When lamin A/C fails, the heart suffers

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Voor mijn vader

CONTENTS

CHAPTER 1

Often Seen but Rarely Recognised: Cardiac Complications of Lamin A/C Mutations

Based on:
JH van Berlo, D Duboc, YM Pinto, Often seen but rarely recognised: cardiac complications of lamin A/C mutations.
European Heart Journal 2004 (25): 812-814

INTRODUCTION

Inherited forms of heart failure

Heart failure is a clinical syndrome, where complaints of breathlessness and fatigue are caused by an inability of the heart to provide enough oxygenated blood to the peripheral tissues. In addition to this morbidity, heart failure is also a quite lethal condition: mortality rates can mount to 50% in five years, making the prognosis for some forms of heart failure as gruesome as for many malignant diseases[1]. The majority of cases of heart failure is caused by ischaemic heart disease, caused by atherosclerotic disease with subsequent acute or chronic ischemic loss of cardiac tissue. Further causes are hypertension, alcohol abuse or valvular dysfunction. However, about 30% of cases remain idiopathic. Of these, 30% appear to be caused by an inherited form[2]. Familial dilated cardiomyopathy (DCM) was first described in 1961 by Battersby and Glenner[3]. They observed that the syndrome of heart failure was transferred in a family in an autosomal dominant fashion.
It lasted until the late 90's before a causative gene mutation had been found in a family with dilated cardiomyopathy[4]. At this moment 16 different autosomal dominant gene mutations have been found to cause DCM[(for review see 5)]. However, usually no follow up papers are published identifying another family with a mutation in the same gene. This led to the idea that in dilated cardiomyopathy every family harboured its gene mutation. When the lamin A/C gene was published to cause DCM, soon thereafter many other investigators identified families with a mutation in the lamin A/C gene, suggesting that many families with inherited cardiac disease may suffer from a mutation in the lamin A/C gene. The disease caused by these mutations usually has devastating consequences.

Heart failure can be caused by lamin A/C mutations

Most cardiologists are quite alarmed when they learn that a patient carries the mutation in the SCN5a gene responsible for "Brugada" syndrome. In contrast, when confronted with a family carrying a lamin A/C mutation, they are often less aroused. Many investigators have published families carrying a lamin A/C mutation that results in a cardiac phenotype[6]. This underscores the fact that mutations in the lamin A/C gene very often affect the heart, often with devastating consequences. However, in contrast with SCN5a and other genes known to cause sudden death, the role of lamin mutations in heart failure as well as sudden death is only now beginning to be appreciated. The high incidence of sudden cardiac death and high number of mutation carriers in need of cardiac transplantation underscores the need to increase our awareness and understanding of cardiac pathologies caused by lamin A/C mutations.

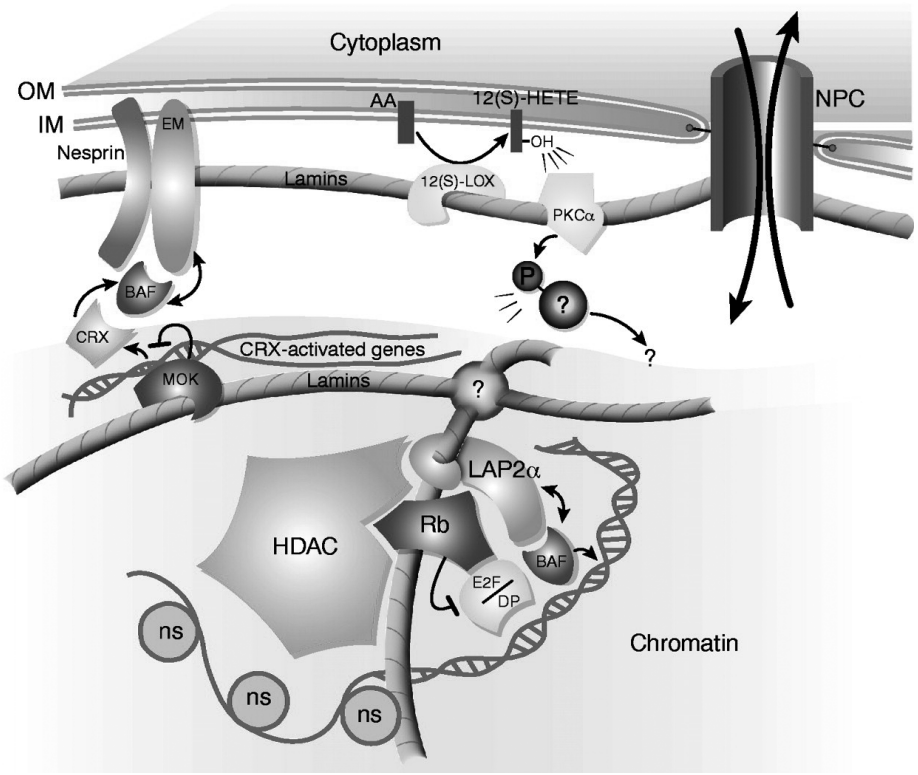

Figure 1
Proposed model of lamin A/C in the nucleus. Depicted are a few of the many interaction partners that have been found. Important structural partners include Emerin (EM), Nesprin and the Nuclear Pore Complex (NPC). Furthermore, some transcriptional regulators bind to lamin A/C, such as BAF, MOK and Rb. The Figure shows a **hypothetical** model how lamin A/C interacts with these regulators and how gene transcription might be influenced by lamin A/C (From Zastrow et al[9] reprint with permission of author)

Pathophysiology of lamin A/C mutations

The lamin A/C gene codes, by alternative splicing, for the nuclear envelope proteins lamin A and lamin C[7,8]. These proteins are located on the inner surface of the inner nuclear membrane and interact with many other proteins and with DNA (see figure 1)[9]. Mutations in lamin A/C have been related to different diseases. At the start of the studies described in this thesis they had been shown to cause Emery-Dreifuss and Limb-girdle muscular dystrophy, Dunnigan's type familial partial lipodystrophy and Dilated Cardiomyopathy[10-13]. In the subsequent years more than 5 other distinct phenotypes have been associated with lamin A/C mutations, e.g. mandibuloacral dysplasia, axonal neuropathy CMT2B1, and restrictive dermopathy[14-16]. Recently, interest in this protein has been further heightened by the finding that lamin A/C mutations also cause some forms of progeria, which originate premature aging and death in the second decade of life[17-19].
Although the broad range of diseases caused by lamin A/C mutations suggests that this protein plays a vital role in diverse biological systems, the precise pathophysiology of lamin

A/C mutations remains enigmatic.
Some light has been shed by the recent development of lamin knockout models. Mice lacking lamin A/C also develop cardiomyopathy, thus providing an animal model to study the cardiac effects of lamin dysfunction[20]. One current hypothesis on how lamin A/C dysfunction may affect cellular and cardiac biology states that disturbed lamin function impairs nuclear stability. In turn, this may hinder nuclear functions, as has been recently suggested[21]. A second hypothesis, not necessarily incompatible with the first one, states that lamins A and C are necessary to direct the proper function of transcription factors, so lamin dysfunction could impair the regulation of gene expression[22]. Finally, it has been proposed that vital cytoskeletal proteins adhere to lamins, and that the loss of lamin function interferes with cytoskeletal functions. Despite these insights, there is still no clear concept explaining how these functions of the lamins explain the cardiac pathology seen in carriers of lamin A/C mutations.

Prevalence of lamin A/C mutations

Nevertheless, the idea that lamin A/C must be important for the heart is irrefutably demonstrated by the finding that mutations in the lamin A/C gene can cause cardiomyopathy. In the last few years more than 16 different genes have been found to cause dilated cardiomyopathy when mutated[23]. When all studies reporting one of these causative genes for hereditary dilated cardiomyopathy are examined together, lamin A/C mutations emerge as the most frequently reported by far, with more than half of all published reports involving patients with a mutation in the lamin A/C gene. Part of the high discovery rate of lamin A/C gene mutations could be due to the specific phenotype of the DCM caused by mutations in this gene, but this does not seem to fully explain the high frequency of reports compared to other genes.
Within the group of hereditary cardiomyopathies, lamin A/C mutations seem to comprise a highly prevalent, if not the most prevalent group. Assuming that 10% of heart failure cases are familial, and that half may be caused by mutations in the lamin A/C gene, as has been suggested, then 2.5% to 5% of the heart failure population harbour a mutation in the lamin A/C gene. This prevalence is probably excessive, but decreasing estimates by a factor of 10 still leaves ~0.5% of all patients with heart failure carrying a mutation in the lamin A/C gene. This means that in the Netherlands, which has 200,000 heart failure patients, 1000 of them may carry a mutation in the lamin A/C gene. Consistent with this idea, since 2001, 150 carriers of a mutation in this gene have been identified in the Netherlands alone. The relative ease with which we encounter these mutations again underscores the notion that lamin A/C mutations are probably more prevalent than currently appreciated.
By comparison, 108 patients with the Brugada syndrome were reported in 1998, 6 to 9 years after the initial publication[24]. The first mutation in the lamin A/C gene was reported in 1999 by Bonne et al. In the 5 years since that report appeared, over 300 patients with a lamin A/C gene mutation causing heart failure have been reported. By comparison, recent estimates of the incidence of Brugada syndrome range from 5 to 66 per 10,000. However, this estimate is based on populations in parts of Southeast Asia and a small region in Japan and is probably an overestimation for the rest of the world[25]. Therefore, although there are fewer clinical clues to arouse suspicions of lamin A/C mutations, their prevalence seems to have caught up with that of the Brugada syndrome.

AIM OF THE THESIS

Aim of this thesis is to investigate in a translational manner the cardiac consequences of mutations or polymorphisms in the lamin A/C gene.

Based on this aim we defined the following goals:

- Identify from literature the specific cardiac phenotype of patients with a lamin A/C mutation
- Identify a molecular mechanism that might explain the cardiac phenotype of lamin A/C mutations
- Describe the cardiac phenotype in one family with a newly found lamin A/C mutation
- Investigate a possible treatment strategy to prevent sudden cardiac death in patients with a lamin A/C mutation
- Investigate whether polymorphisms in lamin A/C are related to heart failure

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CHAPTER 2

Do lamin A/C mutations portend a high risk of sudden death?

Jop H. van Berlo, Willem G. de Voogt, Anneke J. van der Kooi, J. Peter van Tintelen, Gisèle Bonne, Rabah Ben Yaou, Denis Duboc, Tom Rossenbacker, Hein Heidbüchel, Marianne de Visser, Harry J.G.M. Crijns, Yigal M. Pinto. Meta-analysis of clinical characteristics of 299 carriers of LMNA gene mutations: do lamin A/C mutations portend a high risk of sudden death?
Journal of Molecular Medicine 2005 (83): 79-83

ABSTRACT

Objective: To evaluate common clinical characteristics of patients with lamin A/C gene mutations that cause either isolated dilated cardiomyopathy or dilated cardiomyopathy in association with skeletal muscular dystrophy.
Methods: We pooled clinical data of all published carriers of lamin A/C gene mutations as cause of skeletal and/or cardiac muscle disease and reviewed ECG findings.
Clinical characteristics: Cardiac dysrhythmias were reported in 92% after the age of 30. Heart failure was reported in 64% after the age of 50. Sudden death was the most frequent reported mode of death (46%) in both the cardiac and the neuromuscular phenotype. Carriers of lamin A/C gene mutations often received a pacemaker (28%). However, this intervention did not alter the rate of sudden death. Review of the ECG showed typically a low amplitude P wave and prolongation of the PR interval with a narrow QRS complex.
Conclusions: This meta-analysis suggests that cardiomyopathy due to lamin A/C gene mutations portends a high risk of sudden death and that this risk does not differ between subjects with predominant cardiac or neuromuscular disease. This implies then that all carriers of a lamin A/C gene mutation need to be carefully screened with particularly emphasis also on tachyarrhythmias. Prospective studies are needed to evaluate risk stratification and proper treatment strategies.

INTRODUCTION

Twenty to thirty percent of patients with idiopathic dilated cardiomyopathy appear to have a familial form of dilated cardiomyopathy (DCM). Elucidation of the genetics of familial dilated cardiomyopathy showed an important role for mutations in the lamin A/C gene[1,2]. Lamins A and C are alternative splice products of the lamin A/C gene[3-5] and form a lamina on the inner surface of the inner nuclear membrane[6]. Patients carrying a mutation in the lamin A/C gene are seen by neurologists with Emery-Dreifuss[7] (EDMD) or limb girdle muscular dystrophy[8] (LGMD). Recently lamin A/C gene mutations were also described to cause isolated dilated cardiomyopathy or AV-conduction disturbances with or without atrial arrhythmias[9].
Despite the progress made in the genetics of laminopathies, many important clinical issues remain unresolved. Clinical characteristics to suspect cardiac laminopathy are ill described and it is unknown which, if any, clinical parameters bear prognostic significance. To generate hypotheses on common clinical phenotypes we systematically evaluated the clinical characteristics already described for the known carriers of lamin A/C mutations.

METHODS AND DEFINITIONS

Using the keywords lamin and/or Emery Dreifuss and/or limb girdle muscular dystrophy and/or dilated cardiomyopathy in a PUBMED search we identified 21 publications between March 1999 and March 2002 reporting lamin A/C gene mutations or the patient characteristics of the mutation carriers[1,7-26]. We collected all provided patient characteristics and included all carriers and patients with a lamin A/C gene mutation described to cause EDMD, LGMD1B or DCM, regardless presence of dysrhythmias or dilated cardiomyopathy. Dysrhythmias were defined as presence of either conduction system disease or supraventricular or ventricular arrhythmias. Patients with familial partial lipodystrophy, progeria, axonal neuropathy and mandibuloacral dysplasia, caused by mutations in the lamin A/C gene, were excluded, because a similar cardiac phenotype has not been described for these diseases.
To partially address the prevalence of dysrhythmia and sudden death, we checked for data in our own database. Dysrhythmia is defined as presence of either conduction system disease, sinus node dysfunction, atrial premature beats, fibrillation or flutter, ventricular premature beats or ventricular tachycardia. Furthermore, we analysed ECG characteristics of subjects we had previously reported[24].
Data were analysed using SPSS 10.0. For comparison between groups Chi-square was used. For age dependent penetrance the Chi-square for trend was used and for continuous data the Student's t-test was used.

Table 1a
Clinical characteristics of patients with LMNA mutation leading to EDMD or LGMD (with muscular dystrophy) or DCM (without muscular dystrophy)

	With muscular dystrophy	Without muscular dystrophy	All patients
number of patients	**190**	**109**	**299**
age at presentation	5.4	36	
age	26.6	36.3	31.2
Male / female / unknown	104 / 69 / 17	59 / 50 / 0	163 / 119 / 17
nr of unaffected carriers	4	23	27
dysrhythmias*	**108**	**73**	**181**
- sinusbradycardia	8	6	14
- AV block	72	63	135
- atrial fibrillation	19	28	47
- arrhythmia, unspecified	56	11	67
pacemaker implanted	**48**	**36**	**84**
Left Ventricular End Diastolic Diameter	48 mm	54 mm	52 mm
fractional shortening	28 %	28 %	28 %
left ventricular dilation	23	54	77
systolic dysfunction	33	60	93
heart failure	**25**	**53**	**78**

* Includes AV conduction disturbances (1st degree AV block or worse) and / or ventricular arrhythmias (spontaneous VPB's on 12 lead ECG or worse) and / or atrial arrhythmias (APB's, AF)
AV = Atrio-ventricular

DESCRIPTION OF COMMON CLINICAL CHARACTERISTICS

In total we identified from literature 299 carriers of lamin A/C mutations of whom the clinical characteristics are shown in table 1a.
The most prominent and often described characteristic cardiac features of lamin A/C mutations were conduction system disease and arrhythmias. Because not all publications reported these features separately we pooled the data available on presence of conduction system disease, supraventricular and ventricular arrhythmias under the term dysrhythmia. This also reflects that different forms of rhythm disturbances have been described for lamin A/C gene mutations.

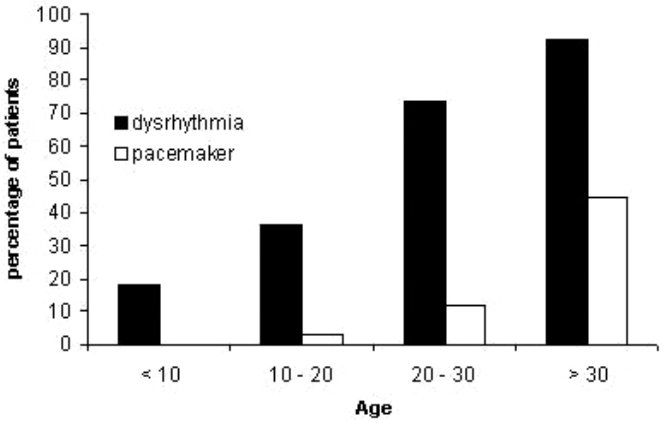

Figure 1a
Age dependent penetrance of dysrhythmias and pacemaker implantation.

Dysrhythmias were reported increasingly with increasing age(figure 1a). To assess age dependent penetrance of dysrhythmias we analysed presence of dysrhythmias in 157 out of the total 299 patients, because these patients were alive at time of publication and age was reported. Dysrhythmias were described in 111 of 157 patients (71%). Below the age of 10, 2 out of 11 patients had developed dysrhythmias (18%), between the age of 10 and 20 it had risen to 36%, further increasing to 74% in patients between 20 and 30 yrs and to 92% of 79 in patients older than 30 yrs ($p<0.001$ with Chi-square for trend). Dysrhythmias were equally distributed between carriers who presented with either a neuromuscular or a predominantly cardiac phenotype (table 1, $p=0.085$ with Chi-square).
A typical ECG shows a low amplitude P wave and PR interval prolongation with a normal QRS duration (figure 2).
The pacemaker implantation rate was exceptionally high: in 40 out of the 157 patients, who were alive with known age, a pacemaker was implanted (25%), indicating the eventual severity of the conduction system disease (figure 1a). The age dependent pacemaker implantation rate was 0% below the age of 10, 3% in patients between 10 and 20, increasing to 12% in patients between 20 and 30 yrs and further increasing to 44% in patients above the age of 30 ($p<0.001$ with Chi-square for trend). In total 32 of the 76 patients who have died had a pacemaker implanted (42%) which is the same implantation rate as in the age group above 30 years ($p=0.782$ with Chi-square).

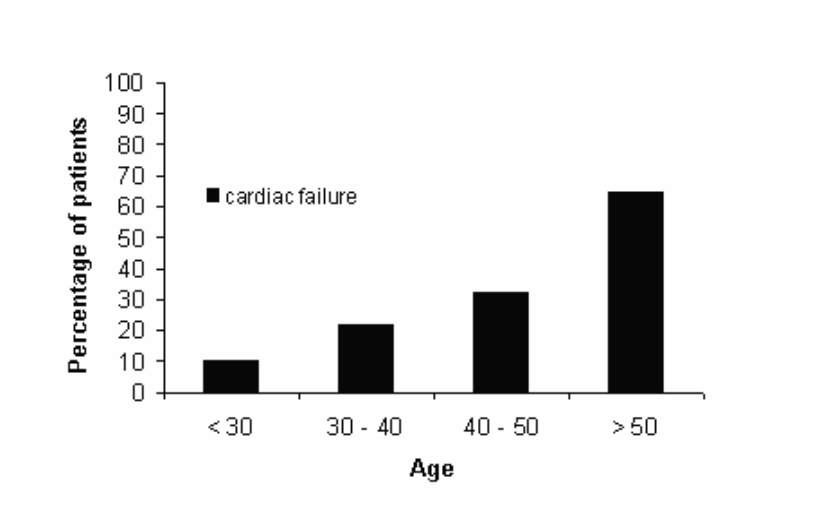

Figure 1b
Age dependent penetrance of cardiac failure.

Heart failure was reported at a later age and less frequently than dysrhythmias (figure 1b). It was reported in 10% in the age group below 30 yrs, increasing to 22% in patients between 30 and 40 yrs, 32% in those between 40 and 50 yrs and 64% above 50 yrs ($p<0.001$ with Chi-square for trend).

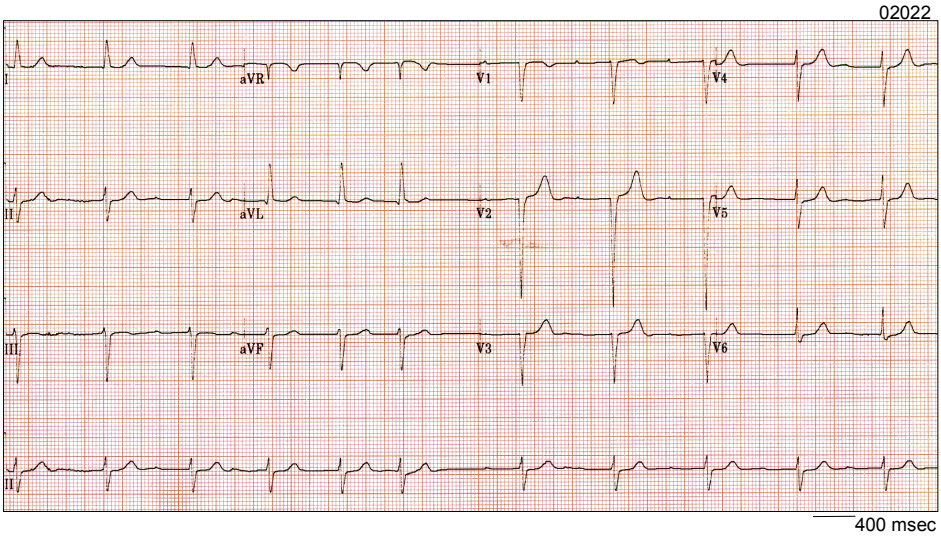

Figure 2
A typical laminopathy ECG, showing 1st degree AV block with low amplitude P waves and a narrow QRS complex.

In total 76 (25%) patients out of the total of 299 published patients were reported to have died at a mean age of 46 yrs. Strikingly, sudden death was far more prevalent (46%) than death due to heart failure (12%) (table 1b). 50% of deceased pacemaker carriers died suddenly, compared to 43% sudden deaths in patients that did not carry a pacemaker (p=0.556 with Chi-square), indicating that a pacemaker does not protect against sudden death in this patient group.

Table 1b
Age and mode of death of patients with LMNA mutation leading to EDMD or LGMD (with muscular dystrophy) or DCM (without muscular dystrophy)

	Muscular dystrophy	No muscular dystrophy	All patients
number of patients died	44	32	76
age at death	45	59	46
sudden death	19	16	35
- with pacemaker	6	10	16
- without pacemaker	13	6	19
due to heart failure	5	4	9
- with pacemaker	1	2	3
- without pacemaker	4	2	6
unclassified death	20	12	32
- with pacemaker	10	3	13
- without pacemaker	10	9	19

Of the 35 patients who died suddenly, 23 (66%) were documented to have died suddenly before the age of 60, while the age was not reported in 8 of these patients. Of the 23 patients that died suddenly before the age of 60, 10 (43%) carried a pacemaker, suggesting a pacemaker does not prolong life.
The risk of dying suddenly is the same in the patient group with a neuromuscular phenotype (43% of patients died suddenly) as in patients with an isolated cardiac phenotype (50% of patients died suddenly) (Chi-square 0.347, p=0.556).
We determined the level of dysrhythmias, ventricular dysfunction and sudden death in 2 of our own large unpublished families with a lamin A/C gene mutation. These 2 families consist of 32 affected individuals. As can be appreciated in table 2 the main phenotypical characteristics of these 2 families are comparable to the published patients. Two family members who survived an episode of out-of-hospital ventricular fibrillation and hence received an ICD show the probable tachy-arrhythmic nature of sudden death.
In the normal population between 25 and 65 years of age the contribution of sudden death to total mortality has been suggested to be 18.9%[27]. The sudden death rate in patients with lamin A/C mutations (46%) compared with the normal population shows an increased risk of sudden death in patients with lamin A/C mutations with an odds ratio of 3.7 (95% CI 2.3 to 5.9, p<0.001).

Table 2
Comparison between published patients and 2 of our unpublished families shows no difference in the main phenotypical characteristics. Figures are numbers or means unless stated otherwise.

	Published patients	Unpublished families	P value
Patients	299	32	
Dysrhythmia	61 %	63 %	0.857
LVEDD	52 mm	54 mm	0.778
FS	28 %	26 %	0.690
Nr of patients died	75	20	
Age of death	46	44	0.655
Sudden death	46 %	65 %	0.145
- with pacemaker	46 %	31 %	0.351
- without pacemaker	54 %	69 %	

LVEDD indicates left ventricular end diastolic diameter, FS indicates fractional shortening

DISCUSSION

This first systematic analysis of all published carriers of lamin A/C mutations suggests that laminopathies causing skeletal and/or cardiac muscle disease carry a very high risk of sudden death. These data suggest that this risk might be unrelated to heart failure and unresponsive to pacemaker therapy. Furthermore, we confirm that laminopathies carry a very high prevalence of dysrhythmias, and suggest that quite characteristic features of the 12-lead surface ECG allow clinical suspicion of laminopathy.

Based on this analysis we propose the hypothesis that lamin A/C gene mutations cause characteristic ECG changes early in course of cardiomyopathy in the setting of EDMD, LGMD1B or DCM and that patients with these mutations carry a grave risk of sudden death, probably due to tachyarrhythmias. We further propose that a myriad of atrial dysrhythmias accompanies disease progression in the fourth decade in almost every patient and that heart failure follows two decades later.
The high penetrance of dysrhythmias has been shown to improve screening of this disease. Screening based on AV block already proved relatively successful[1] and we suggest that further refinement of ECG characteristics might increase this success rate.
The high number of pacemakers implanted indicates that these patients often seek cardiological expertise. However, the prevalence of sudden death is equal in both the pacemaker and non-pacemaker group, suggesting lethal tachyarrhythmias causing sudden death. These data also suggest that pacemaker therapy alone is not sufficient to prevent sudden death. However, when to implant an ICD is an important question to which at this time no satisfactory answer can be given. We propose to perform an electrophysiological examination in each patient before pacemaker implantation or at age 35. In case of inducibility during this study implantation of an ICD should be considered. It might be advisable in

patients who need a pacemaker to use a device suitable to switch to ICD. Whether this strategy of ICD implantation reduces the sudden death rate and increases life span should be studied in a prospective trial.
Importantly, sudden death is reported with equal frequency in patients with muscular dystrophy and patients with isolated dilated cardiomyopathy. Therefore, subjects without manifest cardiac abnormalities or cardiac failure seem still at a high risk of sudden death.

Study limitations

Although this type of study can be hampered by recall and publication bias the high number of sudden deaths, even in carriers of a pacemaker is important information.
In a separate analysis of carriers of sarcomeric mutations we found a sudden death rate of 23% (unpublished data) which makes it very unlikely that the high sudden death rate is found in every genetic form of cardiomyopathy. Therefore, the finding of a high rate of sudden death seems characteristic of laminopathies. A very recent publication found unfavourable prognosis in laminopathy patients compared to idiopathic DCM, but an explanation for this finding was not proposed[28]. Furthermore, the data obtained for the meta-analysis is closely resembled by the findings in our own unpublished patients, suggesting indeed a certain degree of uniformity. More recent publications are also in line with the conclusions made here[29,30].

CONCLUDING REMARKS

In conclusion, based on a systematic analysis of carriers of lamin A/C mutations we propose that these subjects carry a high risk of sudden death. This high risk seems unrelated to heart failure, and is equally prevalent in subjects with a predominant muscular dystrophy and a predominant cardiomyopathy phenotype. The high risk of sudden death is only present in carriers of a lamin A/C mutation and not in carriers of sarcomeric mutations.
Our findings prompt to study cardiac arrhythmias and their treatment in a prospective manner to address our hypothesis and optimise diagnosis and treatment of these patients.

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CHAPTER 3

A-type lamins are essential for TGF-β1 induced PP2A to dephosphorylate transcription factors

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ABSTRACT

Diseases caused by mutations in lamins A and C (laminopathies) suggest a crucial role for A-type lamins in different cellular processes. Laminopathies mostly affect tissues of mesenchymal origin. Since transforming growth factor (TGF) β1 signalling impinges on the retinoblastoma protein (pRB) and SMADs, we tested the hypothesis that lamins modulate cellular responses to TGF-β1 signalling, via regulation of these transcription factors in mesenchymal cells. We here report that A-type lamins are essential for the inhibition of fibroblast proliferation by TGF-β1. TGF-β1 dephosphorylated pRB through PP2A, both of which, we show, are associated with lamin A/C. In addition, lamin A/C modulates the effect of TGF-β1 on Collagen production, a marker of mesenchymal differentiation. Our findings implicate lamin A/C in control of gene activity downstream of TGF-β1, via nuclear phosphatases such as PP2A. This biological function provides a novel explanation for the observed mesenchymal dysfunction in laminopathies.

INTRODUCTION

The LMNA gene encodes different karyoskeletal proteins, lamin A, lamin Adel10, lamin C and lamin C2, which are the products of alternative splicing. These A-type lamins form a meshwork at the inner surface of the inner nuclear membrane known as the lamina. Formation of the lamina involves structural proteins, such as emerin, nesprin, LAP2α and lamin B(1), that bind to lamins A and C. A multitude of other protein classes bind to the lamina, ranging from signalling proteins to chromatin and transcription factors. Among the latter are MOK2, SREBP1a/c and pRB (1). However, the functional relevance of the physical interaction between lamins and transcription factors remains unknown. Lamins are directly involved in mitosis and apoptosis, mainly because the lamina needs to disintegrate in order for mitosis or apoptosis to take place (2). In addition, lamin A/C levels correlate with the proliferative capacity of cells: lamin expression is upregulated in highly differentiated cells, while their expression is lower in cells with a higher proliferative capacity (3, 4).
Mutations in the LMNA gene are implicated in at least 9 clinically distinct diseases which affect different tissues in varying degrees (5-14). Currently two main hypotheses regarding the pathophysiology of laminopathies have been proposed. The first hypothesis stresses a structural role for lamins and proposes that lamins are crucial for structural integrity of the nucleus and hence that mutated lamins may disrupt nuclear architecture. The second hypothesis emphasises the putative role of lamins in transcription and proposes that mutations in lamin A/C cause alterations in gene regulation.
Despite the large variation in diseases caused by lamin A/C mutations, most tissues affected are from mesenchymal origin and increased Collagen and tissue fibrosis are common to most of these diseases (6, 15-18). Based on this notion, we explored the hypothesis that lamins A and C are involved in the regulation of proliferation and differentiation of mesenchymal cells through modulation of critical transcription factors.

RESULTS

Lamin A/C is important for regulation of proliferation.

We first studied cell cycle parameters in mouse embryonal fibroblasts (MEFs) derived from lamin A/C null mutant mice and wild type controls. Lamin deficient MEFs (LMNA -/- MEFs) showed increased proliferation compared to wild type control cells (LMNA +/+ MEFs) determined by MTS assay, which directly measures changes in cell numbers (Figure 1A). To assess which phase of the cell cycle was affected by loss of lamins A and C, cells were pulse-labelled with BrdU. BrdU incorporation data corroborate the notion of an increased proliferative rate: S-phase progression of LMNA -/- MEFs was significantly shortened (Figure 1B). S-phase progression is regulated by progressive phosphorylation of pRB (19). We next examined LMNA wild type and deficient cells for altered pRB-phosphorylation: (serum starved) G1-arrested cells revealed increased phosphorylation of the retinoblastoma protein in LMNA -/- MEFs compared to similarly treated control LMNA +/+ MEFs (Figure 1C). Since phosphorylation of pRB is functionally linked to G1/S transition (20), these findings are consistent with a more rapid transit through S-phase and suggested a loss of normal cell cycle control in cells lacking lamins A and C.

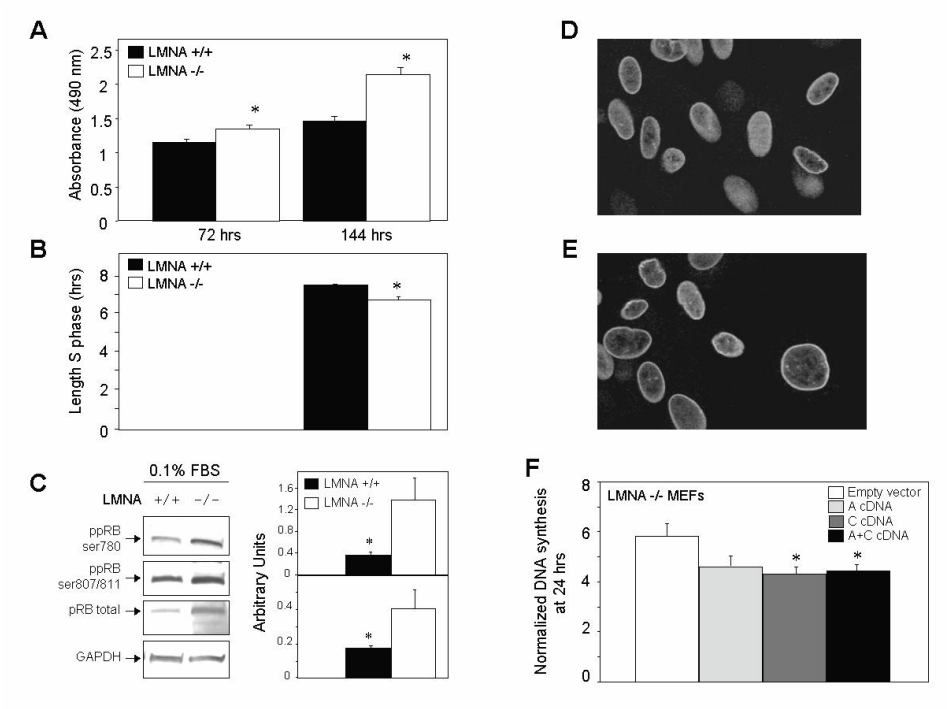

Figure 1. Loss of lamin A/C increases cell proliferation.

(**A**) Proliferation of LMNA -/- (null) MEFs is significantly increased compared to LMNA +/+ (wild type) MEF controls (n=8 for each bar, asterisk indicates $p<0.01$ (t-test)). Cell proliferation was determined 72 and 144 hours after plating of cells by MTS assay (see: *Methods*) of non-synchronised cells in medium supplemented with 10% FBS. (**B**) Pulse chasing with BrdU reveals a significantly shortened S-phase (10%) by in LMNA -/- MEFs as compared to LMNA +/+ MEFs (n=2, 6 respectively, asterisk indicates $p<0.05$ (t-test)). (**C**) Synchronised (serum starved: 0.1 % FBS in figure) LMNA -/- MEFs show increased pRB phosphorylation as opposed to synchronised +/+ MEFs. In LMNA -/- MEFs ppRBser780 and ppRBser807/811 were both significantly higher when normalised for total pRB and GAPDH as indicated by the 2 right panels (upper: ppRBser780/total pRB, n=4; lower: ppRBser807+811/total pRB, n=3). Antisera used - immunodetection: α-ppRB ser780, α-ppRB ser807/811, α-total-pRB (all polyclonal), α-GAPDH 6C5; asterisk indicates $p<0.05$. Reconstitution of (HA-tagged) lamins A (**D**) and C (**E**) expression in LMNA -/- MEFs. Immunostaining with an α-HA antibody reveals normal nuclear localisation of both recombinant lamins A/C (magn. 400x). (**F**) Lamin A/C-reconstituted genetically matched MEFs show a significantly decreased cellular proliferation rate. Genetically matched, serum starved cells (LMNA-KO plus empty vector, A, C or A+C MEFs) were incubated with 10% FBS for 24 hrs. Cell proliferation was determined by measuring [^{3}H]-Thymidine incorporation during the first 6 hrs (t0-t6) of 10% FBS incubation after serum starvation and again during the last 6 hrs (t18-t24). Incorporation rates during (t18-t24) were normalised to those during (t0-t6); (Empty vector= empty vector infected LMNA -/- MEFs, A/C rescue= LMNA -/- MEFs with re-expression of lamin A and/or C, n=6 for all cell types, asterisks indicate $p<0.05$).

The cell lines used above were used before to address the role of lamins in cell biology (21, 22) but were independently immortalised, which may affect cell cycle characteristics of these cells differently. To address the role of LMNA in cell cycle regulation in an unbiased fashion, we therefore, generated a genetically matched cell model: tagged-lamin A and/or C were stably re-introduced in LMNA -/- cells using retroviral expression vectors. Re-expression of lamins A and C, either alone or in combination (latter not shown), resulted in

the expected nuclear localisation of the tagged lamins (Figure 1D and E). We next studied DNA synthesis in these lamin A and/or C-reconstituted LMNA -/- MEFs as a measure for proliferation. Re-expression of lamin A or C alone or in combination significantly reduced proliferation rates to less than 80% of their empty vector-infected controls (Figure 1F). Taken together, the above findings support an important involvement of lamin A/C in control of fibroblast proliferation.

Lamins A and C modulate TGF-β1 signalling

Having established an effect of lamin A/C expression levels on cell cycle regulation, and given the observation that laminopathies (i.e. LMNA dysfunction) affect mainly tissues from mesenchymal origin, we next focussed on transforming growth factor β1 (TGF-β1) signalling. The cell cycle regulatory effects of TGF-β1 on cells depend on differentiation-state and origin of the target cell (23): TGF-β1 stimulates cell proliferation in undifferentiated cells, while it blocks the cell cycle progression in highly differentiated cells (24). Independently, it is known that the level of lamin A/C expression is highly related to the degree of differentiation (3, 4). The analogy between these observations suggested a possible functional relationship between TGF-β1 signalling and the presence of A-type lamins: TGF-β1 may negatively regulate cell cycle progression in the presence of lamins A and C and, conversely, may fail to do so, or stimulate cell proliferation in the absence of lamins A and C. To study lamin-dependency of TGF-β1 signalling in relation to cell cycle control, we used the genetically matched LMNA -/- MEF model, with or without re-constituted lamin A and/or C expression. DNA synthesis was quantified by measuring [^{3}H]-thymidine incorporation during the first 6 hrs after release and again, between 18 and 24 hrs after release, so that an incorporation ratio (late over early interval) of larger than 1 indicates net DNA synthesis. In empty vector infected LMNA -/- MEFs, TGF-β1 does not reduce DNA synthesis (Figure 2A), whereas addition of TGF-β1 strongly reduced DNA synthesis in lamin reconstituted cells. In cells re-expressing lamin C or lamins A + C, the effect of TGF-β1 addition was more pronounced than in lamin A-only reconstituted cells. (Figure 2A). In conjunction with our earlier observation that lack of lamin C or A+C affects MEF proliferation more than lack of lamin A only (Figure 1 F), the above data suggest that, although lamin A contributes to modulation of TGF-β1 induced signalling, loss of lamin C in our model systems is more critical than loss of lamin A. The combined presence of both lamins was most effective in inhibiting the cell proliferation in response to TGF-β1.

To independently prove that the above described differences in proliferation are indeed caused by lamins, we generated genetically matched 3T3 fibroblasts in which lamin A/C protein levels were significantly reduced (knocked-down) by stable RNA interference through retroviral expression of short hairpin RNAs (shRNA) (Figure 2B). Consistent with the MEF-data (Figure 1, Figure 2A), DNA synthesis was increased in 3T3 fibroblasts in which lamin expression was knocked-down (lamin A/C-KD): upon stimulation of (serum starved) G1-arrested fibroblasts with 10% Fetal Bovine Serum (FBS), lamin A/C-KD fibroblasts show significantly higher basal DNA synthesis level compared to empty vector infected fibroblasts (Figure 2C).

In addition, the inhibitory response to TGF-β1 was also dependent on lamins in the 3T3 model system: consistent with our observations in the genetically matched MEF model, indeed, TGF-β1 blocks DNA synthesis in LMNA wild type 3T3 cells, while in the lamin A/C-KD 3T3 cells TGF-β1 induced a 58% increase in DNA synthesis ($p<0.01$ for difference in response, Figure 2D). These data provide independent genetic proof that cell cycle progression is altered in lamin deficient mesenchymal cell types.

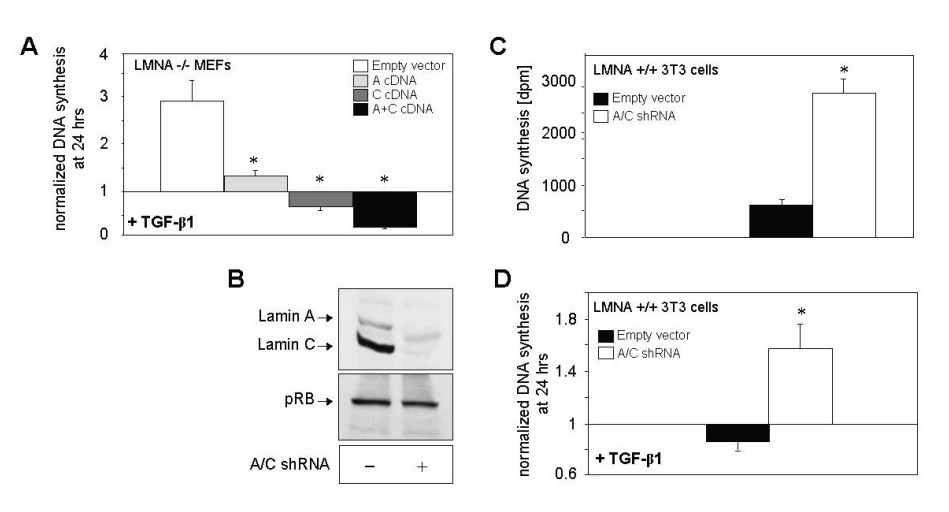

Figure 2. TGF-β1 signalling is modulated by lamin A/C

(**A**) Lamin A/C-reconstituted LMNA -/- MEFs show a lamin dependent decrease in cell proliferation. Genetically matched, serum starved cells (LMNA-KO plus empty vector, A, C or A+C MEFs) were incubated with 1 ng/ml TGF-β1 for 24 hrs; [^{3}H]-Thymidine incorporation was determined during the first 6 hrs (t0-t6) and again during the last 6 hrs (t18-t24) of the experiment. The incorporation during (t18-t24) over that during (t0-t6) was determined; a ratio of 1 implies no increase in DNA synthesis (n=6 for all cell types, asterisk indicates $p<0.01$ in comparison to LMNA). (**B**) Stable knock-down of murine lamin A/C expression in 3T3 fibroblasts. Cells were stably infected with a lamin A/C shRNA-pRetrosuper vector and selected. Upper panel: stable knock-down after 3 months of continuous culturing; antiserum used: lamin A/C (346); lower panel: total pRB levels are unaffected after 3 months of continuous culturing; antiserum used: pRB G3-245. (**C**) Knock-down of lamin A/C increased [^{3}H]-Thymidine incorporation approximately 4.5 fold in 3T3 cells stimulated with 10% FBS (n=6, asterisk indicates $p<0.01$). (**D**) TGF-β1 blocks proliferation in WT 3T3 cells, but not in 3T3-LMNA-KD cells (n=6, asterisk indicates $p<0.001$). [^{3}H]-Thymidine incorporation was determined as described in figure 2A.

Phosphorylation of pRB is altered in absence of lamin A/C.

It is known that pRB is essential for TGF-β1-induced growth inhibition (25). Prompted by our finding that the effect of TGF-β1 on the cell proliferation depends on presence of lamins, we investigated whether pRB phosphorylation was altered in response to TGF-β1 induction. Cell cycle arrest requires hypo-phosphorylation of pRB; we therefore investigated the phosphorylation status of pRB in response to TGF-β1 in relation to presence or absence of lamin A/C. In lamin A/C rescued LMNA -/- MEFs, TGF-β1 induced dephosphorylation of pRB within 15 minutes, while this dephosphorylation was not seen in LMNA -/- MEFs (Figure 3A). Importantly, also in the 3T3 fibroblasts with knock-down of lamin A/C we observed increased levels of ppRB795 compared to control fibroblasts in response to TGF-β1 exposure (data not shown). These findings confirmed that A/C lamins are essential for the pRB mediated arrest of cell cycle by TGF-β1. Recently published data suggest increased degradation of pRB in absence of lamin A/C as a cause for cell cycle deregulation (22), whereas our data suggest altered pRB phosphorylation underlies the observed proliferative defect. Although both independent genetically-matched model systems described in this report, reconstituted genetically-matched LMNA-/- MEFs (Figure 1C) and 3T3 LMNA-KD model (Figure 2B), do not show any effect on pRB stability, both decreased pRB protein

levels and diminished pRB phosphorylation are expected to have a similar effect on cell proliferation.

Protein Phosphatase 2A binds to lamin A/C and is responsible for TGF-β1 induced dephosphorylation of pRB.

The rapid TGF-β1-induced dephosphorylation of pRB suggested the involvement of a protein phosphatase. It is known that TGF-β1 can directly activate PP2A and thereby inhibit cell cycle progression in a post-transcriptional manner (26). Furthermore, it was recently established that PP2A directly dephosphorylates pocket protein family members, among which p107 and pRB (27). Hypo-phosphorylated pRB subsequently binds and blocks sites of DNA replication (28). Because lamin A/C co-localises at sites of DNA replication (29), we hypothesised that PP2A is responsible for the lamin A/C-dependent dephosphorylation of pRB. This also suggested that lamins, pRB and PP2A might physically associate. We first confirmed pRB binding to A-type lamins in LMNA +/+ MEFs under non-restrictive (i.e. 10% FBS) growth conditions, as shown before (30) (Figure 3B). In addition, we here show the novel finding that also PP2A is part of the lamin-pRB interaction complex, as it co-immunoprecipitates with lamins (Figure 3C) and pRB (Figure 3D). The PP2A-pRB interaction was lamin dependent in proliferating cells (Figure 3D), since PP2A-pRB interaction was lost upon re-expression of lamins A and C (Figure 3D, second lane). Our data show that the reduced PP2A-pRB interaction can not be explained by competitive binding of PP2A or pRB to lamin: 1) at 0.1% FBS and in the absence of TGF-β1 (Figure 3E, first and third lane), the PP2A-pRB interaction is clearly not dependent on the absence of lamin A/C, 2) TGF-β1 signalling induces a rapid (within 15 minutes) loss of PP2A-pRB interaction in the presence of lamins under conditions of restrictive growth (0.1% FBS), concomitant with reduced pRB phosphorylation (Figure 3E, second and fourth lane). This suggests that PP2A-pRB interaction is at least in part determined by the phosphorylation status of the complex members. Indeed, in the absence of lamins, TGF-β1 fails to induce ppRB dephosphorylation (Figure 3A); the sustained interaction between pRB and PP2A under conditions of limited proliferation (Figure 3E) further supports the notion that post-translational modifications play an important role in these protein interactions. Whether the reduction in PP2A-pRB association under 10% FBS (Figure 3D) and under 0.1% FBS + TGF-β1 (Figure 3E) is quantitatively similar, and would therefore be the direct cause of TGF-β1 signalling, remains to be further investigated.

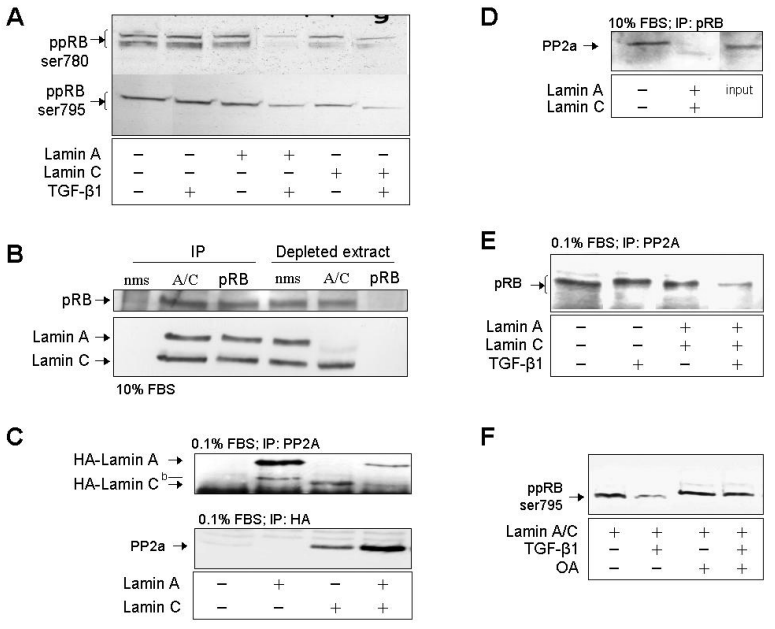

Figure 3. In vivo association between lamin A/C, pRB and PP2A; PP2A dephosphorylates pRB in a lamin dependent fashion.

(**A**) TGF-β1 induces rapid dephosphorylation of pRB or shows constitutively reduced phosphorylated pRB in the presence of lamin A or C, respectively. In contrast, empty vector infected LMNA-/- MEFs do not show TGF-β1 induced pRB dephosphorylation. MEFs were serum starved prior to growth factor stimulation (15 minutes) (**B**) pRB and lamin A/C interact, as demonstrated by reciprocal co-immunoprecipitation (IP) of pRB and lamin A/C in unsynchronised LMNA +/+ MEFs (antisera used – IP: nms (normal mouse serum), α-lamin A/C (sc-7293), α-pRB (polyclonal); immunodetection: α-pRB (polyclonal), α-lamin A/C (sc-7293). (**C**) Protein phosphatase 2A (PP2A) is a new interaction partner of lamin A/C. Co-immunoprecipitation shows that PP2A and lamin A/C interact; immunoprecipitation against PP2A co-precipitates lamin A and C (upper panel) from A-type lamin-reconstituted LMNA -/- MEF extracts; reciprocal immunoprecipitation confirms the specificity of the interaction of PP2A with HA-tagged lamins A/C; band indicated by "*b*" is a non-specific band (input showed similar expression of lamin A and/or C and PP2A (data not shown); lower panel; antisera used – IP: α-PP2A 5H4, α-HA-lamin A/C 12CA5; immunodetection: α-PP2A 6F9, α-HA-lamin A/C 12CA5). (**D**) In asynchronously growing cells, the interaction between PP2A and pRB is dependent on the presence of lamins A and C. PP2A co-immunoprecipitates with pRB in cycling LMNA -/- MEFs, but not in lamin A and C re-expressing LMNA -/- MEFs (antisera used – IP: α-pRB G3-245, immunodetection: α-PP2A 6F9). (**E**) In serum deprived cells, the interaction between PP2A and pRB is rapidly reduced upon TGF- β1 stimulation in lamin A/C reconstituted LMNA -/- MEFs, but not in LMNA -/- MEFs (antisera used – IP: α-PP2A 6F9; immunodetection: α-pRB G3-245) Immunoprecipitation was carried out 15 minutes following incubation with or without TGF-β1 (**F**) A crucial role for PP2A in mediating TGF-β1-induced pRB dephosphorylation. OA (125 nM final concentration) effectively inhibits the rapid dephosphorylation (15 minutes) of pRB induced by TGF-β1, in lamin A+C reconstituted LMNA -/- MEFs. Total pRB was equal throughout the lanes (data not shown; antisera used - immunodetection: α-ppRB ser795 polyclonal.

The physical interaction between PP2A and pRB suggested a relevant role for PP2A in lamin-dependent TGF-β1-induced dephosphorylation of pRB. To show that PP2A is directly responsible for dephosphorylation of pRB we measured dephosphorylation of pRB in the presence of okadaic acid (OA), a known selective PP2A inhibitor at the applied concen-

tration (28). In lamin A/C expressing cells, OA prevented TGF-β1-induced dephosphorylation of pRB (Figure 3F), which underscores the role for PP2A in pRB dephosphorylation in our model system. Taken together, our data provide evidence for a functional association between both A-type lamins, pRB and PP2A, and demonstrate that A-type lamins are needed for proper functioning of nuclear PP2A and post-translational regulation of pRB.

Lamins A and C modulate TGF-β1 induced SMAD phosphorylation.

The above demonstrates a pivotal role for lamins A and C in TGF-β1-induced cell cycle control. We next asked whether A-type lamins are also important for other TGF-β1-mediated cellular processes. TGF-β1 has been implicated in tissue fibrosis in various clinical conditions. TGF-β1 increases Collagen production by inducing phosphorylation of (regulatory) rSMADs (31); phosphorylated rSMADs translocate to the nucleus, where they act as regulators of transcription. TGF-β1 receptors remain activated for a few hours, during which they continuously phosphorylate SMADs. Since fibrosis is a hallmark of many laminopathies (6, 15-18), we set-out to investigate whether lamins are implicated in the genesis of tissue fibrosis via aberrant TGF-β1 signalling. We first studied TGF-β1-induced SMAD phosphorylation in the presence and absence of lamins. In LMNA -/- MEFs, TGF-β1 effectively induces phosphorylation of SMAD2 (Figure 4A) and SMAD3 (not shown) within 15 minutes, which indicated that TGF-β1 can activate downstream effector molecules in the absence of lamins. rSMAD Phosphorylation kinetics are clearly altered in the absence of lamin: a more rapid and intense phosphorylation of SMAD 2 and 3 occurs, which also dissipates faster (Figure 4A). Phosphorylated SMADs act as co-factors which activate Collagen I and III promoters; abnormal phosphorylation of SMAD is expected to alter Collagen expression. To compare Collagen production by LMNA -/- and +/+ MEFs, we used Proline incorporation. This shows that Collagen production by LMNA -/- MEFs is significantly increased compared to wild type MEFs (Figure 4B). TGF-β1 also increased Collagen synthesis in LMNA +/+ MEFs; basal Collagen production was significantly higher in LMNA -/- MEFs and did not further increase in response to TGF-β1. Of relevance, re-expression of lamin A and C in LMNA -/- MEFs, reverted the phenotype to that observed in LMNA +/+ MEFs (Figure 4C). In the presence of lamins A and C, TGF-β1 induces a rapid phosphorylation of rSMAD2, which dissipates slowly between 6 to 24 hrs (Figure 4A). In contrast, in the absence of lamin A and C, phosphorylation of SMAD2 peaks at 1 hour and is sustained only until 3 hrs (Figure 4A). Of notice, in the absence of lamin C, we observed a striking reappearance of SMAD phosphorylation at 24 hrs. This indicated that the kinetics of SMAD2 phosphorylation were significantly and consistently altered in the absence of lamin A and C. Comparison of SMAD 2 and 3 phosphorylation in single lamin reconstituted cells suggested that lamin C plays a more prominent role in regulation of SMAD 2 phosphorylation (Figure 4A and data not shown), while restoration of SMAD 3 phosphorylation kinetics requires both lamin A and C (not shown). This could reflect functional regulatory divergence between these rSMADs.

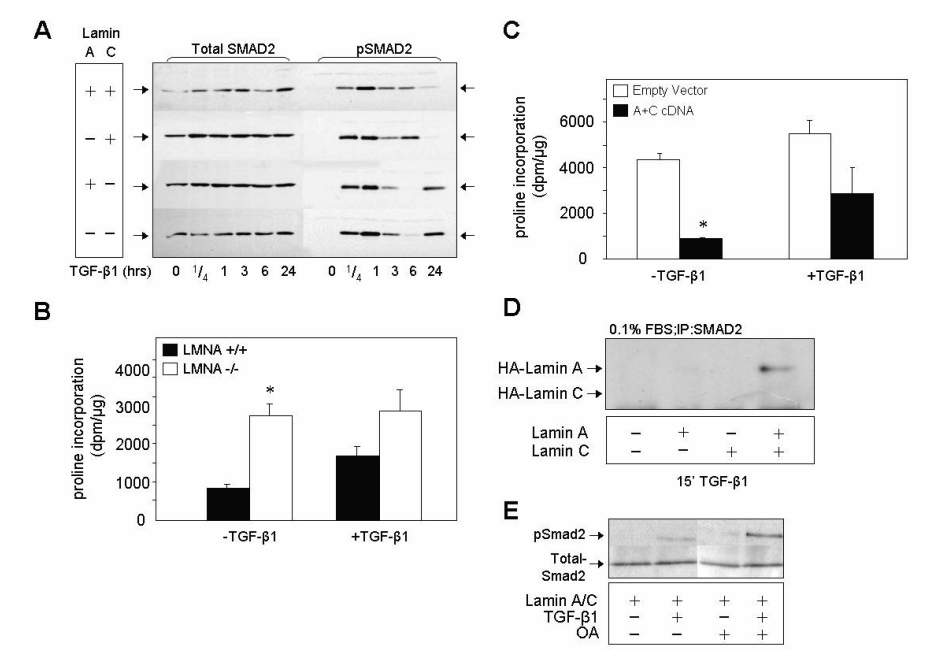

Figure 4. SMAD phosphorylation and Collagen production are increased in the absence of lamin A/C.

(**A**) In presence of lamin A and C, SMAD 2 and 3 (SMAD 3 data are not shown) are phosphorylated within 15 min in response to TGF-β1 stimulation of MEFs. SMAD phosphorylation peaks after 1 hr and gradually decreases to near basal levels at 24 hrs. In absence of both lamins, a more rapid and intense phosphorylation of SMAD 2 and 3 occurs, which also dissipates faster. In lamin deficient MEFs, SMAD 2 and 3 phosphorylation reappears at 24 hrs. (n=3, representative blot is shown, antisera used – immunodetection: α-SMAD2 polyclonal, α-pSMAD2 polyclonal and α-GAPDH 6C5). (**B**) Increased Collagen production in murine fibroblasts in the absence of lamin A/C. Wild type MEFs show a more than two fold induction of Collagen production upon TGF-β1 induction; basal Collagen production is already maximally elevated in LMNA -/- MEFs, and is not further induced upon TGF-β1 stimulation. Collagen production was measured by [^{3}H]-Proline incorporation (no TGF-β1 stimulation: LMNA +/+, LMNA -/- MEFs: n=7, 9 respectively; asterisk indicates $p<0.05$); with TGF-β1 stimulation LMNA +/+, LMNA -/- MEFs: n=7, 9 resp, ($p=0.06$)). (**C**) Lamin A/C-reconstituted MEFs show similar characteristics as wild type MEFs: lamin A and C expression reverts abnormally high Collagen production in LMNA -/- MEFs to normal (i.e. wild type) levels (n=6 for each cell type and each condition). Reconstitution of lamin C expression only in LMNA -/- MEFs reduced Collagen synthesis significantly (to 56% of empty vector control; $p<0.05$) under basal conditions, and further to A/C-reconstituted-levels in the presence of TGF-β1, while reconstitution with lamin A only did not induce a significant reduction of Collagen production (data not shown). (**D**) SMAD2 associates with lamin A/C in reconstituted LMNA -/- MEFs. Only in presence of both lamin A and C, an association between lamin A and SMAD 2 is observed, indicating probable ternary complex formation (antisera used – IP: α-SMAD2 polyclonal; immunodetection: α-HA 12CA5). (**E**) SMAD 2 dephosphorylation is inhibited by Okadaic acid. Reconstituted LMNA -/- MEFs show a TGF-β1 induced SMAD2 phosphorylation, which is augmented by inhibition of PP2A (antisera used – immunodetection: α-total SMAD polyclonal, α-phospho-SMAD polyclonal).

To further explore how lamins A and C are involved in the response to TGF-β1, we tested whether SMADs associate with A-type lamins. Our data show that SMAD2 co-immunoprecipitates with lamin A/C (Figure 4D); the absence of signal in the control immunoprecipitation (LMNA -/- MEFs) shows specificity of the association. Interestingly, the

SMAD2-lamin A/C association is only detected in the presence of both lamin A and C, as re-constitution with either lamin type by itself does not support interaction (Figure 4D). We identified PP2A as a novel interactor of A-type lamins (Figure 3); this positions PP2A in close proximity to phosphorylated SMAD, and potentially implicates PP2A in regulation of SMADs as well. To test this idea, we assessed the effects of OA treatment on SMAD2 phosphorylation. OA-treated cells show both increased basal as well as TGF-β1-induced phosphorylated SMAD2 levels (Figure 4E). These combined results demonstrated that, in analogy to their regulatory role in pRB functioning, A-type lamins are functionally involved in control of SMAD2 phosphorylation. It appears that A-type lamins exert their effect on these nuclear transcription regulators by altering the action of phosphatases, like PP2A, which are crucial for dephosphorylating these transcription factors.

DISCUSSION

We here report that LMNA -/- MEFs display defective transcription factor regulation. Firstly, aberrant pRB phosphorylation, accelerated S-phase transition and increase in cell numbers in genetically matched, synchronised cell cultures point toward a cell cycle regulatory defect in LMNA -/- MEFs. In addition, we find that LMNA -/- MEFs lack the proper inhibitory signalling that normally is activated in response to TGF-β1.This lack of inhibitory signalling may contribute to the increased proliferation of LMNA -/- MEFs. Defective TGF-β1 signalling also results in the increased production of Collagen. These abnormal characteristics of LMNA -/- MEFs are effectively corrected by stable re-introduction of both lamin A and C expression. The observation that the phenotypic rescue is less pronounced in LMNA -/- MEFs re-expressing only lamin A or lamin C may indicate the need for the combined presence of both lamin A and C.
Our findings provide the first evidence that lamins A and C modulate downstream effects of TGF-β1 signalling. Present concepts on the biological role of lamin A/C either highlight their role in maintaining integrity of nuclear structure or a thus far hypothetical role for lamin A/C in gene regulation (32). We here propose a novel hypothesis that links the 'structural' and 'gene regulation'-concepts. It may help to explain why mutations in lamin A/C have such distinct clinical consequences and underscores the important role of lamins A/C in mesenchymal biology (33). Our findings also shed new light on earlier published data. For instance, in a first description of the cellular role of lamins, Lammerding et al. reported that lamins A/C are important to maintain nuclear stability, and revealed altered NFκB activity in LMNA -/- MEFs (21). Our data suggests a possible explanation for the altered NFκB activity in LMNA -/- MEFs as the lack of dephosphorylation of relA, the functional nuclear subunit of NFκB in these cells, may explain the observed altered NFκB activity.

To explain the loss of TGF-β1 modulation at the molecular level, it is conceivable that lamins function to position PP2A as a regulator of incoming TGF-β1 signalling: via PP2A-dependent dephosphorylation pRB, and other transcription regulators like SMAD2, TGF-β1signalling is quenched (see hypothetical model; Figure 5). Absence of A-type lamins interrupts the negative regulatory action of TGF-β1. Although the exact molecular mechanism of this negative action is currently not known, it is possible that A-type lamins provide an important docking site for PP2A or one of its subunits, e.g. PP2A subunit A, B, or C, or alternatively ppRB. A link between failure to respond to the growth inhibitory properties of

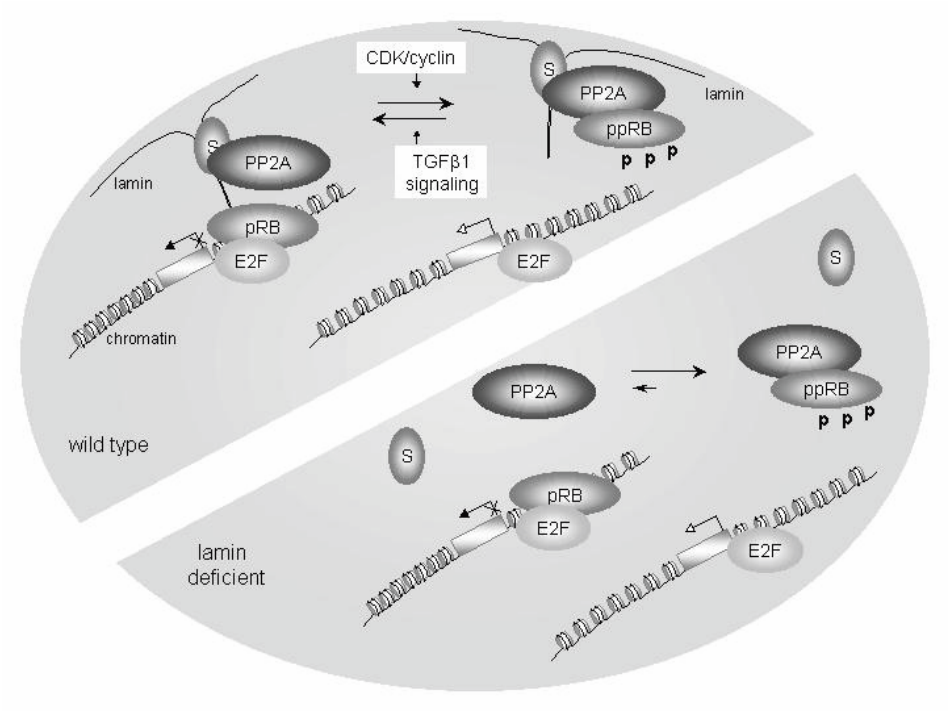

Figure 5. Hypothetical model for lamin dependent gene regulation via PP2A and pRB.
In this hypothetical model, PP2A and pRB are used to illustrate how lamins may affect transcriptional regulation. In resting wild type cells (upper left), both PP2A and hypo-phosphorylated pRB are bound to lamin A/C; however, interaction between PP2A and pRB is low. Mitogenic signalling induces cyclin/CDK-dependent pRB phosphorylation, resulting in the release of transcriptional repression by pRB and activation of *e.g.*E2F-dependent gene expression. TGF-β1 counteracts mitogenic signalling by stimulating PP2A activation: phospho-pRB (ppRB) and PP2A interact and PP2A rapidly dephosphorylates ppRB, thereby restoring its' interaction with lamin A/C and effectively silencing E2F-dependent transcription. The mechanism by which TGF-β1 signalling induces nuclear PP2A activity remains unresolved; it may however depend on binding of and/or activation by crucial regulatory units (symbolised by S in the diagram), like PR72(27). In absence of lamins (lower right), pRB may still bind E2F. However, incoming mitogenic signals that promote E2F-dependent cell cycle progression are ineffectively quenched: in lamin deficient cells, phospho-pRB is inefficiently dephosphorylated, despite increased PP2A/pRB interaction. Consequently, there may not be sufficient hypo-phosphorylated pRB available, or alternatively, pRB may be mislocalised in the nucleus. It is likely that also docking of PP2A to lamin A/C is required for proper catalytic activation. Both are expected to result in failure to repress E2F-dependent gene targets, resulting in increased proliferation of lamin A/C deficient cells. This hypothetical model integrates hypotheses on the role of lamins both regarding structure and transcriptional regulation.

TGF-β1 and loss of A-type lamins is not without precedent. Some metastatic cancer cells show strongly reduced lamin A/C expression, which is paralleled by insensitivity to TGF-β1 induced cell cycle arrest (24). In line with this, we find that in the presence of lamins, TGF-β1 signalling rapidly diminishes the interaction between PP2A and pRB, while ppRB is concomitantly dephosphorylated. In contrast, in LMNA -/- cells, ppRB dephosphorylation is abrogated; the sustained PP2A/pRB interaction suggests this interaction does not lead to dephosphorylation of ppRB. Our observation that PP2A preferentially co-immunoprecipitates ppRB (Figure 3D) and the finding that A-type lamins preferentially interact with

hypo-phosphorylated pRB (34) support such a model. We propose that PP2A/lamin A/C docking is needed to restore pRB functionality (i.e. transcriptional repression and consequent cell cycle control), by rapid dephosphorylation of ppRB in response to TGF-β1. Lack or mutation of A-type lamins may leave PP2A/pRB complexes unable to inactivate relevant targets, as a result of improper sub-nuclear localisation of one or both factors. It is plausible that a transient ternary complex formation between lamin, PP2A and pRB is needed for proper transcriptional repression.
Furthermore, it is known that TGF-β1 can act through SMAD-independent pathways (26). One of the SMAD-independent pathways employs the phosphatase PP2A. TGF-β1 activates a B-subunit of PP2A. This B-subunit than forms a ternary structure with the A/C dimer of PP2A. Most likely, this also happens in our model. TGF-β1 activates both SMADs and simultaneously a B-subunit of PP2A, which then activates PP2A to dephosphorylate nuclear SMAD and/or pRB. Phosphorylation of pRB most likely involves cyclins D1 and E, which activate cyclin dependent kinases (CDK) to phosphorylate pRB. However, dephosphorylation of pRB is probably in part dependent on TGF-β1. This will probably be regulated in a similar manner as the dephosphorylation of SMAD; TGF-β1 activates a B subunit, which in turn activates PP2A to dephosphorylate pRB. Recently PP2A was described as an important element in the equilibrium of pRB phosphorylation(35). Which PP2A subunit is responsible for nuclear PP2A activity is unknown. PR59 is a candidate, as it has been described to associate with pRB(27).

Recent reports suggest that MAN1, an integral protein of the inner nuclear membrane with physical connections to Lamin A/C, is involved in TGF-β1 signalling through binding and possibly regulation of SMAD2 and SMAD3(36, 37). Although whether and how MAN1 is involved in laminopathies has yet to be resolved, these reports corroborate our findings that nuclear membrane associated proteins represent an important site of regulation of transcription factor activity. Furthermore, the finding that MAN1 may regulate SMAD2 and SMAD3 could in part explain the observations in SMAD phosphorylation here described. In absence of lamin C we observed a striking reappearance of SMAD phosphorylation at 24 hours. This latter somewhat surprising finding may be due to a loss of regulation of SMAD phosphorylation via factors like MAN1. MAN1 interacts with lamins. It is possible that MAN1 preferentially binds lamin C, or that it needs at least lamin C to function properly. This might explain why in presence of lamin C, SMAD phosphorylation might be regulated more in accordance with the wild type situation.
Our data suggest a novel mechanism for the pathogenesis of laminopathies. A key element of many diseases caused by mutations in lamin A/C is tissue fibrosis (6, 15-18). The mechanism we describe here proposes that lamin A/C functions as a vital regulatory factor of nuclear processes downstream of TGF-β1 signalling. Without lamin A/C, nuclear signalling is enhanced due to sustained activation (i.e. decreased dephosphorylation) of transcriptional regulators. As a consequence fibroblasts show higher proliferation rates and produce more collagen. In patients with a lamin A/C gene mutation this may result in enhanced cell turnover and increased fibrosis. Interestingly, these are also characteristic clinical features of another lamin A/C related disease: progeria. The enhanced cell turnover, accelerated ageing, and accompanying increased fibrosis, typical for progeria, suggest a common link to lamin dysfunction. In addition, a mouse model for progeria shows increased collagen deposition in the skin and increased numbers of fibrocytes in the heart(38).
Evidently, this conjecture requires experimental confirmation. The important clinical implication is that if fibrosis in laminopathies is confirmed as a primary cause rather than secondary to myocyte loss, then patients might benefit from treatment aimed primarily at reduction of fibrosis.

In conclusion, our study is the first to show that lamin A/C interacts with pRB, SMAD2 and PP2A and that this interaction is crucial for proper regulation of mesenchymal cellular physiology. Loss of lamins results in a loss of dephosphorylation of pRB and SMAD2. The consequence of abnormal transcription factor phosphorylation is increased proliferation and excessive Collagen production by fibroblasts. This novel pathophysiological concept provides a molecular rational for the observed mesenchymal dysfunction in laminopathies, and suggests that lamins may be important for various transcriptional processes by interacting with nuclear phosphatases.

MATERIALS AND METHODS

Cell Culture

LMNA -/- MEFs and LMNA +/+ MEFs were kind gifts of B. Burke and C. Stewart. Cells were cultured in Dulbecco's modified Eagle's Medium (Gibco) containing 10% FBS, 1mM L-glutamine and 0.1% gentamycine at 37ºC in humidified 5% CO_2 / air. Lamin A and C rescued and empty virus infected MEFs were cultured under 1 μg/ml puromycine and 37.5 μg/ml hygromycine. Proliferation experiments were performed under 10% FBS or 0.1% FBS conditions with addition of TGF-β1 as indicated.

Generation of lamin A and C rescued LMNA -/- MEFs; shRNA expressing 3T3 fibroblasts

cDNA of lamin A and C was generated from peGFP-LaminA and pS65-LaminC by PCR of the cDNA using primers 5' CAGTGTCGACCGAGACCCCGTCCCAGCGG 3' for both lamin A and C and 5' CATAGAATTCTTCTAGACAGATTACATGATGCTGC 3' for lamin A and 5' CATAGAATTCTTCTAGAGGCCTCAGCGGCGGCTA 3' for lamin C. This introduced a SalI site with deletion of the original ATG site and an EcoRI site after the stop-codon. The PCR product and pMT-HAX (haemaglutinin-tag; containing an extra upstream XhoI-site) were digested with SalI and EcoRI and the cDNAs of lamin A or C were ligated in frame into pMT-HAX after the HA-epitope. The resulting plasmid was digested with XhoI and EcoRI and HA-epitope tagged lamin A was ligated into retroviral vector pBabe-hygroX and HA-epitope tagged lamin C was ligated into retroviral vector pBabe-puroX; both babe vectors carried an additional XhoI cloning-site. Human 293T packaging cells were transfected with pBabe-HA-lamin A, pBabe-HA-lamin C or empty vector. Supernatant was aspirated and filtered to obtain infectious virus. LMNA -/- MEFs were infected in presence of 2 μg/ml polybrene. Infected cells were selected with 150 μg/ml hygromycine or 2 μg/ml puromycine respectively, resulting in lamin A, lamin C, lamin A/C rescued LMNA -/- MEFs or empty vector control LMNA -/- MEFs.
Post-transcriptional silencing of murine lamin A/C was achieved by the shRNA sequence: gagcttgacttccagaagaacat. This sequence was used in forward (GATCCCCGAGCTTGACTTCCAGAAGAACATttcaagagaATGTTCTTCTGGAAGTCAAGCTCTTTTTGGAAA) and reverse primer (AGCTTTTCCAAAAAGAGCTTGACTTCCAGAGAACATtctcttgaaATGTTCTTCTGGAAGTCAAGCTCGGG) and ligated into pRetrosuper (39). This sequence was stably introduced into 3T3 cells using the pRetrosuper system. Cells were infected either with an empty vector or the shRNA-containing vector. Infected cells were selected with 2 μg/ml puromycine.

Gel electrophoresis and immunoblotting

Cells were lysed with sample buffer (62.5 mM TRIS pH 8.6 containing 2% SDS and 10% β-mercapto-ethanol). Cell lysates were sheared through 23G needle and boiled at 95ºC for 10 minutes. Proteins were separated in 4%-20% gradient gels or specific percentage acrylamide gels. Separated proteins were transferred to PVDF membrane and detected with specific antibodies. Primary antibodies were incubated with HRP-linked secondary antibodies that are visualised with enhanced chemo-luminescence. Primary antibodies were lamin A/C (Santacruz 7293 (346)), pRB, ppRBser780, ppRBser795, ppRBser807/811 (Cell Signalling Technology (CST) 9300 and BD G3-245), GAPDH (RDI 6C5), PP2A (5H4 and 6F9, Covance Research MRT-204R), HA (12CA5), total Smad2 (CST 3102), pSmad2ser465/467 (CST 3103). Secondary antibodies were rabbit anti-mouse (DAKO P0161), Goat anti-rabbit (DAKO E0432) and Goat anti-rat (SCBT 2003), all HRP-linked. Intensity of signals on autoradiograms was quantified by densitometry. Data were compared using the Student t-test. The number of experimental repeats is indicated in the figure legends.

Immunoprecipitation

Cells were lysed on ice in ELB buffer (250mM NaCl, 0.1% NP40, 50 mM Hepes pH 7.0, 5mM EDTA) containing protease inhibitor tablet (Roche 1836153), 0.5 mM DTT and 0.5 mM Na_3VO_4. Cell lysates were sonicated on ice and centrifuged. Lysates were rotated for 1 hr at 4ºC with 1 μg primary antibody. Normal mouse and rat serum were used as negative controls. Fifty percent protein A and 50% protein G sepharose beads were washed with ELB buffer and added to cell lysates for 1 hr at 4ºC. Beads were pelleted and washed 5 times with ELB buffer. Proteins were dissolved in sample buffer and separated by gel electrophoresis.

Proliferation assays

^{3}H-Thymidine-incorporation assay
Cells were trypsinised, counted and seeded at 6000 cells per well in 96-wells plates. After attachment cells were synchronised by serum deprivation for 48 hrs. Transforming growth factor β1 or 10% FBS was added as indicated. Six hours prior to end-point 10 μl 25 nCi/μl [^{3}H]-Thymidine (Amersham) was added. Incubation was stopped by freezing at -20ºC. Cells were spotted on filters, which are placed in liquid scintillation fluid (Filtercount). Scintillations were counted and represented as disintegrations per minute. DNA synthesis was depicted as dpm at a certain time point. Normalised DNA synthesis was dpm at 24 hrs after 6 hours incorporation normalised to dpm at 6 hours after 6 hours incorporation. Statistical analysis was done by Student t-test.

MTS-assay
Cells were trypsinised, counted and seeded at 6000 cells per well in 96-wells plates. At indicated time point 20 μl 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Promega CellTiter 96® AQueous MTS) and 1 μl 0.92 mg/ml phenazine methosulfaat (Merck) was added for three hours. The tetrazolium is reduced by living cells into a coloured formazan product, which was measured with a colorimeter. The quantity of formazan is directly proportional to the number of cells. Absorbance was measured at 490 nm. Statistical analysis was done by Student t-test.

Determination of S phase progression rate

Cells were trypsinised, counted and seeded at 200,000 cells per well in 6-wells plates. After attachment cells were pulse labelled with 10 μM bromodeoxyuridine (BrdU, Sigma B5002) for 30 minutes and chased with 5 μM deoxythymidine (Sigma T1895). Cells were harvested at 0, 1, 2, 3, 4 and 6 hours after start of chasing. Cells were fixed in methanol, washed in PBS, treated with 0.4 mg/ml pepsin in 0.1N HCl and incubated with anti-BrdU. Primary antibodies were counterstained with FITC-conjugated secondary antibody. DNA was stained with propidium iodide. Total DNA content and BrdU status was analysed by flow cytometry. S phase duration was calculated by extrapolating to 100% BrdU incorporation from the studied time points. Statistical analysis was done by comparing extrapolations from repeated experiments by Student t-test.

Quantification of Collagen production

Cells were trypsinised, counted and plated in 6-wells plates. After attachment cells were washed twice with PBS and synchronised in 0.1% FBS containing DMEM for 24 hours. 0.15 mM L-ascorbic acid with or without 10ng/ml TGF-β1 was added for 24 hours, after which 1 μCi/ml [^{3}H]-Proline (Amersham) was added. After 48 hours incubation cells were washed with PBS twice and incubated with 10% trichloroacetic acid for 30 min at 4ºC. Cells were scraped and harvested, pelleted and resuspended in 10% TCA at 4ºC. Cells were pelleted and resuspended in 1 N NaOH. Protein concentration was determined with Bradford assay. Protein solution is dissolved in hi-ionic scintillation fluid and disintegrations per minute are counted in a scintillation counter. Statistical analysis was done by Student t-test.

Indirect Immunofluorescence Microscopy

Cells were trypsinised, counted and plated on cover slips. After attachment cells were fixed in 3.7% formaldehyde and permeabilised in 0.1% triton. Cells were incubated with first specific antibodies against lamin A or lamin C (133a2 and ralC) for 1 hr and with FITC-conjugated secondary antibody for 1 hr. Preparations were mounted in 1,4-Diazabicyclo[2.2.2]octane (DABCO, Sigma). Fluorescent images were photographed using a Nikon Eclipse E800, equipped with a Nikon DXM1200 digital camera. Images were processed using Nikon ACT-1 software.

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CHAPTER 4

Severe myocardial fibrosis caused by a deletion of the start codon containing exon of the lamin A/C gene

Based on:
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Submitted

ABSTRACT

A large multi-generation family with an autosomal dominantly inherited form of cardiac fibrosis with a highly malignant clinical outcome has been investigated to identify the gene causing this disease. Since myocardial fibrosis preceded the clinical and echocardiographic signs in this family we consider the disease a hereditary form of cardiac fibrosis.
Twenty-five family members were clinically evaluated and 5 unaffected and 8 affected family members were included in a genome-wide linkage study. The highest LOD score (2.6) was found in the region of the lamin AC (LMNA) gene. Mutation analysis of the gene both by denaturing gradient gel electrophoresis (DGGE) and sequencing failed to show a mutation. Subsequent Southern blot and multiplex ligation-dependent probe amplification analyses, however, revealed a deletion of the start codon-containing exon. Up- and downstream flanking exons appeared not to be deleted.
In vitro studies demonstrated that the deletion results in the formation of nuclear aggregates of lamin, suggesting that the mutant allele is being transcribed and that it encodes a protein with a dominant negative effect.
We therefore conclude that this novel LMNA deletion causes a distinct, highly malignant cardiomyopathy with early onset primary cardiac fibrosis, due to a dominant negative effect of the shortened protein, secondarily leading to arrhythmias and end-stage cardiac failure.

INTRODUCTION

Dilated cardiomyopathy (DCM) is characterised by dilatation and impaired contractile function of the left ventricle. The mortality rate is high, as 50% of patients do not survive more than 5 years after diagnosis[1]. The incidence is estimated at about 4/10,000 per year making it one of the most important reasons for cardiac transplantation[2]. Up to 50% of cases lack an underlying diagnosis, justifying the classification "idiopathic" DCM (iDCM)[3].
The familial character of iDCM has become increasingly clear as several recent studies have shown that up to 35% of all iDCM patients have at least one affected (first-degree) relative[4-7]. In at least 60% of these familial iDCM cases, an autosomal dominant inheritance pattern is suggested[6-8].
A number of genes involved in different types of inherited cardiomyopathies have recently been elucidated[9]. Among the most frequently reported genetic causes of DCM, identified in up to 30% of cases of DCM in association with cardiac conduction disease (CCD) or supraventricular arrhythmias, are mutations in the gene encoding the lamin A and C (LMNA) proteins.
DCM is clinically highly variable, encompassing the spectrum from frank dilatation with relatively little fibrosis to less dilative forms with more severe derangement of myocardial histology.
Myocardial fibrosis accompanies virtually all forms of cardiomyopathy[10] including inherited forms and is therefore commonly regarded to be a secondary phenomenon. The idea that fibrosis is secondary to heart failure has been fuelled by the fact that monogenetic causes giving rise to extensive primary myocardial fibrosis have not been described yet.
We here demonstrate a large family, known in our clinic with a hereditary form of early onset cardiac fibrosis[11] and address the question whether myocardial fibrosis can be considered a separate disease entity or must be regarded as a subphenotype of DCM.

MATERIALS AND METHODS

Investigated family and cardiological investigations

The investigated family has been known at our hospital for nearly fifty years[11]. As part of our clinical genetic counselling procedure we asked patients to inform relatives about the possibility of genetic counselling and cardiological evaluation. Patients who were investigated gave their written informed consent. To establish the clinical phenotype, electrocardiographic recording, exercise testing, echocardiography, signal averaged electrocardiography and 24-hour electrocardiography were performed. Due to the extensive and early fibrosis that has been described in this family[11], all family members suspected of being affected (as indicated by atrioventricular blockade or supraventricular arrhythmias on 24-hour Holter monitoring), underwent a right ventricular endomyocardial biopsy (EMB) after obtaining a fully normal coronary angiogram that included a left ventricular angiogram.

Histology

Paraffin sections of EMB and post-mortem full-thickness sections of right and left ventricular myocardium were stained with hematoxylin and eosin. A Masson trichrome stain was

used to determine the amount of collagen. Patients with manifest (interstitial) fibrosis were considered as being affected.

DNA isolation

Blood samples were collected from all marked-affected and unaffected subjects of the family involved (Figure 1). Genomic DNA was extracted from peripheral blood leukocytes using the salting out procedure[12].

Linkage analysis

Genome wide mapping was performed using approximately 300 fluorescent microsatellite markers (ISOGEN Bioscience, Maarsen, the Netherlands), spread over the entire human genome with an average resolution of 10 cM. DeCODE, Marshfield and Genethon human-linkage maps (http://www.ncbi.nlm.nih.gov/mapview/) were used as guidance for inter-marker distances.
Standard polymerase chain reaction (PCR) amplification was performed in 20 μL containing 150 ng of genomic DNA, and 10 pM of fluorescently labelled primers. Reaction products from 4 to 8 markers were pooled and were separated on an automatic ABI 377 DNA sequencer or on a MegaBACE 1000. Marker analysis was performed with the GENESCAN version 3.1 and GENOTYPER version 2.5 software (Applied Biosystems, Foster City, U.S.A.), respectively. Linkage analysis was performed using Gronlod[13] with a 5% recombination frequency, a penetrance of 90% and a phenocopy rate of 10%.

Denaturing gradient-gel electrophoresis and sequence analysis of the LMNA gene

Point mutation analysis for the LMNA gene was performed using both denaturing gradient gel electrophoresis (DGGE) and sequencing. Thirteen amplicons were analysed using both methods. For sequencing, PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) and then sequenced using the Thermo Sequenase Kit (Amersham Life Science, Buckinghamshire, U.K.) or the Sequenase kit (USB, Cleveland, U.S.A.).

Southern blots

Genomic DNA (8 μg) was digested either with BamHI and XbaI or with Bgl2 and EcoRV. DNA fragments were separated overnight by gel electrophoresis in a 0.7 % agarose gel and were transferred under denaturing conditions (0.4 M NaOH and 0.6 M NaCl) to a Hybond N+ membrane. The filters were rinsed, air-dried and baked at 80 °C for two hours. Prehybridisation was carried out in 0.5 M phosphate buffer, 6% SDS, 1.0 mM EDTA solution for 30 minutes at 65°C and hybridisation in fresh solution overnight with [^{32}P]dCTP-labeled LMNA cDNA probe (a 2027 bp insert of a LMNA cDNA (IMAGp958B1610Q2)). Filters were washed twice in 2 x SSC, 0.1% SDS at 65°C for 15 minutes and in 1 x SSC, 0.1% SDS for 15 minutes and in 0.3 x SSC, 0.1% SDS for 10 minutes. After washing the blot was exposed to X-ray Kodak XAR film with intensifying screens at -80 °C for several days.

cDNA analysis

2.5 μg RNA, isolated from deletion carrier III-12, was used to make cDNA using the Ready-To-Go You-Prime-First-Strand Beads Kit (Amersham Biosciences, USA) according to the manufacturer's protocol. This cDNA was used to perform a PCR using the exon-2 forward primer (CGGAGATCTCAGAGGCACCGAC) and an exon-3 reverse primer (GCAG-CATCTCATCCTGAAGT). PCR products were purified and sequenced as described above. For the positioning of the BACs relative to the human sequence, we have used the May 2004 human reference sequence (UCSC version hg 16) based upon NCBI Build 35.

Multiplex ligation-dependent probe amplification

For the detection of large deletions or duplications of whole exons, we used the multiplex ligation-dependent probe amplification (MLPA) test[14,15]. The probe mix contains 8 coding exon probes for the LMNA gene (exons 1,2,3,4,6,7,8 and 10, MRC-Holland). In addition, we designed a set containing 3 adjacent non coding exons probes at the 5' region of the LMNA gene and 4 control probes specific for DNA sequences outside the LMNA gene.

Cell culture, immunoblotting and indirect immunofluorescence

Primary human dermal fibroblasts from a patient and an unaffected relative were cultured at 37ºC under 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Cells were counted and plated in 6-wells plates, allowed to attach for 24 hours and lysed with sample buffer. Protein samples were sheared through a 23G needle and boiled at 95ºC for 10 min. Proteins were separated in 8% acrylamid gels and transferred onto PDVF membranes. Primary antibodies against lamins A and C (Jol2, a kind gift from dr. C.J. Hutchison, Durham, U.K.) and GAPDH (RDI TRK5G4-6C5) were incubated overnight at 4ºC in 3.5% Protifar plus (Nutricia, The Netherlands) in TRIS-buffered saline, 0.1% Tween. Secondary antibody, rabbit anti-mouse IgG HRP-linked, was visualised with Enhanced-chemo-luminescence.
For immunofluorescence studies cells were plated on coverslips, allowed to attach for 24 hours and fixed in 3.7% formaldehyde. Cells were permeabilised with 0.1% Triton and stained with primary antibody against lamin A (133a2) and counterstained with FITC-conjugated rabbit anti-mouse IgG (F0232, DAKO, Heverlee, Belgium). Immunofluorescently labelled cells were counterstained with the DNA dye 4'-6-diamidino-2-phenylindole (DAPI). Nuclear aggregates of 100 separate nuclei were counted.

Table 1
Clinical characteristics of mutation carriers.

			referred for									LV dilatation							
No in pedigree	**Sex**	**Age diagnosis**	**Palpitations**	**Dyspnea**	**CVA / Thrombosis**	**Screening**	**CCD (Age)**	**AF (Age)**	**VPB (/Hr); VT's**	**NSVT**	**Age echo**	**LV function**	**LVEDD**	**LVESD**	**Age at death**	**Cause of death**	**Myocardial PA**	**Biopsy / Autopsy (age)**
III-3	F	34	+				1st degree AVB (34)	+ (34)	58; 2	+	44	nl	38	27	60	Pneumonia due to ALS	+ extensive fibrosis + CS	B (54)/A (60)
III-7	F	50	+				AVB (50)	+ (50)	NA		NA				53	Progressive HF	+ extensive fibrosis + CS	A (53)
III-8	F	22	+				LBBB (22)	+ (35)	NA		40	pr	45	39	46	Progressive HF	+ fibrosis	B (40)
III-11	M	49					NA	NA	NA		NA				49	SCD	+ extensive fibrosis + CS	A (49)
III-12	F	42			+		LBBB (42)	+ (42)	284; 1	+	42	pr	55	45	-	-	+ fibrosis	B (48)
III-14	M	40				+	1st degree AVB (40)	+ (48)	15; 0	-	36	nl	54	37	-		No abnormalit-ites	B (47)
III-15	F	20	+				1st degree AVB (27)	-	318 ; 0	-	43	nl	46	27	-	-	+ fibrosis	B (43)
III-17	M	43			+		Aspec IV CD	+ (44)	90; 3	+	45	pr	50	38	49	Progressive HF	+ fibrosis + CS	B (45) / A (49)
III-18	M	40	+	+			LBBB (40)	+ (40)	474 ; 4	+	40	pr	52	45	41	SCD	+ fibrosis + CS	B (40) / A (41)
III-19	F	45	+				1st degree AVB (47)	- (47)	70; 0	+	47	nl	46	25			NA	
IV-2	F	42	+			+	1st degree AVB (42)	+ (42)	3 ; 0	-	42	nl	50	29	-		+ fibrosis	B (42)
IV-5	F		+			+	-	-	1; 0	-	39	nl	51	24			+ fibrosis	B (39)
IV-6	F	35	+			+	-	-	1; 0	-	36	nl	46	30			+ fibrolipomato-	B (36)

																fibrolipomatosis	
IV-7	M	30		+	1st degree AVB	-	4; 0	-	32	nl	51	40	-	-	NA		
IV-8	M	33		+	-	-	63; 0	-	33	nl	55	35			+ distinct fibrosis	B (33)	
IV-9	F	26		+	-	-	2; 0	-	26	nl	41	28	-	-	+ distinct fibrosis	B (26)	
IV-11	M	26		+	1st degree AVB (26)	-	9; 0	-	26	nl	48	32	-	-	NA	-	
IV-14	M	20	+		LBBB		1547; 4	+	20	pr	69	61	HTX	-	+ diffuse fibrosis	B (20) post HTX	

AF= atrial fibrillation, ALS= amyotrophic lateral sclerosis, AVB= atro-ventricular block, CCD= cardiac conduction disease, CS= conduction system, HF= heart failure, HTX= heart transplantation, IV CD= intra-ventricular conduction delay, LBBB= left bundle branch block, LV= left ventricular, LVEDD= left ventricular end diastolic dimension, LVESD= left ventricular end systolic dimension, NA= not applicable, NI= not investigated, NL= normal, NSVT= non-sustained ventricular tachycardia, PA= pathological anatomy, PR= poor, SCD= sudden cardiac death, VPB= ventricular premature beat

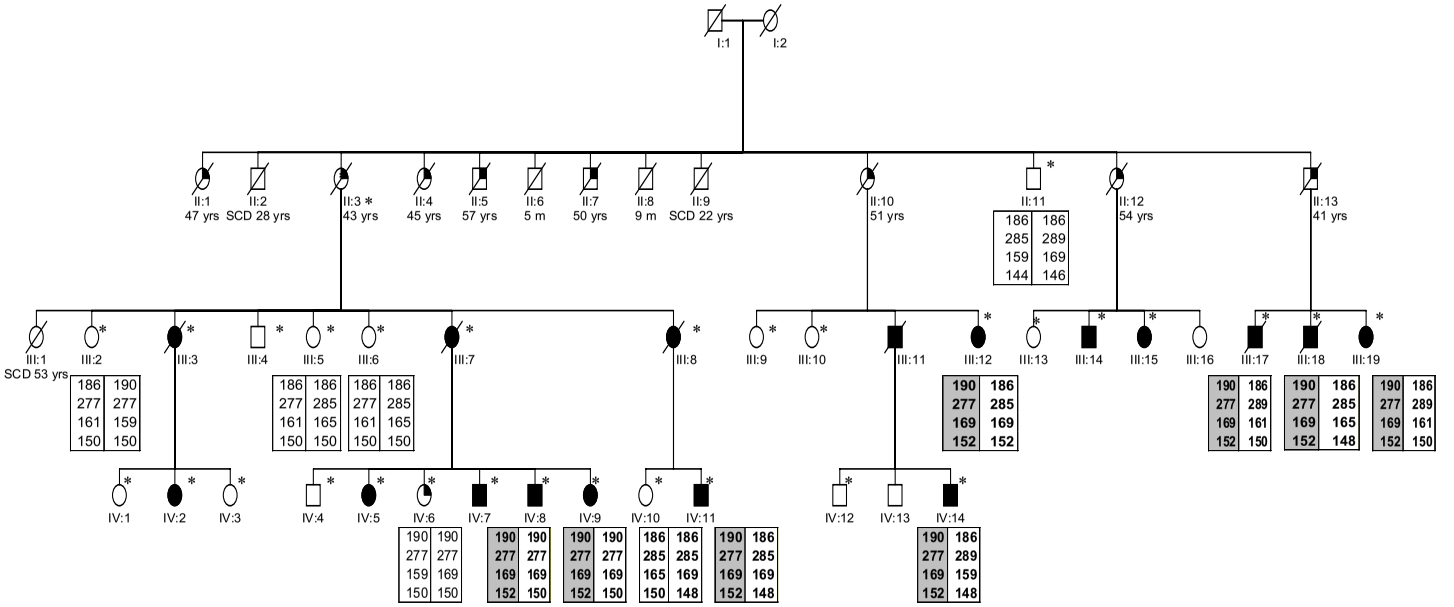

Figure 1

Pedigree of the family. Black filled symbols indicate clinically and genetically affected persons (deletion carriers). Top-right black filled symbols indicate clinically affected on either personal examination, from medical records or literature (11). Open symbols indicate clinically (and except for III-19 also genetically) unaffected individuals and line through symbols, deceased individuals. # indicates that the DNA was included in linkage analyses. SCD=sudden cardiac death.

RESULTS

Clinical and histopathological evaluation

We evaluated 29 individuals from three generations (Figure 1); generation II has been described previously[11]. Twenty-five family members visited us for screening starting in 1995 (11 males, 14 females). We studied the clinical records and postmortem examinations of four additional persons (three female) who had died (III-3, III-7, III-8 and 11)(Figure 1). No signs of neuromuscular disease or lipodystrophy were noted. CPK values were within normal limits. Two patients (III-17 and 18) died during follow-up.
Of the 29 family members we studied, 18 were suspected to be affected either on clinical grounds or because of premature death. Of these 18 subjects, 15 were histologically investigated: 13 underwent EMB (four of these with additional post-mortem or post transplantation examinations). In two patients only post-mortem investigations were available.

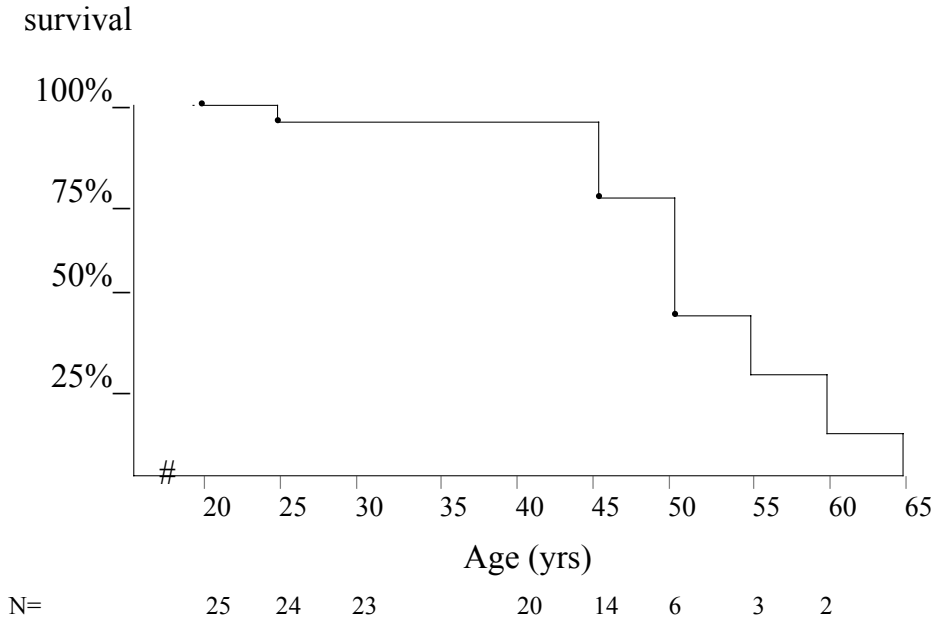

Figure 2
Kaplan-Meier curve with end points cardiac death or cardiac transplantation.

Myocardial fibrosis was found in 14 of these 15 histologically investigated subjects. In four of these (III-12, III-15, IV-8, IV-9 see Table 1) distinct pathological fibrosis was identified compared to more extensive fibrosis in remaining 10 patients. In patient IV-6, EMB showed fibrolipomatosis that could not be distinguished from arrhythmogenic right ventricular cardiomyopathy. Histological post-mortem examination of full thickness myocardium in 5 patients (III-3, III-7, III-11, III-17 and III-18), all demonstrated extensive areas of interstitial and replacement fibrosis throughout the left and right ventricular myocardium and extensive

fibrosis of the cardiac conduction system. The definite diagnosis was decided based on a positive biopsy or post-mortem histological proof of fibrosis.
More importantly, fibrosis (III-15, IV-2, 5, 8 and 9) or fibrolipomatosis (IV-6) was detected in six subjects who did not exhibit abnormalities on echocardiography; in four of them (III-15, IV-2, 5 and 6) palpitations were the only complaint. In asymptomatic subjects, a first degree AV-block or supraventricular tachycardias raised the suspicion that they might be affected, which resulted in the treating physician ordering an EMB, which revealed fibrosis.
Thus, out of all 14 of the affected family members with pathological interstitial myocardial fibrosis, five had cardiac fibrosis and one had fibrolipomatosis without any important loss of left ventricular function. The clinical data and survival curve for these patients are shown in Table 1 and Figure 2.

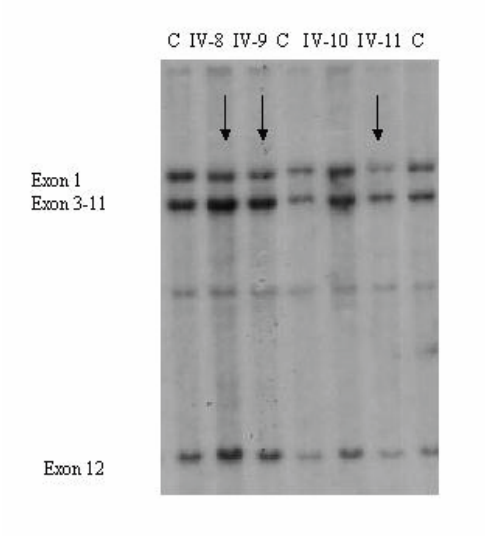

Figure 3
Southern blot showing reduced intensities of the signal corresponding with exon 1 of the LMNA gene in affected patients (IV-8, IV-9, IV-11) compared to controls (C) and a non-affected family member (IV-10).

Genetic analysis

Genome wide linkage screening yielded the highest LOD score for the region containing the LMNA gene. Although linkage was not completely conclusive since the obtained experimental LOD score was 2.6 and no marker was linked without recombinants, the gene was screened for point mutations using both DGGE and direct sequence analysis. These analyses did not reveal any sequence variation. Subsequent Southern blot analysis using two different double digests showed a deletion of the exon containing the start codon of the LMNA gene (Figure 3). PCR on cDNA revealed two PCR products of approximately 1050 and 400 bp in length (data not shown). Subsequent sequence analysis showed cDNA containing all exons whereas the shorter band was lacking exon-1 and 1 of the LMNA gene (Figure 4). This resulted in a deletion of 674 bp, which explains the two different-sized bands on the agarose gel. The deletion of exon 1 was confirmed by MLPA analysis, whereas the other coding exons had been retained. Because of technical difficulties in the design of the primers, a deletion of exon-1 could not be confirmed.

From 16 of the 18 clinically affected family members DNA samples were available. The LMNA deletion was identified in all but one (IV-6) of these 16 persons.

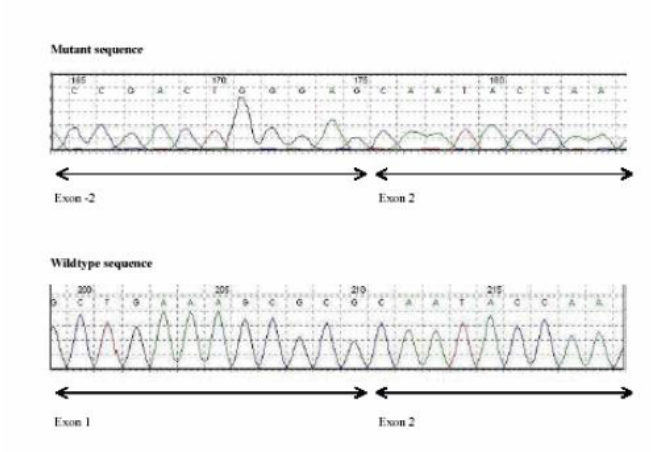

Figure 4
Forward cDNA sequence of III-12 showing the last 11 nucleotides of exon –2 followed by the first 9 nucleotides of exon 2 (upper panel) and the wild-type sequence in the lower panel

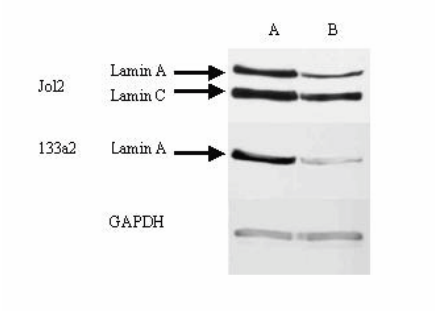

Figure 5
Western blot analysis showing decreased expression of both lamins A and C in fibroblasts from mutation carrier (B), as compared to a non-carrier family member (A). No alternative product from the mutated allele was seen. GAPDH serves as a control that equal amounts of protein have been loaded.

Cell culture studies

To assess the effect of this novel deletion on the expression of lamins we performed Western blot and indirect immunofluorescence assays. Western blot analysis of lamins A and C clearly showed decreased expression of both normal-sized lamins A and C in cells from a mutation carrier, when compared to the expression of control cells (Figure 5). Indirect immunofluorescence clearly showed an increased number of nuclear aggregates in cells from a lamin mutation carrier but hardly any aggregates in control cells (Figure 6). (0.54 (standard

error of mean (SEM) 0.10) in control cells vs. 6.05 (SEM 0.32) in cells from a lamin mutation carrier ($p<0.0001$)).

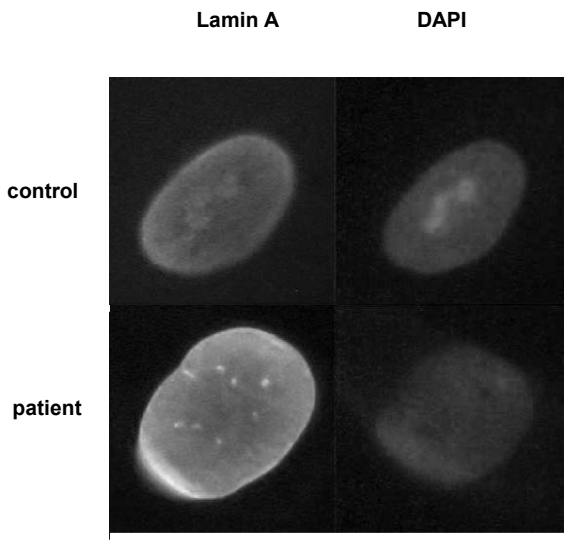

Figure 6
Indirect immunofluorescence with lamin A antibody showed nuclear lamin aggregates in fibroblasts from mutation carrier, but not in control fibroblasts.

DISCUSSION

In a family with early onset myocardial fibrosis, we identified a deletion of the 5' end of the LMNA gene including the start codon containing exon. This deletion causes a striking form of cardiac fibrosis to occur early in the development of the cardiomyopathy. More importantly, in six patients cardiac fibrosis was present before cardiac function was clearly affected as judged by echocardiography. Myocardial fibrosis was also noted in the majority of the first families described by Fatkin et al. having dilated cardiomyopathy due to LMNA gene mutations[16], but primary fibrotic forms of cardiomyopathy caused by LMNA or other mutations still has not been described. This early occurrence of severe cardiac fibrosis in the absence of cardiac function loss in the presented family, suggested that the observed deletion caused a primary form of cardiac fibrosis. If fibrosis would were secondary to a loss of myocytes, it would then be expected that the diffuse and widespread severe cardiac fibrosis should be accompanied by a widespread loss of myocytes with impaired cardiac function. However, since cardiac function remained preserved despite massive and diffuse cardiac fibrosis, it is highly probable that the fibrosis is a primary phenomenon. Our finding of nuclear aggregates of lamin in cultured fibroblasts from a mutation carrier suggests a dominant negative effect of this deletion. This raises the possibility that also other dominant

negative LMNA gene mutations may cause primary fibrosis, thus forming the underlying pathology in this cardiac phenotype.

Pathophysiologic effect of the LMNA deletion containing the start codon

The LMNA gene encodes lamins A and C by means of alternative splicing. These proteins are major components of the nuclear lamina, a fibrous network underlying the inner surface of the nuclear envelope[17]. Mutations in the lamin A/C gene (http://www.DMD.nl/lmna_home.html) have been reported in a variety of disorders, such as autosomal dominant and recessive Emery-Dreifuss muscular dystrophy (MIM 181350 and 150330 respectively)[18,19], limb-girdle muscular dystrophy type 1B (MIM 159001)[20], autosomal recessive Charcot-Marie-Tooth disease type 2B1(MIM605588)[21], Dunnigan-type familial partial lipodystrophy (MIM 151660)[22], mandibuloacral dysplasia (MIM 248370)[23] the Hutchinson-Gilford progeria syndrome (MIM 176670)[24,25], atypical Werner's syndrome (MIM 277700)[26] and lipoatrophy with diabetes, hepatic steatosis, hypertrophic cardiomyopathy and leukomelanodermic papules[27]. Finally mutations in the LMNA gene have been identified in patients with iDCM with conduction disease[16] or "pure" DCM[28,29]. The exact pathophysiological mechanism of LMNA mutations leading to such an impressive heterogeneous spectrum of disorders, has not yet been elucidated. However, myocardial fibrosis as a secondary phenomenon has been described before in different laminopathies[16,30,31].
We identified a deletion of the LMNA exon containing the start codon. Such a deletion is predicted to affect mainly the head-domain of lamin i.e. may lead to a shortened protein lacking the head domain. Assuming that the deletion does not interfere with the splicing of the other exons, potential new translation initiation sites lie at cDNA position 559 and 598 (amino-acids 187 and 200 respectively). When these proteins are produced, they lack the N-terminal region of lamin A and C, resulting in a smaller protein lacking the head domain as we also found here. Previously, targeted mutation studies in Chinese hamster ovary (CHO) cells have shown that functional disruption of the head domain has dominant negative effects, as these aberrant proteins can form aggregates of lamin in the nucleus[32]. In line with this in-vitro observation, we also found an increased number of lamin nuclear aggregates in the nucleus of cultured fibroblasts of a carrier of this deletion, as opposed to the virtual absence of these aggregates in a non-deletuion carrier family member. This suggests that the mechanisms identified *in vitro*[32] do cause cardiac pathology *in vivo* via this dominant negative effect.

Do lamin A/C mutations cause a specific cardiac phenotype?

The large pedigree made it possible to clinically evaluate patients at the earliest sign of being affected, i.e. when demonstrating subtle cardiac (supraventricular) arrhythmias, or cardiac conduction disease. LMNA mutations can give rise to cardiac conduction disease[31]. The histopathological substrate for the occurrence of conduction disease, however, remains unclear. We were, however, able to demonstrate that pathological myocardial fibrosis occurs at the first signs of conduction delay in a relatively large number of the family members affected. At post-mortem examinations, extensive fibrosis of the conduction system was especially notable, since in six of the patients cardiac fibrosis was present before cardiac function was clearly affected as judged by echocardiography.
Another distinct finding was the small extent of dilatation for a mutant gene often described as a cause for DCM. Despite severe cardiac complications in laminopathies, this observation has been made before[33-37]. Therefore these combined data suggest that LMNA muta-

tions may cause a distinct cardiomyopathy characterised by fibrosis and rather limited cardiac dilatation, which suggests a distinct disease entity within the spectrum of idiopathic "dilated" cardiomyopathy. This implies that distinct forms of cardiomyopathy can be encountered and that LMNA mutations may give rise to a type of cardiomyopathy in which primary fibrosis drives the specific pathophysiology on a highly malignant course. Myocardial fibrosis, therefore, may not occur as a separate disease entity, but rather as a subphenotype of DCM.

This study has also shown that it is difficult to establish conclusive linkage when the phenotype cannot be established beyond a doubt on the basis of clinical criteria and age. Only after the identification of a large intragenic deletion in the LMNA gene, in the region of the highest LOD score among all other genomic loci, was the associated phenotype able to be established, demonstrating both age-dependent penetrance and the occurrence of a phenocopy.

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CHAPTER 5

Primary prevention of sudden death in patients with lamin A/C gene mutations

Based on:
Christophe Meune, Jop H. van Berlo, Frédéric Anselme, Gisèle Bonne, Yigal M. Pinto and Denis Duboc. Primary prevention of sudden death in patients with lamin A/C gene mutations.
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Abstract

Aim: Atrial fibrillation, atrioventricular conduction defects, heart failure and sudden death are common manifestations in patients with lamin A/C gene mutations. Recent reports emphasised that ventricular arrhythmias may be responsible of sudden death; we hypothesised they could be prevented by internal cardioverter defibrillator (ICD).

Methods and results: 19 consecutive patients (41.7±13.4 years, 14 men, ejection fraction 58.1±11.6%) with documented lamin A/C mutations, referred for permanent pacing, were included in this prospective observational study and ICD was implanted. Arrhythmic events were recorded by the electronic memories of the implanted devices; factors potentially related with ventricular tachyarrhythmias were analysed by uni- and multivariate analysis.
After a mean follow-up of 33.9±21.0 months, 8 patients received appropriate ICD therapies. Ventricular tachyarrhythmias were documented in 11 patients, including 6 patients with ventricular fibrillation. The arrhythmias were treated with antitachycardia pacing in 1 patient, and by automatic shocks in 7 patients. The events were not sustained in 3 patients. No factor was found to be predictive of arrhythmic events, including spontaneous or induced ventricular or supraventricular tachyarrhythmias, ambient drug therapy, or cardiac function.

Conclusion: In patients with lamin A/C mutations and significant sinus dysfunction or atrioventricular conduction disturbances, life-threatening ventricular arrhythmias were common and adequately treated by prophylactic ICD.

INTRODUCTION

Lamins A and C, encoded by the *LMNA* gene, are major structural components supporting the nuclear envelope[1]. Lamin A/C mutations cause several diseases, including Emery-Dreifuss muscular dystrophy[2], dilated cardiomyopathy (DCM) with conduction system disease[3,4], limb-girdle muscular dystrophy type 1B[5], familial lipodystrophy of Dunnigan type[6], Charcot-Marie-Tooth disease type 2[7], mandibuloacral dysplasia[8], Hutchinson-Gilford progeria[9,10], atypical Werner's progeroid syndromes[11], and a recently described restrictive dermopathy[12]. We and others have previously highlighted the role of lamin A/C mutations in sudden death and heart failure[3,4,13-17]. Several studies have pointed out that different lamin A/C mutations cause very similar cardiac phenotypes, characterised by atrial fibrillation, conduction system disease requiring pacemaker implantation, sudden death and heart failure[3,4,13,15,17]. Some reports have emphasised that sudden death may be the first disease manifestation[4,15]. Additionally, it may be due to ventricular arrhythmias and therefore occur despite the implantation of a pacing system[4,14,16,17]. While there is consensus regarding secondary prevention by implantable cardioverter defibrillators (ICD), the efficacy of this strategy in the primary prevention of sudden death, in carriers of lamin A/C mutations, has not been studied. Based on the natural history of the disease, we have proposed to implant ICD instead of pacing systems in patients presenting with lamin A/C mutations and cardiac conduction disturbances, regardless of history of spontaneous or inducible ventricular tachyarrhythmias. We report the initial results of this prospective therapeutic strategy.

PATIENT ENROLLMENT AND METHODS

This was an observational prospective study conducted at 4 medical centers highly skilled in the management of patients with genetically determined cardiac and muscular disorders. Our study complies with the Declaration of Helsinki; the study protocol was reviewed and approved by the Ethics Committee of each participating institution, and all patients granted their informed consent. Between March 1999 and January 2004, all patients with documented lamin A/C mutations and referred for permanent cardiac pacing to a participating center were systematically offered the implantation of an ICD. A history of ventricular tachyarrhythmia, spontaneous or inducible by programmed ventricular stimulation, was not a selection criterion, and patients were enrolled solely on the presence of lamin A/C mutations associated with cardiac conduction defects. Clinical information, collected before and after the implantation of the cardioverter defibrillator, were prospectively stored in a computer database and reviewed by 2 experts unaware of the patients' clinical status. Data collected included demographic characteristics, specific gene mutation, type of conduction defects requiring implantation of a pacemaker, left ventricular ejection fraction (LVEF), drug regimen, presence of ventricular arrhythmias previously identified on 24-h ambulatory electrocardiographic monitor and, when available, results of signal-averaged electrocardiogram and electrophysiologic testing before cardioverter defibrillator implantation. The electronic memories of the pulse generators were regularly interrogated and downloaded to determine whether ventricular episodes of ventricular tachycardia (VT) or ventricular fibrillation (VF) had occurred. VF was defined as regular or irregular tachycardia with regard to QRS or electrographic polarity, amplitude, morphology and sequence, with a mean cycle length of 240ms or less[18]. VT was defined as regular (monomorphic) or irregular (polymorphic) tachycardia with regard to QRS or electrocardiographic polarity, amplitude and morphology with a mean cycle length of more than 240ms. In case of shock delivery by the ICD, event mark-

ers and electrogram recordings were thoroughly analyzed by 2 investigators to verify the appropriateness of therapy.

Statistical analyses

Differences between groups were examined by use of the Mann-Whitney and Wilcoxon's signed-rank tests for continuous variable, and Fisher's exact test for categorical variables. Factors potentially related with the occurrence of ventricular tachyarrhythmias, including sex, LVEF, left ventricular dilatation, presence of spontaneous or induced ventricular arrhythmias and medical treatment, were identified by univariate analysis with Fisher's exact test for categorical variables, and simple logistic regression analysis for continuous variables. A *P* value less than 0.05 was considered statistically significant
In addition, survival without appropriate cardioverter defibrillator intervention was estimated using Kaplan Meier method. (Statview software Abacus Concept, Berkeley, CA).

RESULTS

Between March 1999 and January 2004, 19 consecutive patients were enrolled, including 9 related patients from 4 separate families, and 10 unrelated patients.Their baseline characteristics, including muscular and non-muscular phenotypes, gene mutations and cardiac findings before ICD implantation are presented in Table 1. The primary clinical presentation of these patients was as follows: 9 patients presented with Emery-Dreifuss muscular dystrophy, one with limb-girdle muscular dystrophy, one with dilated cardiomyopathy with cardiac conduction system disease, one with shoulder's muscle amyotrophy; the remaining 7 patients were screened as family of the probands.
No patients experienced symptoms of syncope, transient loss of consciousness or sustained palpitations.
Indication for permanent pacing was as follows: paroxystic 3rd degree atrio-ventricular (A-V) block in 4 patients, 2nd degree A-V block in 4, infra-nodal 1st degree A-V block in 4, nodal 1st degree A-V block in 3, sinus dysfunction in 3, and isolated mild bradycardia in a patient but with associated atrial fibrillation and symptoms of limitation at rest.
Spontaneous ventricular arrhythmias were recorded during 24-h ambulatory electrocardiographic monitoring in 11 patients, consisting usually of isolated ventricular premature complexes. No patient had late potentials on the signal-averaged electrocardiogram. Ventricular tachyarrhythmias were induced in 3 of 15 patients who underwent programmed ventricular stimulation (Table 1).

Table 1
Baseline characteristics of overall population (n=19) versus patients who experienced ventricular arrhythmias (VA) during follow up (n=11)

	All patients	VA during follow up
Men/Women, n	14/5	9/2
Age, years (mean ± SD)	41.7 ± 13.4	41.4 ± 15.5
Muscular phenotype, n		
Emery-Dreifuss	9	8
Limb-Girdle	1	1
Dilated Cardiomyopathy	1	0
Shoulder's muscle amyotrophy	1	1
Gene mutation, n		
c.28ins, p.Thr10delinsThrfsX30	2	0
c.1_356Del, p.Met1 (Del exon 1)	3	0
c.778_780delAAG, p.Lys260del	1	1
c.16C>T, p.Gln6X	5	5
c.746G>A, p.Arg249Gln	1	1
c.1129 C>T, p.Arg377Cys	2	0
c.1130 G>A, p.Arg377His	1	1
c.1357 C>T, p.Arg453Trp	1	1
c.149 G>C, p.Arg50Pro	1	1
c.178 C>G, p.Arg60Gly	1	0
c.976 T>A, p.Ser326Thr	1	1
Supraventricular arrhythmias on 24-h ECG		
None	4	1
Atrial premature complexes in runs	4	2
Paroxysmal atrial fibrillation	10	8
Permanent atrial fibrillation	1	0
Ventricular arrhythmias on 24-h ECG		
None	8	4
Isolated premature ventricular beats	8	5
Ventricular premature complexes in runs	2	2
Ventricular tachycardia	1	0
Left ventricular ejection fraction, %	58.1 ± 11.6	62.4 ± 9.6
Late potentials on signal-averaged ECG	0/14	0/10
Inducible ventricular tachyarrhythmia, n	3/15	2/9

Table 2
Cardiac status of patients with versus without ventricular arrhythmia (VA) during follow up

	VA (n=11)	No VA (n=8)	P-value
LVEF, %	62.4 ± 9.6	52.1 ± 12.0	NS
LVEF < 45% (n)	1	2	NS
Prior treatment with ACE-i (n)	1	2	NS
Prior treatment with β-blocker (n)	2	1	NS
Prior antiarrhythmic treatment* (n)	5	1	NS
Inappropriate ICD shock (nr of pts)	1	0	NS

VA = ventricular arrhythmia during follow up, LVEF = left ventricular ejection fraction, ICD = implantable cardioverter defibrillator, * Including β blocker agent and/or amiodarone

An ICD with dual chamber pacing system was implanted in all: no complication occurred. After a mean follow-up of 33.9±21.0 months, ventricular tachyarrhythmias were documented in 11 patients (Table 1). Appropriate cardioverter defibrillator shocks were delivered in 7 patients, 6 of whom had VF. One patient who had sustained VT was treated with antitachycardia pacing. The remaining 3 patients had non-sustained VT and no therapeutic was delivered by the device (Table 2). In addition, one patient received inappropriate shocks, while having also confirmed ventricular tachyarrhythmias during follow-up. Three patients in our cohort had reduced EF <45%, one experienced ventricular arrhythmias and was adequately shocked by the device whereas the 2 remaining had no ventricular arrhythmias during follow-up. It is noteworthy that 6 patients with LVEF>55% received appropriate ICD shocks during follow-up. Figure 1 is representative of follow-up after ICD implantation and time from implantation to first delivered automatic shock.
The cardiac status was not different between patients with documented ventricular tachyarrhythmia during follow-up as compared to those that remained free of ventricular tachyarrhythmia (Table 2). Additionally, no tested variable, including demographic criteria, EF, drug regimen, spontaneous or induced ventricular arrhythmias before implantation emerged as significant by multivariate analysis.
Finally, it is noteworthy that, in the overall patient population, the mean LVEF fraction decreased significantly, from 58.1±11.6% at baseline, to 46.4±15.5% at the end of follow-up (*P*=0.022), with no difference between patients with or without ventricular tachyarrhythmia during follow-up.

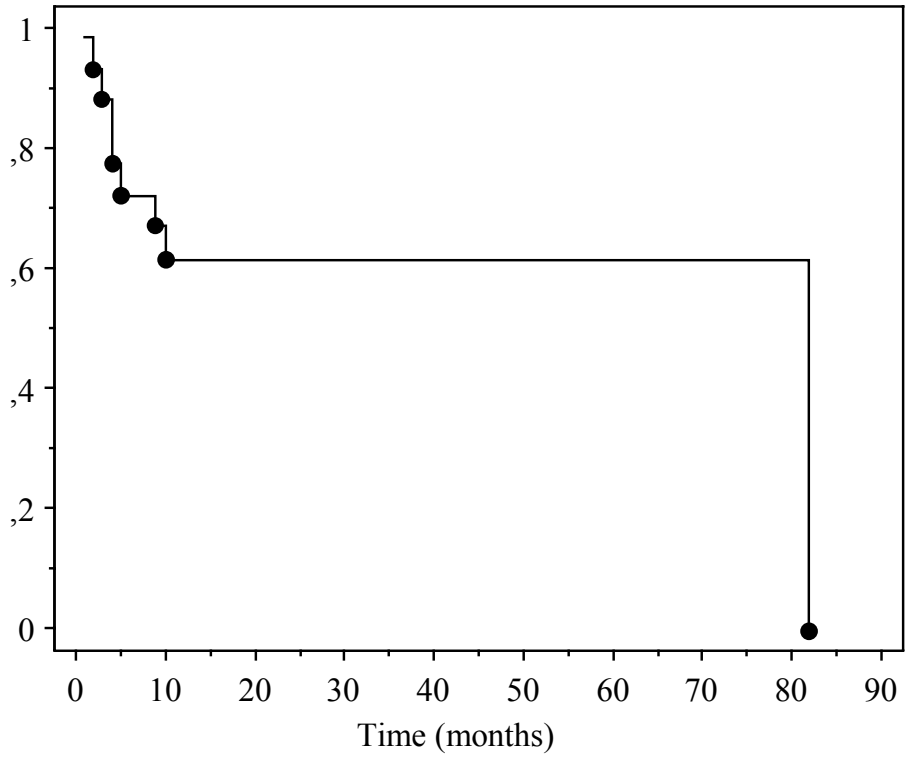

Figure 1
Survival without automatically delivered shock by internal cardioverter defibrillator (Kaplan-Meier estimation)

DISCUSSION

The main findings of this study are 1) at a mean follow up of less than 3 years, nearly 60 percent of patients with lamin A/C mutations and cardiac conduction abnormalities developed ventricular tachyarrhythmias, 2) malignant ventricular arrhythmias, prompting the delivery of appropriate ICD shocks, occurred in lamin A/C mutated patients with altered cardiac conduction but preserved cardiac function and no inducible ventricular tachyarrhythmias, and 3) prophylactic ICD implantation may increase the survival of patients with lamin A/C mutations in need for pacing, since life-threatening arrhythmias occurred in 7 patients.

The role of lamin A/C mutations in the development of heart diseases has been recently highlighted, but may be still underestimated[16]. The exact mechanisms behind the development of these disorders remain unknown, though current hypotheses include nuclear instability and dysregulation of gene expression[1,19-21]. While lamin A/C mutations may be associated with different skeletal muscle abnormalities, they are also responsible for very similar cardiac phenotype, characterised by atrial fibrillation, cardiac conduction disturbances, followed by ventricular arrhythmias coexisting with or preceding heart failure[3,4,13-15,17].

Sudden death, which accounts for approximately 50 percent of all-cause mortality in pa-

tients with lamin A/C mutations, may be due to conduction system disease as well as ventricular arrhythmias, which are not preventable by the implantation of a pacing system[3,4,16,17]. The high incidence of ventricular tachyarrhythmias observed in our patients with lamin A/C mutations and A-V conduction disturbances or sinus dysfunction is concordant with these earlier studies.

A reliable prevention of ventricular tachyarrhythmias is of vital importance. ICD are effective in the primary and secondary prevention of sudden cardiac death in patients with or without coronary artery disease and a depressed LVEF[22,23] but their effectiveness in the primary prevention of sudden death in patients with lamin A/C mutations has not been previously reported. In this study, we found no factor positively or negatively predictive of the long-term incidence of ventricular tachyarrhythmias. In particular, muscular phenotype, a preserved LVEF, negative electrophysiologic testing, or treatment with a beta-adrenergic blocker, angiotensin-converting enzyme inhibitor or amiodarone did not predict the absence of tachyarrhythmic events during long-term follow-up (Table 1,2). ICD implantation was uncomplicated in all patients, and we documented after a mean follow-up of almost 3 years, cardiac tachyarrhythmias in 11 patients. Moreover, if we restrict our analysis to "malignant ventricular arrhythmias", we documented an astonishing high percentage of patients adequately resuscitated by the device as 6 patients had VF and 1 patient had sustained VT >240 beats/min during follow-up[24]. Taken together, these observations may suggest that patients with lamin A/C mutations and conduction system defects should be viewed as candidates for ICD rather than pacing alone, regardless of other factors, including LV function or history of life-threatening arrhythmias.

The optimal timing of ICD implantation cannot be ascertained by our study. On one hand, we demonstrated that ventricular tachyarrhythmias may occur early in the course of the disease, before LV dysfunction. Moreover, 3 patients not included in this study while having no conduction defect, underwent implantation of ICD on the basis of sinus bradycardia and the background of family history of sudden cardiac death and gene mutation. One of these had ventricular tachyarrhythmia during follow-up and was treated by automatic shock. Ventricular tachyarrhythmia may therefore be the first manifestation of the disease, which suggests that the device should probably be implanted soon after lamin A/C mutation identification. On the other hand, all of our patients included in the study had conduction system defects and permanent pacing have been planned. Therefore, pending the results of a larger study, we recommend that all patients with lamin A/C mutations planned for permanent pacing undergo ICD implantation. While this strategy should markedly improve the survival of this patient population, they may later develop LV dysfunction as the disease progresses[15,16], as was indeed observed in our own study. The design of a preventive treatment to delay the development of heart failure remains a major challenge in these patients.

The precise mechanisms of ventricular tachyarrhythmias in this disease remains undetermined. They may develop in the context of advanced heart failure and fibrosis, but occur also in patients with preserved LVEF[14]. Focal areas of myocardial fibrosis may be the source of arrhythmias, while having no effect on EF.

Study limitations

Our study was limited by the absence of a control group. It was our opinion that the random assignment of patients to an untreated group would be unethical, given the known high incidence of sudden death and life-threatening arrhythmias associated with this disease. Therefore, we chose a prospective observational design to evaluate the impact of prophylactic implantation of ICD on long-term survival.

The aim of our study was not to compare various strategies in the prevention of arrhythmias (devices versus amiodarone i.e.). In fact we did not document any factor associated with the absence or presence of ventricular arrhythmias, including β-blocker agent or amiodarone treatment, but our study may be underpowered for such analysis due to our small population. Moreover, as we only selected candidates for permanent pacing, we assume that our strategy of ICD implantation rather than a simple pacing system may not dramatically increase the risk of the procedure. However our study may be viewed as a preliminary report allowing future studies comparing ICD to drug therapy.
Our study may also suffer from referential bias, as we only included patients from our tertiary centres.
Important factors, which remain unanswered by this study, include the optimal timing of ICD implantation, the need of antiarrhythmic drug therapy before and after device implantation, and the impact of preventive treatment of heart failure.

CONCLUSION

The incidence of life-threatening ventricular tachyarrhythmias in patients with lamin A/C mutations and conduction system defects was high. These tachyarrhythmias, which cannot be prevented by permanent cardiac pacing, occurred in patients with different muscular phenotypes and with preserved LV function. These life-threatening ventricular tachyarrhythmias were adequately treated by the ICD. Until the development of a study comparing ICD implantation to drug therapy, ICD implantation rather than pacemaker implantation may be considered in these patients.

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CHAPTER 6

A common risk haplotype in the Lamin A/C gene is associated with heart failure

Based on:
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Submitted

Abstract

Introduction- Mutations in the lamin A/C gene (LMNA) cause familial heart failure. Single nucleotide polymorphisms (SNPs) in LMNA have been shown to increase risk for insulin resistance, obesity and metabolic syndrome. We tested whether SNPs in LMNA modify cardiac phenotypes in cardiac patients and in probands from the general population.

Methods and results- We performed a comprehensive SNP analysis of LMNA and its surrounding genomic region to obtain a high resolution linkage disequilibrium (LD) and haplotype structure. The coding region of LMNA resides entirely on one haplotype block with exceptional high levels of LD. Two highly different clades of haplotypes exist within that block (hα, 91% and hβ, 9%). We found indication for positive evolutionary selection of clade hα-haplotypes.

In the general population (n=702) clade hβ-haplotypes were associated with diminished posterior wall thickness (p=0.030) and movement (p=0.027) and with diminished velocity time integral over the aortic valve (p=0.002). In a large general population sample (n=4034) clade hβ-haplotypes were associated with heart failure (OR 1.63, p=0.035) and with presence of a pacemaker (OR 3.66, p=0.018).

In a sample of familial heart failure patients (n=106), where mutations in LMNA had been excluded, carriers of at least one hβ-haplotype were diagnosed with heart failure 10 years earlier (p=0.01) and presented with more severe cardiac dysfunction.

Conclusions- We identified a risk haplotype in the LMNA gene which was associated with increased risk of heart failure in the general population. In familial heart failure the risk haplotype caused earlier and more severe presentation of heart failure.

INTRODUCTION

In recent years, lamin A/C (LMNA) gene mutations have gained much attention due to the multitude of diseases they potentially cause. At least 9 different disorders can be attributed to mutations in the LMNA gene, among them certain types of muscular dystrophy, lipodystrophy, premature aging syndromes, and dilated cardiomyopathy[1-10]. In patients with familial forms of dilated cardiomyopathy (DCM), LMNA mutations are the most frequently reported [11], making LMNA mutations an important cause of hereditary heart failure. The mechanism by which mutations in the LMNA gene lead to cardiac disease has not been elucidated yet.

Given the cardiomyopathy caused by mutations in lamins A and C, it is intuitive to postulate that common polymorphisms in the LMNA gene may modulate cardiac disease in a broader sense. Some authors identified association between a common synonymous single nucleotide polymorphism (LMNA-H556H, rs4641, MAF=0.21) in LMNA and dyslipidemia and insulin resistance in Japanese[12], obesity indexes in Inuit[13], subcutaneous adipocyte size in Pima Indians[14], plasma leptin levels and physical indices of obesity in Canadian Oji-Cree[15]. These reports however only investigated the role of a single SNP in the LMNA gene. Recently a haplotype combining 5 SNPs within the genomic region of the LMNA gene was shown to associate with the metabolic syndrome[16].
To investigate more comprehensively whether polymorphisms in the LMNA gene are associated to cardiac disease we analysed the linkage disequilibrium (LD) structure of polymorphisms within the LMNA gene and its surrounding genomic region and constructed haplotype blocks based on the knowledge about the LD structure. Next we tested the association of haplotypes to cardiac phenotypes in patients with familial dilated cardiomyopathy (n=106) as well as in a general population sample (n=4034).

METHODS

Study Population

General population sample
Between 1999 and 2001 the KORA (Cooperative Health Research in the Region of Augsburg) survey was performed in the region of Augsburg, Southern Germany. The survey was population based, originating out of and designed similar to the WHO MONICA surveys[17]. Out of the total study population (n=4261), a randomly selected sample of 702 individuals was studied between 2002 and 2003 specifically for cardiovascular diseases. All individuals underwent resting 12 lead ECG recordings and 2D and M-mode ultrasonography. Two expert investigators judged ultrasounds independently and averaged measurements were used.
In these 702 individuals we studied LMNA haplotype block structure as well as the association between LMNA and ECG and ultrasound phenotypes. In 4034 individuals we studied the association between one haplotype and heart failure or the presence of a cardiac pacemaker. Heart failure or pacemaker therapy was ascertained by a questionnaire, where we regarded only subjects to have heart failure if they reported to have received treatment for it in the last 12 months.
Blood samples were drawn after informed consent had been obtained. All studies involving

humans were performed according to the declarations of Helsinki and Somerset West and were approved by the local medical ethics committee.

DCM Patients
We studied 120 patients that had been diagnosed with idiopathic DCM and had been referred to the University Hospital Maastricht and University Medical Center Groningen for further diagnostic and genetic work-up. Carriers of LMNA gene mutations were detected by MLPA and sequencing and were excluded (n=14), resulting in 106 probands that were included in the present study. All probands gave informed consent for DNA investigation.

Apes
We studied the frequency of LMNA haplotypes in 18 apes from 5 different primate species to check for evolutionary selection of LMNA haplotypes. The ape species included 4 Pan troglodytes, 2 Pan paniscus, 6 Gorilla gorilla, 4 Pongo pygmaeus and 2 Pongo abelii. The sample included 11 females, 5 males and 2 apes of unknown sex. For genotyping we used the same PCR primers as for humans.

Hapmap data
For comparison of LD structure within LMNA we used data on four different ethnic populations, central Europeans (CEU), Han Chinese (CHB), Japanese (JPT) and Yorubans (YRI) from hapmap version 16[18].

DNA

DNA of all participants was isolated using standard techniques. In brief, white blood cells were isolated by lysis of red blood cells. Subsequently DNA was purified by incubation with SDS and proteinase K and precipitated by using a high salt concentration and isopropanol. The precipitated DNA was dissolved in TE (10mM Tris, 0.1 mM EDTA pH7.8) solution and stored at 4°C until further use.

SNP selection and genotyping

We selected 116 SNPs for genotyping from publicly available SNP databases[19]. Resequencing of the LMNA coding regions in 80 individuals did not reveal any additional synonymous or nonsynonymous exonic SNPs. Within exons and intron-exon boundaries of the LMNA gene all available SNPs were selected. In the other regions one SNP was selected every 2-5 kb covering a genomic region of 280 Kb.

In all probands from the general population and in the apes genotyping was performed by MALDI-TOF mass spectroscopy on the Sequenom system as published previously[20]. In brief, we used primer-extension genotyping of the SNPs by amplifying the SNP flanking region and annealing a specific primer to the amplicon. Depending on the genotype the primer was extended by one or several base pairs, which resulted in different masses of extension products. The extended primers were purified and spotted on a chip. The MALDI-TOF differentiates predefined masses of each genotype and the computer generates automated genotype calling.
In the affected population sample we focused genotyping on the haplotype block comprising the entire coding region of LMNA and genotyped only a single SNP (LMNA_D446D, rs505058), a synonymous, exonic SNP that tags the 2 main clades of haplotypes (hα, hβ)

within that haplotype block. Genotyping was performed by amplification of the flanking region (forward primer 5'-gag-atc-cac-gcc-tac-cgc-aag-3' and reverse primer 5'-agc-caa-aga-gtc-cag-gag-cca-3') followed by restriction fragment length polymorphism (RFLP) analysis (FokI) and gel electrophoretic analysis. In the presence of the more frequent clade of haplotypes (hα) restriction digestion led to 5 fragments while in the presence of risk clade (hβ), only 4 fragments were obtained. SNPs were considered successfully genotyped if they had a call-rate of more than 0.8, the significance level of the test of deviation from Hardy Weinberg Equilibrium exceeded 0.01 and the minor allele frequency was larger or equal to 0.05.

Statistics

LD measures D' and r^2 were calculated as implemented into the Haploview software[21]. Haplotype blocks were defined based on the confidence interval of D' as suggested by Gabriel et al. [22].

For categorical traits the maximum likelihood estimates of the odds ratios corresponded to haplotype or allele relative risks in a multiplicative model, i.e. the genotype relative risk of the rare homozygote was expected to be the square of the genotype relative risk of the heterozygote, and the frequent homozygote was taken as the reference.

SNPs were tested for association to quantitative traits by linear regression analysis using phenotypes as dependent variables. Significance levels were determined for both the one-degree (1df) and the two-degree of freedom test (2df). In the 1df-test the independent variable was derived by transforming SNP's genotypes (AA, Aa, aa) to a relational scale by counting the number of minor alleles (0, 1, 2) assuming a strictly codominant model with identical trait increases between genotypes. This test has a relatively higher power to detect weak effects and was our primary test used. In the 2df-test a SNP was decomposed into two variables representing the two genotypic changes and both were included into a bivariate regression. This test accounts for dominance and recessivity by allowing the trait increase of each genotypic change to take an individual value. It was used to specifically quantify each genotype's effect and significance level in the total sample. The average trait increase per allele was calculated as the mean of both genotypic changes weighted by the genotype frequencies and the variance attributable to a SNP was calculated as the adjusted r^2 value from the bivariate regression analysis.

The association of haplotypes with quantitative phenotypes was inferred by the haplotype trend regression method, which uses a 1 df linear regression analysis[23].

In order to correct for multiple testing, we evaluated the table-wide significance for each data set by a randomisation procedure. The affected status of the individuals was permuted and the single-marker association analyses repeated 1000 times. The table-wide 5% significance level is the level at which 5% of the permuted data sets show at least one lower p-value, no matter at which marker.

Table 1
Genotyping information of 56 SNPs used in association study

Nr	SNP ID	DNA position Hg 17	distance (bp)	localisation	functional status	predicted hetero-zygosity	mAF	HWE	Nucleotide exchange
1	rs2275081	152,717,465		3' of ARHGEF2	locus-region	0.11	0.071	0.386	A/G
2	rs1010033	152,730,953	13,488	ARHGEF2	Intron	0.20	0.068	0.912	A/T
3	rs2297649	152,748,317	17,364	ARHGEF2	Intron	0.40	0.259	0.991	C/A
4	rs1889532	152,764,575	16,258	5' of ARHGEF2	X	0.46	0.264	0.422	T/C
5	rs6670493	152,769,976	5,401	5' of ARHGEF2	X	0.38	0.264	0.781	G/C
6	rs2886070	152,787,044	17,068	3' of SSR2	X	0.48	0.387	0.822	A/G
7	rs1111102	152,806,529	19,485	5' of SSR2	X	0.38	0.257	0.073	A/C
8	rs3820592	152,819,086	12,557	C1orf6	mrna-utr	-	0.275	0.696	T/C
9	rs2297792	152,824,517	5,431	C1orf6	coding-nonsynon	0.29	0.389	0.772	A/G
10	rs2275073	152,843,893	19,376	RAB25	locus-region	0.32	0.182	0.852	A/C
11	rs2275075	152,853,383	9,49	5' of RAB25	locus-region	0.33	0.183	0.830	A/G
12	rs3814314	152,858,256	4,873	5' of RAB25	X	-	0.210	0.290	A/T
13	rs3738593	152,859,046	790	5' of RAB25	locus-region	0.16	0.185	0.273	C/T
14	rs6691151	152,862,611	3,565	5' of RAB25	Intron	-	0.148	0.960	C/T
15	rs4661146	152,873,223	10,612	3' of LMNA	Intron	0.26	0.147	0.898	C/G
16	rs6661281	152,887,918	14,695	3' of LMNA	X	0.46	0.372	0.071	T/C
17	rs915180	152,892,156	4,238	3' of LMNA	X	0.50	0.378	0.118	C/T
18	rs955383	152,895,103	2,947	3' of LMNA	X	0.34	0.265	0.672	C/T
19	rs2485662	152,896,541	1,438	3' of LMNA	locus-region	-	0.284	0.360	A/G

20	rs2485661	152,896,569	28	3' of LMNA	locus-region	-	0.075	0.293	G/A
21	rs547915	152,898,448	1,879	LMNA	Intron	0.14	0.095	0.712	A/G
22	rs582690	152,901,329	2,881	LMNA	Intron	0.10	0.074	0.305	G/C
23	rs584025	152,901,599	270	LMNA	Intron	-	0.074	0.652	G/A
24	rs503815	152,902,682	1,083	LMNA	Intron	-	0.073	0.345	T/C
25	rs501791	152,902,946	264	LMNA	Intron	0.23	0.070	0.420	A/G
26	rs610918	152,902,993	47	LMNA	Intron	0.15	0.068	0.569	A/G
27	rs653969	152,905,712	2,719	LMNA	Intron	-	0.069	0.510	A/G
28	rs521354	152,905,904	192	LMNA	Intron	0.10	0.064	0.715	C/T
29	rs693671	152,906,268	364	LMNA	Intron	0.28	0.068	0.604	A/G
30	rs672200	152,907,516	1,248	LMNA	Intron	-	0.066	0.547	T/G
31	rs593987	152,909,460	1,944	LMNA	Intron	-	0.067	0.660	A/G
32	rs528636	152,911,693	2,233	LMNA	Intron	-	0.098	0.504	C/T
33	rs666869	152,912,903	1,21	LMNA	Intron	-	0.071	0.418	C/T
34	rs509551	152,913,122	219	LMNA	Intron	0.22	0.071	0.393	G/T
35	rs508641	152,913,212	90	LMNA	Intron	0.10	0.072	0.386	A/G
36	rs577492	152,913,812	600	LMNA	Intron	0.14	0.098	0.496	A/G
37	rs2485668	152,914,622	810	LMNA	Intron	-	0.069	0.442	A/G
38	rs538089	152,918,101	3,479	LMNA	coding-nonsynon	-	0.096	0.572	C/T
39	rs534807	152,919,001	900	LMNA	Intron	-	0.095	0.317	G/A
40	rs505058	152,919,258	257	LMNA	Coding-nonsynon	0.37	0.100	0.405	A/G
41	rs476000	152,919,442	184	LMNA	Intron	0.18	0.098	0.539	C/T
42	rs553016	152,919,936	494	LMNA	Intron	0.15	0.100	0.415	C/T
43	rs4641	152,920,607	671	LMNA	coding-synon, intron	0.35	0.253	0.135	C/T

44	rs520973	152,921,092	485	LMNA	Intron	-	0.099	0.446	G/A
45	rs520910	152,921,121	29	LMNA	Intron	-	0.098	0.448	A/G
46	rs7339	152,922,049	928	LMNA	mrna-utr	-	0.097	0.484	G/C
47	rs6669212	152,923,386	1,337	5' of LMNA	locus-region	-	0.162	0.740	G/A
48	rs545731	152,924,363	977	5' of LMNA	X	-	0.089	0.256	C/T
49	rs1468772	152,929,561	5,198	3' of FLJ12287	X	0.41	0.277	0.274	A/C
50	rs3738582	152,936,303	6,742	3' of FLJ12287	locus-region	0.42	0.195	0.766	C/G
51	rs510441	152,943,041	6,738	FLJ12287	Intron	0.34	0.245	0.517	A/G
52	rs7695	152,960,399	17,358	FLJ12287	mrna-utr	0.45	0.361	0.927	T/C
53	rs3738581	152,960,631	232	5' of FLJ12287	locus-region	0.22	0.400	0.286	A/G
54	rs2842857	152,981,809	21,178	5' of FLJ12287	mrna-utr	0.46	0.350	0.170	T/C
55	rs2241109	152,986,155	4,346	3' of PMF1	Intron	-	0.282	0.317	C/T
56	rs2241107	152,995,783	9,628	3' of PMF1	locus-region	-	0.429	0.061	A/G

Nr indicates number, mAF indicates minor allele frequency, HWE indicates Hardy-Weinberg equilibrium p value.

RESULTS

From the 116 SNP assays (see Figure 1 for genomic region that was studied) set up for genotyping in the general population sample, 56 fulfilled our quality limits while 60 SNPs were excluded from further analysis. Included SNPs are shown in Table 1. Twenty-four SNP assays failed to reach a call rate of 80%, 20 were monomorphic in the tested samples, 8 showed a minor allele frequency below 5% and 8 deviated significantly from Hardy-Weinberg equilibrium (HWE).

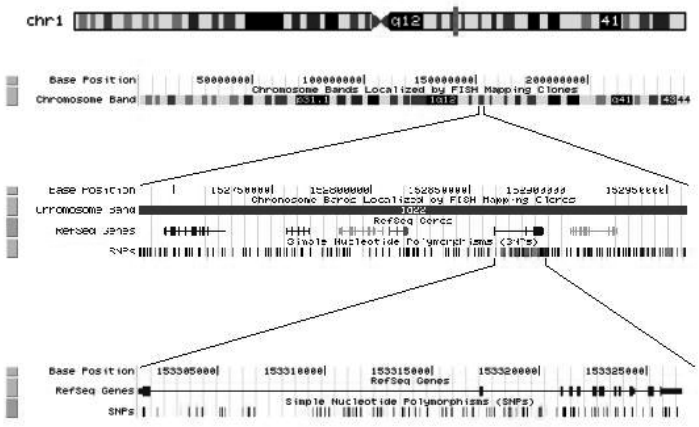

Figure 1
Genomic localisation of the studied region. Upper panel depicts position on chromosome 1, second panel depicts chromosome band, third panel depicts 280 Kb genomic region included in this study, lower panel depicts the LMNA gene

Linkage disequilibrium (LD) and haplotype structure in the LMNA gene region based on the genotype data from the 702 individual sub-sample are shown in Figures 2 and 3. Analysis revealed 8 haplotype blocks in the 280 kb region. Haplotype block 5 (26 kb length) comprised the entire transcriptional unit of the LMNA gene and showed a remarkably high level of LD, both D' and r^2, i.e. has been transmitted over evolutionary time frames virtually without any intragenic recombination.

Cladistic analysis of the haplotypes within this haplotype block revealed that it contains 2 higly different clades of haplotypes (Figure 4). In the general population sample of 702 individuals major clade haplotypes (hα) had a frequency of 91% while minor clade haplotypes (hβ) had a frequency of only 9%.
To test whether there has been recent evolutionary selection of one of the two clades we used the hapmap resource to investigate clade frequency differences between populations from other ethnic origins. In addition we genotyped 18 different apes from 5 primate species for the presence of hα or hβ.
Genotyping of 7 tagging SNPs in the LMNA gene revealed that only hβ haplotypes were present in the apes with a frequency of 100%.

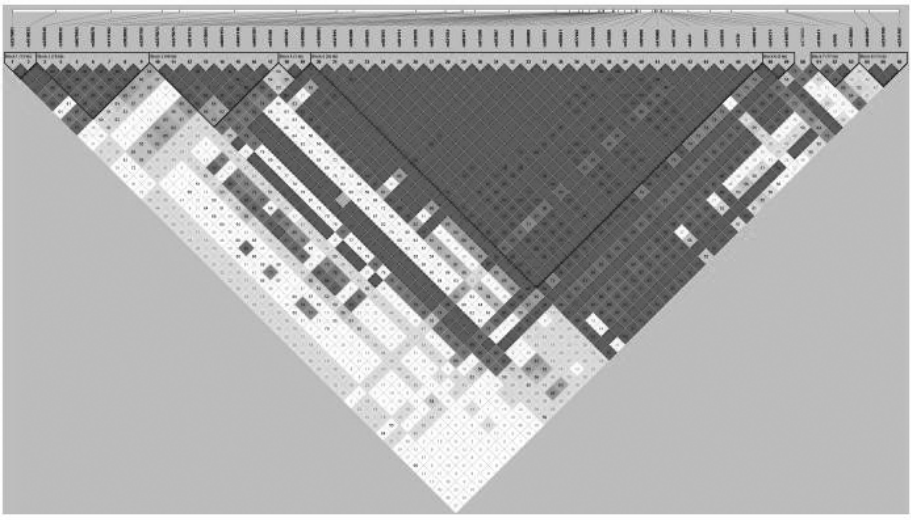

Figure 2
LD structure of the genomic region surrounding the LMNA gene. Dark color indicates high levels of LD (D').

From 8 SNPs typed by the Hapmap project within the LMNA gene in four different ethnic populations, genotyping data is given in Table 2. Genotyping data of SNPs within haplotype block 5 showed obvious differences in different ethnic populations. Similar to recent publication[24] indicating Chinese to be the most recent branch on the evolutionary tree of humans we found 100% allele frequency of hβ in apes, 75% in Yorubans, 8% in Europeans and 2% in Chinese. This strongly indicates positive selection of LMNA clade hα-haplotypes during evolution.

Table 2
Genotyping data of different haplotype tagging SNPs within haplotype block 5 from the HapMap resource. Given are the Allele frequencies of SNPs in clade hβ.

SNP ID	Tagging clade	YRI	CEU	HCB	JPT	This study
rs547915	hβ	0.708	0.076	0.022	0.034	0.095
rs582690	hβ.1	0.575	0.050	0.000	0.000	0.074
rs501791	hβ.1	0.358	nd	nd	nd	0.070
rs2485676	hβ.1	0.417	0.050	0.000	0.000	nd
rs622834	hβ.1	0.417	0.051	0.000	0.000	nd
rs528636	hβ	0.758	0.076	0.022	0.034	nd
rs577492	hβ	0.767	0.075	0.022	0.034	0.098
rs476000	hβ	0.758	0.075	0.022	0.034	0.098

CUE indicates CEPH (Utah residents with ancestry from northern and western Europe), HCB indicates Han Chinese in Bejing, China, JPT indicates Japanese in Tokyo, Japan, YRI indicates Yoruba in Ibadan, Nigeria, nd indicates genotyping was not done for this SNP.

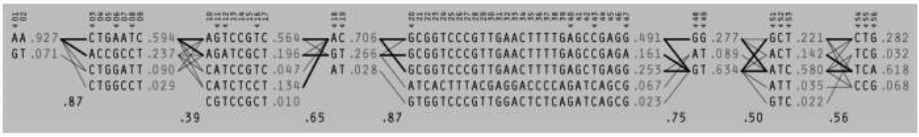

Figure 3
Haplotype block structure of the genomic region surrounding the LMNA gene. Haplotype block 4 contains part of the promotor region while haplotype block 5 contains the entire coding region of the LMNA gene. Haplotype block 5 displays 5 different haplotypes.

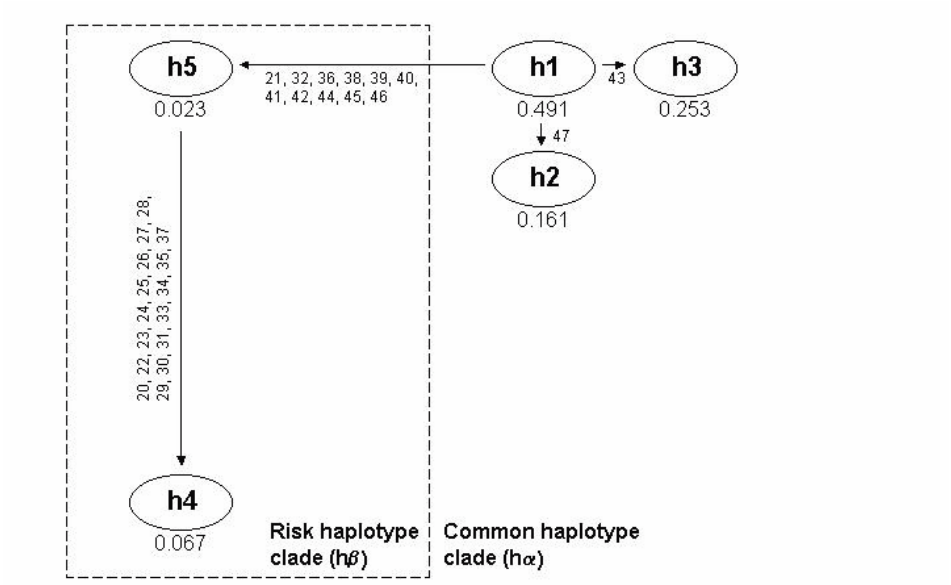

Figure 4
Cladistic analysis of the haplotypes from haplotype block 5 revealed 2 main clades. H indicates haplotype, length of arrows indicates evolutionary distance between haplotypes, numbers below arrow refer to the SNP numbers in Table 1 and numbers below haplotypes indicate haplotype frequency.

To test whether LMNA polymorphisms were associated with functional cardiac changes we first investigated cardiac ultrasound phenotypes in the general test population (n=702). In the general population sub-sample with echocardiography data available (n=702) clade hβ-haplotypes were significantly associated with decreased posterior wall motion and thickness as well as a reduction of velocity time integral over the aortic valve (VTI) (Table 3). As PWD and VTI are correlated (Pearson correlation=0.260, p=0.013), clade hβ seems to confer a risk for diminished posterior wall motion and consequently lower VTI.
In a cardiac cycle, the posterior wall is activated latest[25], and therefore, any disturbance in posterior wall motion and thickness will affect stroke volume, as reflected by VTI.

Table 3
Phenotyping information measured and associations with haplotype clade hβ.

Baseline characteristics	Mean	SD	P value linear regression (1df)	P value ANOVA (2df)
Age (yr)	59.8	12.4	0.824	0.974
Male (nr)	350		0.885	0.853
heart rate (bpm)	66.7	11.4	0.869	0.376
PR interval (ms)	174	20.4	0.425	0.715
QRS duration (ms)	94.2	14.6	0.126	0.310
QTc (ms)	419	24.7	0.492	0.135
LVEDD (mm)	47.5	6.39	0.238	0.490
LVESD (mm)	30.4	7.01	0.260	0.521
IVSD (mm)	10.3	2.84	0.930	0.950
LVPWD (mm)	8.7	2.69	0.030	0.071
LVEF (%)	66.3	12.0	0.883	0.862
Anterior wall motion (grade)	0/1/2/3	635/4/1/0	0.859	0.978
Apical wall motion (grade)	0/1/2/3	632/3/1/1	0.820	0.944
Posterior wall motion (grade)	0/1/2/3	620/11/7/1	0.027	0.045
E-wave (m/s)	62.6	15.9	0.478	0.650
A-wave (m/s)	65.1	17.2	0.519	0.762
Velocity Time integral at Aortic valve (cm)	23.6	6.45	0.002	0.002

SEM indicates standard error of the mean, QTc indicates QT interval corrected for heart rate following Bazett's formula, LVEDD indicates left ventricular end diastolic diameter, LVESD indicates left ventricular end systolic diameter, IVSD indicates interventricular septum diameter, LVPWD indicates left ventricular posterior wall diameter, LVEF indicates left ventricular ejection fraction, Wall motion scores indicate 0=normal, 1=hypokinetic, 2=akinetic, 3=dyskinetic.

With the knowledge from previous studies and our population based results in the test population, we hypothesised that hβ-haplotypes may also increase the risk for developing heart failure and tested, whether they were more prevalent in patients with heart failure. In the entire survey (n=4034), the frequency of hβ-haplotypes was 8.6%, similar to the 9% in the test population (Table 4a, OR 0.852, 95% CI 0.704-1.032, p=0.101). Among individuals with self-reported heart failure, individuals with hβ-haplotypes were overrepresented (OR 1.63, 95% CI 1.03-2.57, p=0.035, Table 4b). As patients with LMNA mutations frequently are treated with a pacemaker[11], we also tested whether clade hβ was associated to pacemaker implantation. Subjects with the clade hβ more frequently carried a pacemaker (OR 3.66, 95% CI 1.16 – 11.58, p=0.018, Table 4c).

Table 4a
Genotyping data of general population sample.

Risk Clade genotype	Frequency	Percentage of population
hα - hα homozygous	3372	83.6%
hα - hβ heterozygous	628	15.6%
hβ - hβ homozygous	34	0.84%
Total	4034	100%

Table 4b
Genotype distribution of general population depending on presence of heart failure.

	Heart failure	
Genotype	**Yes**	**No**
hα - hα homozygous	80	3206
hα - hβ heterozygous	24	584
hβ - hβ homozygous	1	32

Table 4c
Genotype distribution of general population depending on presence of pacemaker

	Pacemaker	
Genotype	**Yes**	**No**
hα - hα homozygous	7	3345
hα - hβ heterozygous	5	619
hβ - hβ homozygous	0	33

We next tested whether clade hβ modified hereditary forms of heart failure. We hypothesised that in hereditary forms of heart failure where a yet undiscovered singular genetic event drives the pathophysiology, this disease process may be modified by the LMNA hβ clade. We excluded carriers of a pathogenic LMNA mutation from the analysis.

The frequency of clade hβ-haplotypes was not different in patients with DCM (AF=0.095) when compared to the general population test sample (AF=0.100) (OR 0.957), indicating that the occurrence of familial forms of heart failure is not related to this clade, and that this clade is not in linkage disequilibrium with mutations that independently cause idiopathic DCM.
Patients carrying at least one copy of clade hβ, presented with symptoms of DCM at an

average age of 39 yrs, whereas patients, who do not carry the risk haplotype, presented with DCM at a mean age of 49 yrs (n=20 vs n=86, p<0.01).
We analysed the electrocardiogram and echocardiography at presentation of these patients, which was present at that time in n=34 and shown in Table 5. This comparison showed that carriers of the risk haplotype not only presented with DCM at a younger age, but also tended to have more severely depressed cardiac function at presentation. Patients with the LMNA risk haplotype tended to have increased end diastolic (LVEDD) and end systolic dimensions (LVESD) and higher heart rates. This indicates that hereditary heart failure caused by genes other than LMNA may be adversely modified by the presence of the LMNA risk clade hβ

Table 5
Clinical characteristics of heart failure patients having and not having the risk clade hβ. Of the 7 probands carrying clade hβ, 6 were heterozygotes while one was homozygote.

Phenotype	Probands *not* having hβ (n=27)	Probands having at least one copy of hβ (n=7)	P value
Age at first Echocardiography (yrs)	50.4	33.3	**0.003**
LVEDD (mm)	62.6	70.4	**0.022**
LVESD (mm)	53.0	58.7	0.099
FS (%)	16.0	17.0	0.412
LA (mm)	47.9	47.2	0.438
LVPWD (mm)	8.6	8.0	0.176
Ventricular rate (min^{-1})	78.0	97.3	**0.010**
PR interval (ms)	166.7	198.8	0.086
QRS duration (ms)	102.1	111.6	0.248
QTc (ms)	439.5	444.3	0.419

LVEDD indicates left ventricular end diastolic diameter, LVESD indicates left ventricular end systolic diameter, FS indicates fractional shortening, LA indicates left atrial size, LVPWD indicates left ventricular posterior wall diameter

DISCUSSION

We have identified a common LMNA risk haplotype, which we show to be associated to a moderately but significantly increased risk for heart failure in the general population.
We furthermore found that the risk haplotype modified heart failure in subjects with hereditary heart failure. In this latter population, where LMNA gene mutations had been excluded, the risk haplotype aggravated the disease phenotype and caused the disease to manifest itself at a younger age of onset.

Since the pathophysiology of cardiomyopathy due to lamin A/C mutations is still unresolved, it remains unclear how a variation in lamin A/C could modify the pathophysiology of other forms of heart failure. Nevertheless, the newly described lamin hβ-haplotype seems to recapitulate the features of cardiomyopathy induced by lamin mutations, as it was associ-

ated not only with impaired cardiac function but also with the incidence of pacemaker therapy. This suggests that the lamin haplotype is associated not only with a predisposition towards heart failure but also towards impaired cardiac conduction.

This study exemplifies the advantages of using a more robust LD-based strategy to investigate a candidate gene for common gene variants modifying cardiac disease under a complex disease model. In a test population we performed a large LD- based analysis of the haplotype block structure of the genomic region within and flanking the LMNA gene, a major disease gene of monogenic heart failure (DCM) and a good polygenic candidate.
Our strategy has important advantages over the analysis of a low number or even only one SNP per candidate gene. Analysing the entire genomic region flanking a gene with a high density of SNPs decreases the likelihood of false negative findings in association studies as most of the relevant common genetic variance of that gene is analysed. In regions of high LD the study of haplotype blocks[26], instead of single SNPs is justified without the need of extensive correction for multiple testing.

We found increased frequency of the risk clade hβ in heart failure patients in the general population. The risk clade also increased the risk for pacemaker implantation. We had initially expected an increased frequency of the risk haplotype in familial DCM cases indicating an increased predisposition towards manifestation of the disease but did not detect such an effect. This indicates that the mere manifestation in patients predisposed by rare mutations to familial forms of heart failure is not significantly influenced by the risk haplotype. Nevertheless, familial DCM patients who were heterozygous for this haplotype presented at an age 10 years younger than patients without this haplotype. Moreover, the severity of heart failure at presentation tended to be worse in presence of the risk haplotype. We therefore conclude that the risk clade modifies the pathophysiologic role of separate unrelated gene mutations that cause heart failure, much like we initially anticipated.
Our finding that carriers of the LMNA risk haplotype present earlier with more depressed cardiac function may be related to the fact that LMNA mutations have also been found to cause premature aging syndromes, known as progeria[7,8,27]. This latter finding strongly suggests that LMNA function is related to aging processes. Combined with our findings it is attractive to speculate that the rare haplotype is in LD with a variant that decreases LMNA function, and accelerates cardiac aging, thereby causing the earlier presentation. Obviously, this interpretation awaits confirmation by additional functional studies.

The functional mechanisms, their predictive role and their potential usefulness as predictive gene tests in patients with incident or prevalent heart failure will have to be determined in future prospective studies. The remarkable haplotype block structure of the LMNA gene and its phenotypic effects we describe here, will greatly facilitate future studies and understanding of its role in cardiovascular disease.

In conclusion, we describe a first comprehensive haplotype analysis of the LMNA gene. In the normal population, we defined haplotype blocks by using the algorithm published by Gabriel et al[22]. We identified a LMNA risk haplotype, which was associated to heart failure and pacemakers. This risk haplotype showed significant association with posterior wall motion and thickness and VTI over the aortic valve in the general population. In a population with familial DCM it was associated to presentation at an earlier age with worse cardiac function at time of presentation.

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CHAPTER 7

General discussion

ROLE OF LAMIN A/C

In recent years, the identification of various diseases all caused by mutations in lamin A/C have generated a heightened interest in this enigmatic protein. While much of the role of lamin A/C in cellular biology has become increasingly clear, many questions remain, part of which has been addressed in this thesis.
The lamin A/C gene can give rise to different splice isoforms that are expressed in different tissues. There appeared to be a specific germline splice isoform lamin C2[1]. Next to that, 3 different splice isoforms have been found in different tissues, lamin A, lamin C and lamin Adel10 (lamin A without exon 10)[2-4]. Whether these different isoforms have different functions is not known, but the expression of these isoforms seems to be linked to development and differentiation[5,6]. Before the discovery of mutations in the lamin A/C gene leading to various diseases, the most important role for lamin A/C was thought to be a structural, giving support to the nucleus. Its characteristics have been extensively studied in mitosis[7-9]. The lamina needs to be disintegrated before mitosis can occur. This occurs through post-translational modification of the lamina. Lamin A and C are phosphorylated by p34/cdc2, which induces disassembly of the lamina allowing mitosis to proceed[10]. After mitosis lamins play an important role in nuclear envelope assembly[11-13]. At least some lamin molecules need to be present for proper assembly, but a normal lamina is not a prerequisite[14]. The regulation of a normal nuclear envelope assembly may be determined by the interaction of lamins with other associated proteins.
Due to the fact that the role of lamin A/C has been mainly seen as a structural one, many studies have been performed identifying binding partners. Emerin showed to be one of the most interesting ones[15,16]. This binding partner was particularly interesting as emerin, when mutated could causes X-linked Emery-Dreifuss Muscular Dystrophy. A large family with the autosomal dominant form of this disease showed linkage to the part of the genome where the lamin A/C gene resides[17]. When this became apparent lamin A/C was an obvious candidate gene that was later proven to be responsible for the disease, showing that both mutations in lamin A/C and in Emerin can cause muscular dystrophy.

ROLE OF LAMIN A/C IN PATHOPHYSIOLOGY OF LAMINOPATHIES

The discovery that muscular dystrophies and dilated cardiomyopathy can be caused by mutations in lamin A/C further established the important role of lamin A/C[17-19]. To better understand the possible role of lamins A and C, the gene has been knocked out in mice[20]. These mice showed an obvious phenotype of growth retardation at 2-3 weeks, altered gait at 3-4 weeks and death by week 8. These findings further underscored the importance of lamins for cellular biology. Critical processes in which lamins are involved include nuclear assembly, apoptosis, DNA replication and possibly transcription.
An apparent hypothesis on how mutations in lamin A/C could lead to heart failure and muscular dystrophy is that the nucleus could become instable[21]. In tissues where cells are subjected to external or internal forces these fragile nuclei could break, resulting in cell death and consequently muscle wasting. Recently some groups indeed found evidence of decreased nuclear stiffness. Lammerding et al. found evidence of decreased nuclear stiffness and altered nuclear mechanics in lamin deficient cells[22]. Next to the decreased stiffness they also found altered nuclear mechano-transduction in lamin deficient cells. The explanation for the altered mechano-transduction in lamin deficient cells was given in altered NF-κB activation. The response to stimuli that induce activation of NF-κB was similar, but the

binding of NF-κB to DNA was significantly enhanced in lamin deficient cells. However, the induction of NF-κB-dependent luciferase by Il-1 was significantly impaired in lamin deficient cells. How this difference could be explained was not further investigated, but the authors suggested an important role of lamins in transcriptional activation after transcription factor binding. A second paper by Broers et al. confirmed the decreased mechanical stiffness of lamin deficient cells[23]. The explanation for decreased stiffness in the latter paper was given in altered interaction between the cytoskeleton and the nucleus depending on presence or absence of lamins.

The structural hypothesis is very plausible for dystrophies of contractile tissue, such as skeletal muscle and heart muscle. However, in the case of lipodystrophy caused by lamin A/C mutations this hypothesis is less intuitive. Therefore a second hypothesis was proposed[21]. The background of this hypothesis is the discovery that different transcription factors and even DNA bind to lamin A/C[24]. Furthermore, the protein is present in nuclear foci where DNA transcription occurs[25]. This led to the hypothesis that lamin A/C is important for the regulation of gene transcription. This hypothesis could explain more diseases that are caused by mutated lamins. Our group described in chapter 3 a possible model how lamins could regulate gene transcription. The model we presented puts lamin in a central position for several post-translational modifications and protein-protein interactions depending on these modifications. More specifically, we found that lamins are important for the dephosphorylation of transcription factors. Two transcription factors showed decreased dephosphorylation in absence of lamins. We furthermore identified protein phosphatase 2A as a novel binding partner of lamins, and showed that altered dephosphorylation dependended on nuclear PP2A activity.

A third hypothesis how lamins could cause disease when mutated was brought forward by investigators of premature ageing. Two recent papers identified pathways that might play an important role in the pathophysiology of laminopathies. Liu et al. showed that presence of pre-lamin A due to absent Zmpste24 caused a higher sensitivity to DNA damage[26]. Zmpste24 is the enzyme that is responsible for the maturation of pre-lamin A. The presence of pre-lamin A is supposed to have a dominant negative effect, as is the case for the mutated lamin A in Hutchinson-Gilford progeria. They furthermore showed that recruitment of p53 binding protein1 and Rad51 to sites of DNA lesion is impaired, which results in delayed checkpoint response and DNA repair. Varela et al. showed marked upregulation of p53 target genes in absence of proper pre-lamin A maturation[27]. This upregulation resulted in senescence phenotype at the cellular level and accelerated aging at the organismal level.

PHENOTYPE OF LAMINOPATHIES

From 1999 until this moment at least 11 different phenotypes (diseases or syndromes) have been associated to mutations in the lamin A/C gene (see Table 1). Overall, these phenotypes might be grouped into 4 different groups.

- Muscular dystrophies
- Lipodystrophies
- Premature Aging syndromes
- other, such as Axonal Neuropathy and Restrictive Dermopathy

Whether these groups of laminopathies represent one specific pathophysiological entity is not known. One possible hypothesis is that the different diseases are all phenotypic variations of one common pathological pathway. In case of laminopathies, this would most probably be premature aging. How this type of premature aging causes disease is not clarified yet. Two recent papers identified disturbances in DNA repair systems and the p53 apoptosis pathway in premature aging due to mutations in lamin A/C. Whether these cellular mechanisms also play a role in the other laminopathies remains to be investigated.

Close clinical observation may often provoke a novel insight into the disease. To further delineate the cardiac phenotype of laminopathies and to use this to generate a possible pathophysiogical explanation for the cardiac disease, we collected all papers describing patients with a mutation in lamin A/C responsible for a cardiac phenotype. We showed in chapter 2 that most common clinical course of the cardiac disease is first the occurrence of conduction system disease or atrial arrhythmias. The penetrance of these dysrhythmias is very high after the age of 30. Only 20 years later do most patients develop heart failure. However, before the age of 50 most patients had already died, and usually suddenly. From this disease course we hypothesised that fibrosis might play an important and early role in the pathophysiology. In chapter 4 we described a family with a new lamin A/C mutation where indeed high levels of fibrosis are seen. This family even showed fibrosis on cardiac biopsy before the disease could clinically be diagnosed, indicating a rather primary type of fibrosis. Whether absence of lamins could induce fibrosis was investigated in chapter 3.

Treatment of carriers of lamin A/C gene mutations

The elucidation of the biological role of lamin A/C might deliver rational therapies for laminopathies in due time. Until then, it is important to identify patients with this type of heart failure or muscular dystrophy as soon as possible. In chapter 2 we suggested some clinical characteristics whereby lamin A/C mutation carriers might be identified. One of the most important characteristics of cardiac laminopathy is the extremely high risk of sudden death. We showed that this high risk is not influenced by the implantation of a pacemaker, suggesting that patients die due to tachy-arrhythmias. We hypothesised that the high risk of sudden cardiac death could be treated by implantation of an implantable cardioverter defibrillator (ICD). In chapter 5 we describe the use of ICDs for primary prevention of sudden death in patients with a lamin A/C mutation. During 34 months follow up the ICD treated ventricular fibrillation or other ventricular tachycardias in 8 of the 19 included patients. Strikingly, the patients still had normal ejection fraction at entry of the study. This further strengthened our observation that sudden death seems to be unrelated to heart failure in laminopathies. Therefore it is crucial to identify carriers of a lamin A/C mutation as soon as possible, before they develop heart failure or die suddenly.

Table 1
Overview of different phenotypes caused by lamin A/C gene mutations

Disease	Phenotype	Reference
Emery-Dreifuss Muscular Dystrophy	Contractures and muscle weakness, with conduction system disease (and cardiomyopathy)	Bonne et al Nat Genet 1999[17]
Limb-girdle Muscular Dystrophy	Pelvic girdle weakness, later contractures and conduction system disease	Muchir et al Hum Mol Genet 1999[19]
Dilated Cardiomyopathy	Heart failure with conduction system disease	Fatkin et al NEJM 1999[18]
Familial partial Lipodystrophy	Loss of subcutaneous fat, insulin resistance and diabetes, accumulation of fat above shoulders	Cao and Hegele Hum Mol Genet 2000[28]
Mandibulo-acral Dysplasia	Postnatal growth retardation, craniofacial anomalies, skeletal malformations, mottled cutaneous pigmentation	Novelli et al Am J Hum Genet 2002[30]
Hutchinson-Gilford Progeria	Premature aging, dwarfism, alopecia, osteoporosis with atherosclerosis	Eriksson et al Nature 2003[31,32]
Atypical Werner Syndrome	Scleroderma-like skin, cataract, subcutaneous calcification, premature atherosclerosis, diabetes mellitus, premature ages facies	Chen et al Lancet 2003[33]
Atypical forms of Progeria	HG-progeria, but without all typical features	Csoka et al J Med Genet 2004[34]
Charcot Marie Tooth type 2 (Axonal Neuropathy)	Motor and sensory axonal neuropathy with diabetes	De Sandre-Giovannoli et al Am J Hum Genet 2002[29]
Restrictive Dermopathy	Early neonatal lethal course.	Navarro et al Hum Mol Genet 2004[35]
Syndrome of generalized lipoatrophy, insulin resistant diabetes, disseminated leukomelanodermic papules, liver steatosis and cardiomyopathy	Generalized lipoatrophy, insulin resistant diabetes, disseminated leukomelanodermic papules, liver steatosis and cardiomyopathy	Caux et al J Clin Endocrinol Metab 2003[36]

Lamin A/C as modifier gene

Apart from clearly pathogenic mutations given the profound effects of lamin A/C dysfunction, variations in the gene may be relevant for human disease. We hypothesised that polymorphisms in the lamin A/C gene could modify the evolution of heart failure caused by other diseases. Some recent reports showed an association between a lamin A/C polymorphism and insulin resistance, adiposity and plasma leptin levels in different populations[37-40]. These studies all showed association between the phenotype and a single polymorphism. A more recent paper showed association of a haplotype block of five lamin A/C polymorphisms with the metabolic syndrome[41].
Because lamin A/C can cause heart failure when mutated, we studied in chapter 6 a pos-

sible role for lamin A/C as modifier gene in heart failure. In contrary to other studies, we first investigated the haplotype block structure of the lamin A/C gene. We showed that the entire gene resides on one haplotype block. Through cladistic analysis we identified two main clades of haplotypes, that were confirmed through evolutionary analysis. In a general population sample we found an association between one clade of haplotypes and the incidence of heart failure. In a population of patients with inherited heart failure, presence of this clade of haplotypes was associated to earlier presentation of disease with a more devastating type of heart failure.
How polymorphisms in the lamin A/C could alter disease course or influence risk of a disease is not known. A possible explanation could be accelerated aging in the affected tissues due to altered lamin function or expression. Future studies are needed to confirm the associations described in this thesis and to explain how lamin A/C polymorphisms can influence the incidence of heart failure.

Concluding remarks

The findings described in this thesis show 1) that cardiac disease caused by mutations in lamin A/C are relatively prevalent, 2) such mutations cause a very malignant disease with a high rate of sudden death which is 3) amenable to prophylactic ICD treatment. Our studies of the pathophysiology of lamin A/C show that lamins are important for transcription factor regulation. How this role of lamins is related to laminopathies remains to be investigated. We propose that the study of lamin mutations can reveal new transcriptional pathways that are important in the development of heart failure, also common types of heart failure such as occurs after a myocardial infarction or due to hypertension. Given that mutations in lamin A/C also cause premature aging, the elucidation of the pathophysiology of laminopathies will furthermore give insight into the biology of aging. It is therefore important to further investigate how mutations in lamin A/C affect the regulatory mechanisms of transcription. Possibly these investigations will result in opportunities for rational therapies. Then in time we might be able to prevent or postpone heart failure and sudden death in laminopathies.

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Summary

Inherited forms of heart failure form an important part of the total heart failure population. Familial heart failure has been recognised as a disease entity from the 60's. However, before the first gene was found to cause familial heart failure, or DCM, it took 30 years. At this point, 15 years after the first discovery of a gene mutation that leads to dilated cardiomyopathy, mutations have been found in about 20 different genes. However, we are still not able to find a gene mutation in more than 60-70% of the families with inherited heart failure. Main problem could be that heart failure is a common final pathway for many different errors or flaws in myocardial architecture or its molecular biology. How all these different mutations in different genes lead to heart failure is unknown.

We hypothesised that careful clinical observation might identify special forms of heart failure that can only be caused by a few different genes, when mutated. In this thesis we focussed on mutations in the lamin A/C gene. At the start of this thesis we knew lamin A/C mutations could cause 4 different diseases. Three of these involved muscular and/or myocardial tissues. We identified the common clinical cardiological characteristics of these 3 diseases. The prognosis of all three is determined by the cardiac involvement in the disease. Early in the disease course patients usually show mild dysrhythmias that can progress to severe conduction system disease. Heart failure usually begins years after the first signs of cardiac involvement. However, some patients show rapid progressive disease with need of cardiac transplantation at young age. We furthermore found that many patients die suddenly and this sudden death occurs despite the presence of a pacemaker.

Based on the clinical characteristics of patients we hypothesised that primary fibrosis in the heart might play a key role in the cardiac pathophysiology. We tested this hypothesis with basic science. With the use of cells derived from the lamin A/C knock-out mouse, in which the gene that codes for lamins A and C is genetically removed, we showed that absence of lamin A/C indeed induced enhanced proliferation and increased collagen production in fibroblasts. We furthermore could show that the mechanism that might be responsible for these pro-fibrotic characteristics in absence of lamin A/C is depending on the growth factor Transforming Growth Factor Beta 1 (TGF-β1). Stimulation with TGF-β1 leads to rapid dephosphorylation of different transcriptional modulators, such as SMAD and the retinoblastoma protein (pRB). Absence of lamin A/C resulted in aberrant dephosphorylation of these factors. Moreover, we were able to show that the protein that might be responsible for this dephosphorylation in reaction to TGF-β1 could be the protein phospatase 2A (PP2A). Inhibition of PP2A resulted in a lamin-deficient type of dephosphorylation of pRB and SMAD. Finally, we were able to show that lamin A/C, PP2A and the transcriptional factors involved need to be in close contact. We found in vivo interaction between these different factors.

Having established that fibrosis can result from lamin A/C deficiency on a cellular level it was very illustrative that in one family with a fibrotic cardiomyopathy a lamin A/C mutation was found. This mutation leads to a deletion of the start codon containing exon of the lamin A/C gene. We were unable to show aberrant protein, but were able to show the DNA deletion from mRNA extracted from patient material. The characteristics of this family fit the described characteristics in the beginning of this thesis. On a cellular level we were able to show decreased expression of lamins A and C in mutation carriers. We furthermore could show increased lamin containing nuclear speckles in mutation carriers. What the relevance of these speckles are remains to be investigated, but it could hint towards aberrant protein products or perhaps towards accelerated aging in cells of mutation carriers.

Due to increased myocardial fibrosis patients could be at increased risk for tachyarrhythmias. As discussed before a pacemaker alone is not enough to prevent sudden cardiac death in these patients. Moreover, we showed that indeed patients with a cardiac laminopathy can die from fast arrhythmias and that these can be treated by the implantation of an ICD. We showed this in patients that were in need of a cardiac pacemaker. Whether all patients with a cardiac laminopathy could benefit from an ICD implantation remains to be investigated.

Finally, we showed that variations in lamin A/C that are not per se disease-causing pose patients at increased risk of developing heart failure. These variations lie on a single haplotype block that spans the entire lamin A/C gene and is conserved in evolution. There seems to be a negative evolutionary selection for the haplotype that increases the risk of heart failure. Furthermore, patients that already have familial dilated cardiomyopathy that is not the result of a lamin A/C gene mutation show a worse phenotype when they are carriers of the risk lamin A/C haplotype. They develop heart failure 10 years earlier with worse cardiac function.

In short, this thesis deals with the cardiac consequences of lamin A/C gene mutations and polymorphisms. Lamin A/C mutations cause a malignant type of DCM with sudden cardiac death at a young age. Possible pathophysiological mechanism acts through increased myocardial fibrosis via aberrant dephosphorylation of transcriptional regulators in response to TGF-β1. In a family with a lamin A/C gene mutation we could show extensive myocardial fibrosis before the development of heart failure when the only sign of the disease is mild conduction system disease. Implantation of an ICD could prevent sudden cardiac death in patients with cardiac laminopathy.
Finally, lamin A/C polymorphisms increase the risk of heart failure.

Samenvatting

De erfelijke vormen van hartfalen (of gedilateerde cardiomyopathie (DCM)) vormen een belangrijk onderdeel van de totale populatie van hartfalers. Familiair hartfalen wordt al als entiteit herkend sinds de jaren 60. Het heeft echter to de 90-er jaren geduurd voor het eerste causale gendefect werd gevonden. Op dit moment, 15 jaar na de ontdekking van dit eerste gen, zijn er gendefecten in ongeveer 20 verschillende genen gevonden. Desondanks, vinden we in zeker 60-70% van de patiënten nog geen mutatie. Een probleem bij het vinden van mutaties is dat gedilateerde cardiomyopathie het eindstadium is van een heleboel verschillende aandoeningen. Hierdoor is er nauwelijks onderscheid te maken tussen verschillende vormen van gedilateerde cardiomyopathie.

Wij veronderstellen dat nauwkeurige observatie van de klinische kenmerken van patiënten wel in staat kan zijn sommige vormen van DCM van elkaar te onderscheiden. In dit proefschrift hebben we ons gericht op hartfalen als gevolg van lamine A/C mutaties. Bij de start van het onderzoek dat ten grondslag ligt aan dit proefschrift was bekend dat deze mutaties 4 verschillende ziekten konden veroorzaken. In 3 van deze ziekten waren hart en/of skeletspieren aangedaan. De prognose wordt bij alle drie door de cardiale betrokkenheid bepaald. Deze betrokkenheid begint met vrij milde ritme- en geleidingsstoornissen op jonge leeftijd. In een periode van 10 tot 20 jaar zijn deze progressief tot pacemaker implantatie nodig is. Hartfalen ontwikkelt zich gewoonlijk pas jaren na de eerste verschijnselen van de ziekte. Desondanks is er een kleine groep patiënten die een snel progressief beloop heeft met vrij vaak noodzaak tot harttransplantatie. Verder hebben we gevonden dat veel patiënten plotseling overlijden en dat deze plotse dood optreedt ondanks de implantatie van een pacemaker.

Gebaseerd op de klinische kenmerken van patiënten met een lamine A/C mutatie veronderstellen we dat primaire fibrose een belangrijke rol zou kunnen spelen in de cardiale pathofysiologie. We hebben deze hypothese onderzocht met behulp van basaal wetenschappelijk onderzoek. Met behulp van cellen uit de lamine knock-out muis, waaruit het gen dat codeert voor lamine A/C genetisch is verwijderd, waren we in staat aan te tonen dat fibroblasten inderdaad versnelde proliferatie en verhoogde collageen depositie vertoonden, twee kenmerken van fibrose. We konden bovendien aantonen dat het waarschijnlijke mechanisme dat ten grondslag ligt aan deze pro-fibrotische karakteristieken in afwezigheid van lamine A/C afhankelijk is van transforming growth factor beta (TGF-β1). Stimulatie met TGF-β1 leidt tot snelle defosforylatie van transcriptie regulatoren, zoals SMADs en het retinoblastoom eiwit (pRB). Afwezigheid van lamine A/C leidt tot verminderde defosforylatie van deze factoren. Verder toonden we aan dat deze defosforylatie waarschijnlijk het gevolg is van PP2A activiteit. Inhibitie van PP2A leidt namelijk tot een zelfde gebrek aan defosforylatie als afwezigheid van lamine A/C. Deze verschillende factoren, pRB, SMAD en PP2A staan in nauw contact met lamine A/C. We hebben in vivo interactie tussen deze verschillende factoren gevonden.

Ter illustratie van deze pro-fibrotische kenmerken als gevolg van afwezigheid van lamine A/C werd in een familie met een fibrotische cardiomyopathie een lamine A/C mutatie gevonden. Deze mutatie leidt tot een deletie van het exon dat het start codon van lamine A/C bevat. We hebben geen misvormd eiwit kunnen aantonen, maar we hebben wel de DNA mutatie geïdentificeerd op het mRNA van een patiënt. De karakteristieken van deze familie komen overeen met de boven beschreven karakteristieken. Op cellulair niveau vonden we verminderde lamine A/C expressie met bovendien een toename van het aantal lamine A/C bevattende kernfoci. De exacte rol van deze foci is onbekend, maar het zou kunnen wijzen

op de vorming van misvormd eiwit of wellicht op versnelde veroudering in cellen met deze lamine A/C mutatie.

Door toename van myocardiale fibrose zouden patiënten een verhoogd risico op snelle hartritmestoornissen kunnen hebben. Zoals eerder aangegeven is een pacemaker bij deze ziekte niet in staat om plotse dood te voorkomen. Wij waren in staat om aan te tonen dat patiënten met een cardiale laminopathie inderdaad snelle hartritmestoornissen kunnen krijgen en dat deze behandeld kunnen worden met de implantatie van een defibrillator. Dit hebben we in ieder geval aangetoond in patiënten die een pacemaker nodig hebben. Of alle patiënten hierbij baat hebben zal nog verder onderzocht moeten worden.

Tenslotte hebben we aangetoond dat variaties in lamine A/C die niet per se een ziekte veroorzaken wel het risico op het krijgen van hartfalen vergroten. Deze variaties liggen op één haplotype blok dat het complete lamine A/C gen omvat en geconserveerd is tijdens de evolutie. Er lijkt een negatieve selectiedruk te bestaan tegen het haplotype dat het risico op hartfalen verhoogd. Bovendien bleek dat patiënten die al een erfelijke vorm van hartfalen hebben, niet ten gevolge van lamine A/C mutaties, een ernstigere vorm van hartfalen ontwikkelen als ze drager zijn van het risico haplotype. Ze ontwikkelen hartfalen 10 jaar eerder en krijgen een ernstigere vorm van hartfalen.

Kort samengevat laat dit proefschrift zien wat de cardiale consequenties zijn van mutaties en polymorfismen in het lamine A/C gen. Lamine A/C mutaties veroorzaken een maligne vorm van hartfalen met plotse dood op jonge leeftijd. Een mogelijk pathofysiologisch mechanisme werkt via toename van fibrosevorming door veranderde defosforylatie van transcriptionele regulatoren in reactie op TGF-β1. In een familie met een lamine A/C mutatie zagen we uitgebreide cardiale fibrose voor het ontstaan van hartfalen met slechts milde geleidingsstoornis als uiting van de ziekte op dat moment. Implantatie van een ICD kon plotse dood voorkomen in patiënten met cardiale laminopathie.
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Curriculum vitae

Jop van Berlo was born January 2nd, 1976 in Nijmegen, the Netherlands. He attended Gymnasium Bernrode in Heeswijk-Dinther, where he graduated in 1994. The same year, he started his medical training at the Catholic University Leuven, Belgium. In 1995 he continued at the Faculty of Medicine, Maastricht University. During third and fourth year he worked as student assistant at the departments of Clinical Genetics and Cardiology, trying to make sense out of the data of the Human Genome Project. After graduation as an MD he started his PhD in 2002 at the department of Cardiology with a focus on inherited cardiomyopathies. He received the dr. E. Dekker grant "Arts voor aanvang specialistenopleiding" to support his research. At the 40th anniversary scientific day of the Netherlands Heart Foundation he won the posterprize in the category Pathogenesis. In 2005 he started as a clinical resident in Cardiology. He will continue striving for his goal to become a good scientist by starting post doctoral training in the USA.

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