gene in Dilated Cardiomyopathy

Initially, when the first genetic causes of cardiomyopathies were identified, it was a common understanding that while hypertrophic cardiomyopathy (HCM) was a disease caused by sarcomere dysfunction, DCM was a disease localized to the cytoskeleton (Towbin, 1998). Instead, it is clear now that the complexity of DCM is well beyond this simplistic hypothesis: genetic research over the past decade has shown that mutations in virtually every structure and pathway of the cardiac myocyte may lead to a dilated and poorly contracting ventricle. Currently, over 40 genes have been associated with DCM; most contributing to a low frequency of the prevalence of DCM, with a few notable exceptions accounting for 5–25% of cases (Sinagra *et al*., 2012). However, until recently, the translation of this knowledge into the clinical practice was significantly limited by the lack of DNA sequencing technology capable of interrogating dozens of genes in an efficient and affordable manner. According to current guidelines, to be considered for clinical genetic testing, disease genes should be associated with a change in management either in the patient or in his relatives at risk, and a test sensitivity of at least 40% (Ackerman *et al*., 2011). However, in DCM the expected test sensitivity was approximately only 20% (Lakdawala *et al*., 2012a), and the technical limitations of the traditional sequencing methods in screening such a large number of low frequency genes made virtually impossible a comprehensive genetic testing. Only recently, the discovery of a high frequency gene (titin) and the availability of high throughput technologies in clinical laboratories (next-generation sequencing) have made clinical testing feasible and comprehensive also in DCM.

The identification of the role of the sarcomeric gene titin has been a major advance in the study of DCM (Herman *et al*., 2012; McNally, 2012). The giant titin (*TTN*) gene (~350 exons) encodes the largest-known human protein (~35,000 amino acids, 2 μm long). It is highly expressed in the heart, where it functions as a giant spring, provides passive force, and regulates sarcomere contraction and signaling. Because of the giant size of the gene, after the initial reports of Gerull *et al*. (2002), *TTN* mutations were known in only a few families with DCM, but the *TTN* mutation frequency and therefore clinical impact in the overall DCM population were unknown.

Recently, a multicenter group of investigators from Harvard, University of Colorado, University of Trieste, the Imperial College of London, Mayo Clinic at Rochester, and the University of Washington Seattle answered this question (Herman *et al*., 2012). The authors studied a large cohort of subjects (792) including 312 subjects with DCM, 231 subjects with HCM, and 249 controls: patients were screened either by next-generation sequencing in the Harvard's laboratory, or by direct sequencing in the Human Genome Center of Seattle. The results were surprising: of the 72 unique "radical" mutations (Figure 1) found in the *TTN* gene (25 nonsense, 23 frameshift, 23 splicing, and 1 large CNV), the majority were in the DCM group (over 20%), while in HCM the frequency was similar to the control group (in only 1% and 3%, respectively). In DCM, *TTN* mutations were over-represented in the titin A-band and absent from the Z-disk and M-band ($P \leq 0.01$), suggesting a domain-specific dominant-negative effect, rather than haploinsufficiency: in other words, the mutant protein is incorporated in the sarcomere, but unable to function normally. Furthermore, DCM *TTN* mutations truncated the protein's C-terminal kinase domain probably impairing signaling, and some of them were predicted to disrupt recoil. Genotype-phenotype analysis showed a high penetrance (>95%) after 40 years, but no other significant difference except some unusual nuclei on histology and a worse prognosis in male than female carriers ($P=4\times10^{-5}$). Finally, *TTN* mutations were found both in ~25% of familial DCM and in 18% of sporadic DCM, a difference that was not statistically significant.

The important role of *TTN* in the pathogenesis of the dilated phenotype is not surprising: in a recent work, Taylor *et al*. (2011) found a significant *TTN* mutation frequency in arrhythmogenic right ventricular cardiomyopathy (ARVC). Furthermore, functional investigations on a novel DCM gene, *RBM20*, showed that this protein is a key component of the titin pathway by allowing the alternative splicing and post-translational regulation of *TTN* (Guo *et al*., 2012). Overall, these discoveries suggest an important role of this gene in the origin of dilated cardiomyopathies.

Advances in the Phenomics of Dilated Cardiomyopathy: from Better Diagnosis to Better Risk Stratification

Recent studies have also brought more insights into the association between the genotype and the phenotype in DCM, information that is eventually critical in the management of genetic testing as we discuss below. McNair *et al*. (2011) investigated the frequency and type of cardiac sodium channel (*SCN5A*) mutations in a large DCM cohort. The frequency of rare variants was found in the expected range of 2% and not surprisingly, the carriers of *SCN5A* putative mutations had a remarkable tendency toward an arrhythmogenic trait, including conduction defects, atrial fibrillation, and ventricular tachycardia, while the prevalence of arrhythmia was significantly lower in non-carrier DCM patients. In the clinical setting, these features may be “red flags” of channelopathy-related DCM and may represent a useful tool in the clinical setting when estimating the pathogenicity of a novel *SCN5A* rare variant in patients with DCM.

Another recent study has examined the incidence of malignant arrhythmia in a cohort of carriers of lamin A/C (*LMNA*) gene mutations (van Rijsingen *et al*., 2012). *LMNA* encodes the two intermediate filaments lamin A and C, which provide the backbone network supporting the nuclear wall in cells in heart, muscle, and other organs. The phenotype resulting from *LMNA* mutations can range from an isolated cardiomyopathy to a muscular dystrophy, and frequently these clinical features can produce “overlapping phenotypes,” again a precious information when evaluating the possible pathogenic role of a rare variant in a patient with DCM. *LMNA* mutations occur in approximately 8% of cases and in these cases a poor prognosis and a risk of sudden death were known (Meune *et al*., 2006; Taylor *et al*., 2003). In their study, van Rijsingen *et al*. (2012) collected a large multicenter European cohort of DCM patient carriers of *LMNA* mutations and examined their arrhythmic events during a mean follow up of 4 years. The investigators found that life-threatening events occurred in 18% of cases, and that they were associated with 4 independent risk factors for malignant ventricular arrhythmia (MVA): non-sustained ventricular tachycardia, left ventricular ejection fraction less than 45%, a truncation mutation, and the male gender. Finally, they discovered that the four risk factors had a cumulative effect which increased as more risk factors were present, ranging from a normal survival if zero or one risk factor was present, to 50% mortality in two years at less than 40 years of age if all four were present, even in the absence of severe left ventricular dysfunction (Figure 2).

Finally, another recent study has focused on the phenotype of early “subclinical” stages of disease (LVEF ≥55%) (Lakdawala *et al*., 2012b). Lakdawala *et al*. studied a cohort of 62 subjects belonging to families with DCM caused by sarcomeric gene mutations: of those, 12 were subclinical carriers, 21 had overt signs of disease, and 29 were non-carriers. Detailed echocardiographic investigations showed that subclinical carriers have subtle but significant changes in systolic myocardial tissue velocity, longitudinal, circumferential and radial strain, and longitudinal and radial strain rate, but no changes in diastolic parameters compared to the non-carriers. Interestingly, these changes had an opposite pattern compared to hypertrophic cardiomyopathy subclinical carriers, where the initial signs of disease are those of diastolic dysfunction. These findings suggest that an accurate state-of-the-art clinical

examination can contribute to the comprehensive assessment of carriers of rare variants suspected to be causal of the disease.

New High-throughput Technologies and Genome-wide Approaches

A dramatic advance in sequencing technology, called next-generation sequencing, recently brought the efficiency of the assay from sequencing 1 gene in 1 week to 50 genes overnight, dropping the cost and the turnaround time and increasing the sensitivity of genetic testing in genetic disorders such as DCM. Next-generation sequencing in the research setting has been used to sequence the whole exome, which is the portion of genome coding for all proteins of the human body. This approach has already produced important results in the study of DCM, when the causal gene was unknown. Theis *et al*. (2011) identified, by linkage analysis and exome sequencing, *GATAD1* as a gene for a rare form of autosomal recessive DCM. *GATAD1* binds to a histone modification site that regulates gene expression. Norton *et al*. (2011) discovered, by genome-wide copy number variation analysis in an autosomal dominant DCM family, a mutation in another novel disease gene, the heat shock protein cochaperone BCL2-associated athanogene 3 (*BAG3*). They confirmed the presence of *BAG3* gene rare variants in ~2% of their DCM study population.

From Bench to Bedside: Clinical Impact of Genomic Advances

Current guidelines may provide an important help to clinicians in selecting the appropriate genetic testing approach in cardiomyopathies. In 2009, on behalf of the Heart Failure Society of America, a group of U.S. experts published the “Practice Guideline” for the Genetic Evaluation of Cardiomyopathy (Hershberger *et al*., 2009). In 2010, a group of European experts published a position statement on genetic counseling and testing in cardiomyopathies on behalf of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases (Charron *et al*., 2010). Finally, in 2011, the Heart Rhythm Society (HRS) and the European Heart Rhythm Association (EHRA) published an expert consensus statement on the state of genetic testing for the channelopathies and cardiomyopathies (Ackerman *et al*., 2011). Therefore, the clinician can currently count on a number of international updated guidelines to provide an appropriate management to patients with genetic heart muscle diseases: here we summarize the most important principles (Ackerman *et al*., 2011; Charron *et al*., 2010; Hershberger *et al*., 2009). Class I recommendation (”is recommended”) for genetic testing include: (a) index cases with cardiomyopathy, and (b) family members for the mutation identified in the proband when therapy/protective measures/lifestyle adaptations may be adopted. Interestingly, Class IIa recommendation is when results of genetic testing may be useful for reproductive counseling or instances in which genetic testing is requested by the patient who wants to know his/her mutation status. Genetic counseling should be part of proper management of genetic cardiomyopathies and is recommended for all patients and relatives with the familial heart diseases and should include discussion of the risks, benefits, and options available for clinical testing and/or genetic testing. Importantly, treatment decisions should not rely solely on the genetic test result but should be based on an individual’s comprehensive clinical evaluation. All guidelines agree that at the current status of knowledge, it is preferable for pre-genetic test counseling, genetic testing, and the interpretation of genetic test results to be performed in centers experienced in the genetic evaluation and in family-based management of the heritable cardiomyopathies (Ackerman *et al*., 2011; Charron *et al*., 2010; Hershberger *et al*., 2009).

Family screening is an important part of a correct clinical approach to DCM. Besides the opportunity to perform genetic testing, family screening with clinical examination of family members at risk, with the possibility of catching early stages of the disease, may have

significant prognostic impact. Moretti *et al*. (2010) compared the natural history of subjects with non-familial DCM to affected relatives of DCM families identified by family screening. As shown in Figure 3, the survival free from death or heart-transplant in sporadic DCM (solid line) was worse compared to the relatives of familial DCM (dotted line). This study suggests that family screening itself, with or without an identified mutation, can effectively identify DCM patients at an earlier stage of disease and improve survival.

Novel technologies now offer unprecedented possibilities to test a large number of genes. Newer DNA sequencing technology (whole-exome and whole-genome sequencing) can now complete the sequencing of an entire human genome several times over in a matter of days -- orders of magnitude faster than the nearly thirteen years required for the initial first-pass done by the Human Genome Project consortium. This technology, which is already used in clinical genetic testing, will undoubtedly add new challenges to the difficulty of distinguishing signal from noise. For instance, the pan cardiomyopathy panel of Harvard Partners laboratory can now test 51 cardiomyopathy genes with a turn-around time of only 8–12 weeks and a cost not significantly different from the cost of sequencing a modest number of genes a few years ago (http://www.hpcgg.org/).

Discerning Rare Benign Variations Versus Pathogenic Mutations in DCM

Meder *et al*. (2011) have recently tested the power and limitations of next generation sequencing as a gene mutation screening tool with a proof-of-concept study. They screened 10 patients with either HCM or DCM by next-generation sequencing for 47 known or putative DCM genes. They found a remarkable number of novel variants (~60,000) including ~21,000 novel deleterious variants such as insertions/deletions. They were able to successfully identify 6 known disease-causing mutations in their study patients. However, they also identified several novel variants (~30) predicted by all consensus criteria to be “disease-causing mutations,” in 9/10 subjects, underlying the difficulty to discriminate between benign and pathogenic variants.

Indeed, the Human Genome Mutation database contains >100,000 mutations in ~4,000 genes reported as pathogenic, mostly missense mutations. However, the unexpected variability of the human genome represents a challenge in the clinical setting. Two large studies have considered this problem: how frequent in the general population are variants that are considered “pathogenic”? And are those variants true pathogenic mutations with low penetrance or just rare benign variations?

Norton *et al*. (2012) analyzed the presence of mutations previously reported as causative for DCM in the cohort of 2,439 subjects from the NHLBI GO Exome Sequencing Project (ESP). Reported pathogenic mutations in 30 DCM genes were present in 17% of the ESP population. Of these mutations, 42% had previous functional data to support their pathogenic role, suggesting that they were unlikely to be false-positives. Based on these findings, the authors proposed a series of novel criteria for the evaluation of rare variants in Mendelian disorders, including a novel more stringent cut-off for the allele frequency of ≤0.04%, suggesting that higher frequencies are of less certain pathogenicity.

In another study, Tennessen *et al*. (2012) examined the exome sequencing data of the 2,440 subjects of the NHLBI GO ESP cohort. In this study the authors examined over 15,000 protein-coding genes to understand the contribution of rare variants to the risk of disease. They found that each subject had ~14,000 SNVs, of which 200 were novel, mainly rare nonsynonymous variants, and that these rare variants predicted to disrupt the protein function of over 300 genes for each person. As the authors note, this abundance of variation

is consistent with the explosion of human population growth. They concluded that to detect the effect of rare variants very large sample size and population-specific data are needed.

Another interesting perspective is that offered by Bick *et al*. (2012), who screened for mutations of 8 sarcomeric genes associated with cardiomyopathy by next-generation sequencing in 2 large cohorts, the Framingham and Jackson Studies Cohorts, for a total of 3,600 subjects. They found rare missense sarcomeric variants in 11% of population, and likely pathogenic variations in 0.6%, twice the previous estimates. Of the 22 individuals found to have likely pathogenic variants, only 4 had cardiac hypertrophy, raising the question if the other variants have low penetrance or are single-nucleotide polymorphisms (SNPs). However, when the authors investigated the risk for cardiovascular events in carriers of any rare missense sarcomeric variants, they found that this was significantly increased (HR 2.3), raising the question that some of these variants are indeed pathogenic.

Overall, these recent studies indicate once more that integration of genetic testing with functional assays, robust bioinformatics, large control cohorts, and expert clinical evaluation can better assist clinicians in discerning pathogenic versus benign rare variants.

Concluding Remarks: the Ying-Yang of the Current Advances

Yang

In the past few months, a number of important scientific and technological advances have changed our approach to the genetics of DCM. We now have approximately 50 cardiomyopathy genes, 40 of which associated with DCM genes, and a novel powerful assay for genetic testing, next-generation sequencing. The frequency for each gene from a low yield (<1% to ~8%) with the identification of *TTN* is now up to 25% therefore the expected mutation detection rate is >40% of cases. This indicates that genetic testing can now be considered a class I recommendation according to current guidelines (Ackerman *et al*., 2011). Phenomic studies, such as the identification of red flags/overlapping syndromes (muscular dystrophy, conduction disease, arrhythmias, and other cardiomyopathies in the family), biomarkers, and epigenetic factors are useful diagnostic tools (Piran *et al*., 2012). Novel data for better risk stratification (such as for *LMNA*) are able to modify patient management and justify once more genetic testing (class I recommendation) and therapeutic intervention (such as AICD).

Ying

However, the new discoveries and tools generate new challenges for geneticists and clinicians. The unexpected remarkable human genetic variation makes genetic testing a *dynamic* process: testing reports need to be updated and possibly changed based on new data about rare genetic variants. To ensure that a rare variant is appropriately classified [benign, pathogenic, or of uncertain significance (VUS)] it is evident now that over 2,000–3,000 ethnically matched controls may be needed to control for genomic variability. Fortunately, there are some databases now available to begin to meet these needs: the 1000 Genome Project, the NHLBI Exome Sequencing Project, and the dbSNP.

To reliably assess the pathogenic role of a mutation, more stringent criteria are needed: variants should be rare (new cut-off allele frequency in DCM <0.04%), “radical” (in/del, splice, nonsense), and segregate within the family, compared to large cohorts of ethnically matched controls, analyzed with robust bioinformatics approaches, and possibly confirmed by functional assays. In this regard, induced-pluripotent stem cells (iPS) repositories of patients with DCM could play an important role.

Clearly this high level of complexity requires an expert genetic team for counseling, managing, delivering, and following up over time the results of genetic testing (www.genetest.org; Lakdawala *et al*., 2012a; Maron *et al*., 2012; Norton *et al*., 2012). Genetic testing is in fact particularly important for screening of family members potentially at risk and must start from successful genotyping of the proband. It may be useful for the identification of non-carriers (no need of precautions and surveillance), although exceptions may exist (multiple mutations, mutation/VUS, laboratory errors). Genetic testing may identify genotype^{+} phenotype^{-}, useful for prevention strategies, sport recommendations, and indications for defibrillator implantation and therapies as discussed above. Finally, genetic testing may be requested by couples to guide reproductive decision-making including utilization of pre-implantation genetic diagnostic strategies.

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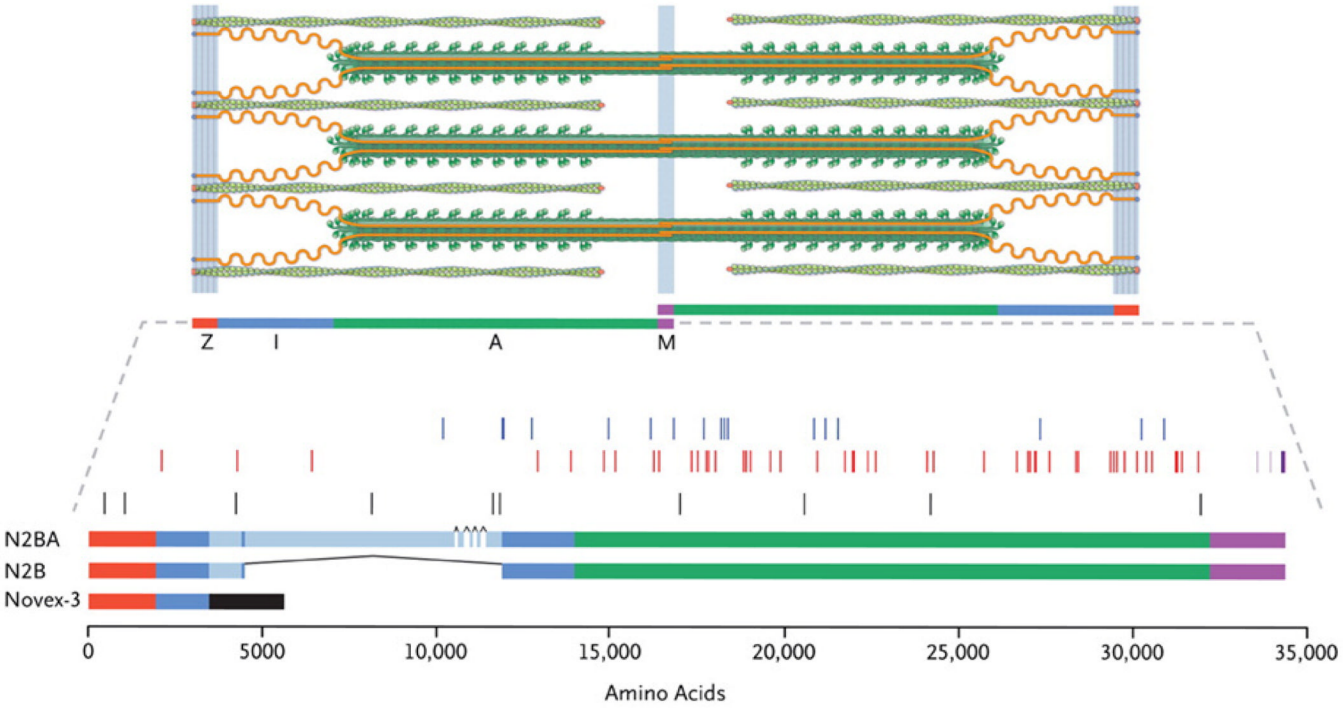
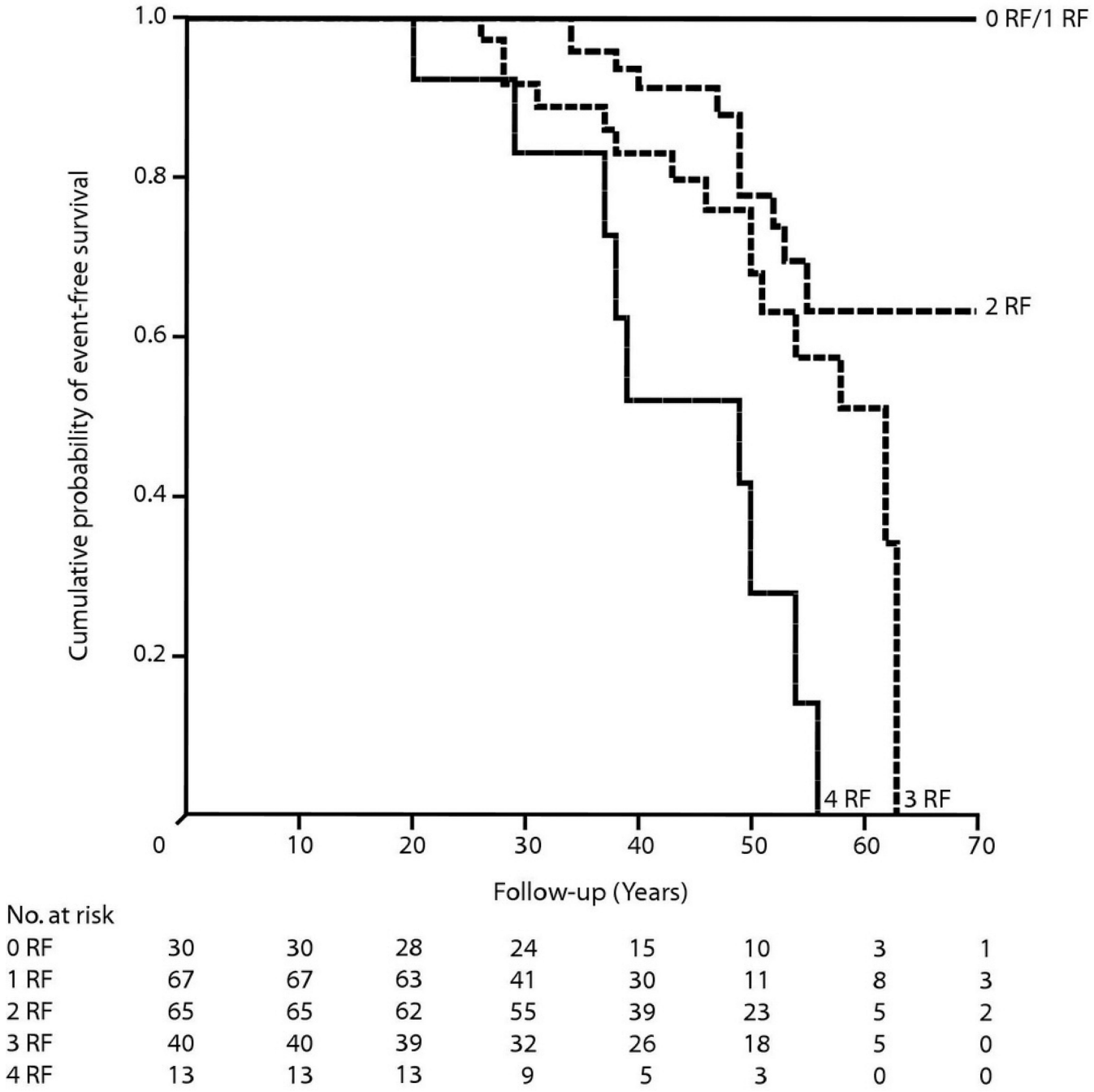


Figure 1.

Titin mutations in DCM. Top: the cardiac sarcomere including titin (orange), the thick filaments (green rods with globular heads) and thin filaments (green coiled ovals), Z-disk (red), I-band (blue), A-band (green), and M-band (purple). Middle: DCM *TTN* splicing and copy-number mutations (blue), nonsense and frameshift mutations (red); truncating mutations in controls and subjects with hypertrophic cardiomyopathy (black), truncating mutations in congenital myopathy (light purple) or limb-girdle muscular dystrophy (dark purple). Bottom: TTN isoforms and sequence variants. From Herman *et al*., with permission (Herman *et al*., 2012).



No. at risk

0 RF	30	30	28	24	15	10	3	1
1 RF	67	67	63	41	30	11	8	3
2 RF	65	65	62	55	39	23	5	2
3 RF	40	40	39	32	26	18	5	0
4 RF	13	13	13	9	5	3	0	0

Figure 2.

Survival in patients with *LMNA* mutations. Kaplan-Meier event-free survival from the date of first visit stratified by 4 independent risk factors (RF): non-sustained ventricular tachycardia, left ventricular ejection fraction <45% at the first visit to the cardiologist, being male, and truncation mutations. The event was defined as occurrence of malignant ventricular arrhythmias (appropriate implantable cardioverter-defibrillator treatment, cardiopulmonary resuscitation, or sudden cardiac death). From van Rijsingen *et al*., with permission (van Rijsingen *et al*., 2012).

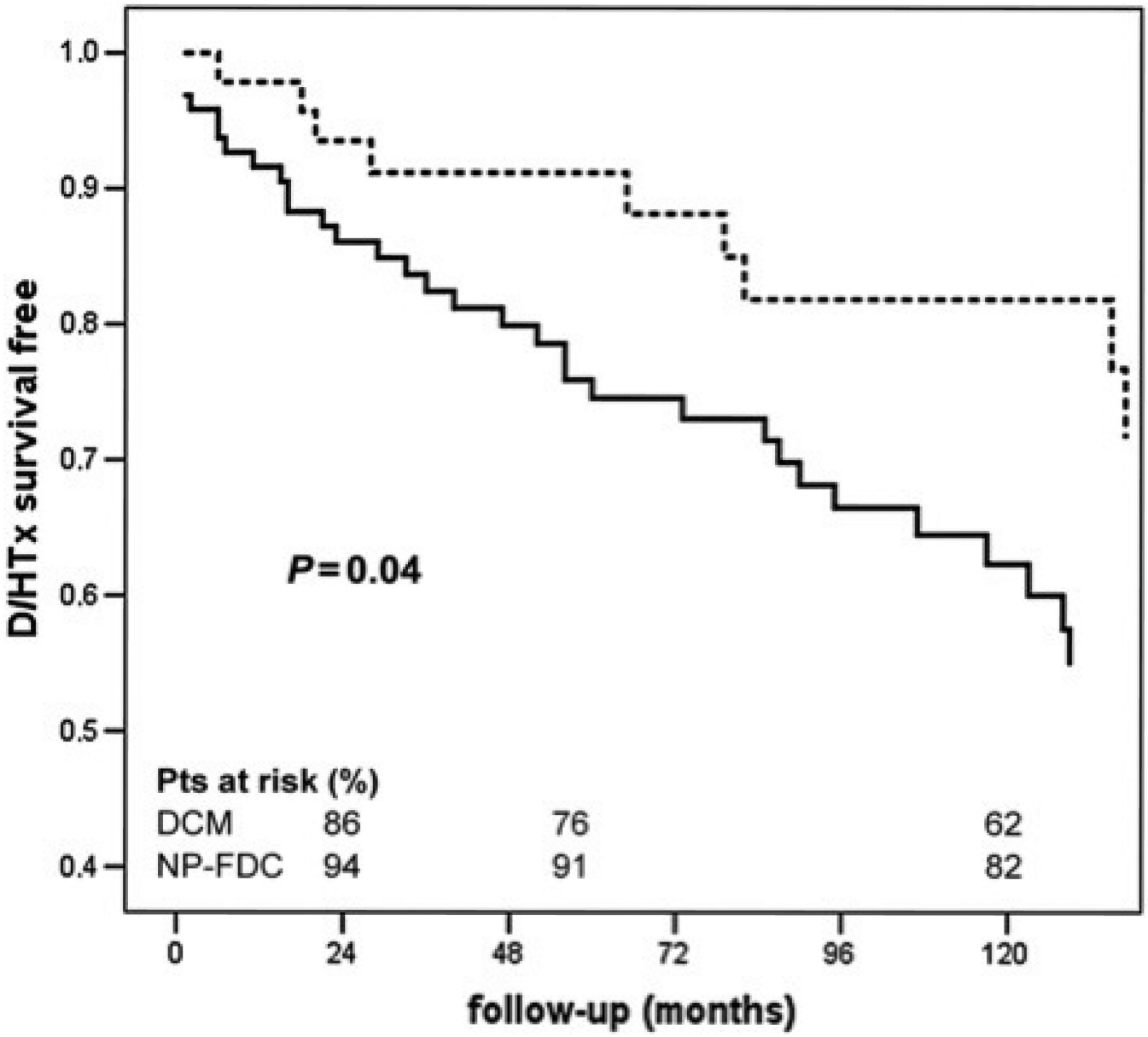


Figure 3.
Survival in patients with sporadic DCM and familial DCM. Analysis of survival free from heart-transplant (HTx) in sporadic (solid line) and non-proband familial DCM (dotted line). D/HTx, death/heart transplant. From Moretti *et al*., with permission (Moretti *et al*., 2010).