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Chapter 5

Relation Between Genotype and Left-Ventricular Dilatation in Patients With Marfan Syndrome.

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Submitted

Abstract

Aims Cardiovascular manifestations in Marfan syndrome (MFS) are related to aortic and valvular abnormalities. However, left ventricular (LV) dysfunction can occur, even in the absence of aortic surgery or valvular abnormalities. We evaluated genetic characteristics of patients fulfilling the MFS criteria with LV dilatation without previous aortic surgery or valvular abnormalities.

Methods and results 182 MFS patients without valvular abnormalities or previous aortic surgery, with a complete fibrillin 1 (*FBN1*) gene analysis, were studied. *FBN1* mutations were identified in 83% of patients. Twenty-nine patients (16%) demonstrated LV dilatation (left ventricular end diastolic diameter corrected for age and body surface area >112%). *FBN1*-positive patients carrying a mutation most likely leading to haploinsufficiency, more often had LV dilatation than missense-mutation carriers (14/75 versus 5/75; $p<0.05$). It was mainly patients carrying large deletions/null-alleles or frameshift mutations who contributed to this effect. Finally, *FBN1*-negative MFS patients significantly more often demonstrated LV dilatation than *FBN1*-positive patients (10/31 versus 19/151; $p<0.05$).

Conclusion LV dilatation in MFS patients is more often seen in those patients without an FBN1 mutation and in those with a mutation most likely leading to haploinsufficiency (in particular large deletions/null-alleles or frameshift mutations) compared to missense-mutation carriers.

Introduction

Marfan syndrome (MFS; MIM#154700) is an autosomal dominant disorder of the connective tissue, caused in a majority of cases by mutations in the gene encoding fibrillin-1 (*FBN1*; MIM#134797). Fibrillin-1 is a 350 kDa glycoprotein and the major component of the elastin-associated extracellular microfibrils. Fibrillin-1 has a modular organisation typical of many extracellular proteins and is composed of many cysteine-rich repeat motifs, the most common one being the epidermal-like growth factor (EGF) module (Figure 1).[1] *FBN1* is a large gene located on chromosome 15q, containing 65 coding exons. *FBN1* mutations are scattered throughout the gene and are usually unique to individual families. More than 600 *FBN1* mutations have been reported so far and about 25-30% of cases represent *de novo* mutations (for the *FBN1* mutation database see: www.umd.be). With current techniques for molecular analysis, the mutation detection rate reaches over 80% in patients fulfilling the diagnostic (Ghent) criteria for MFS.[2-4] Recently, mutations in the transforming growth factor-β receptor 1 and 2 genes (*TGFBR1* and *TGFBR2*) have been

identified in around 5% and 10%, respectively, of MFS patients who did not carry an *FBN1* mutation.[5-9]

MFS affects the cardiovascular system amongst others, with the main cardiovascular manifestations being aortic dilatation/dissection and valvular abnormalities (aortic and/or mitral regurgitation). In addition, mild left ventricular (LV) dilatation (i.e. increased LV end diastolic dimension [LVEDD]) and mild LV systolic and diastolic impairment has been described in MFS, even in patients without significant valvular abnormalities or previous aortic surgery.[10-12] In the study by Chatrath and co-workers, 7 of the 36 adult patients (19%) with MFS without significant valvular abnormalities and without previous aortic surgery showed increased LVEDDs.[10] Using the same criteria, we recently confirmed this observation by demonstrating the presence of an increased LVEDD (in the absence of significant valvular pathology or previous aortic surgery) in 29 of 183 MFS patients (16%).[11] It should be noted that De Backer *et al*. also provided evidence for mild, but significant impairment of the LV systolic and diastolic function in 26 MFS patients who did not have valvular pathology or aortic surgery.[12] Our goal in this study was to investigate whether a relationship exists between LV dilatation in patients with MFS and the presence/absence of a mutation in the *FBN1* gene, the predicted effect of the mutation on the protein, the affected module, and the type or location of a mutation within the gene.

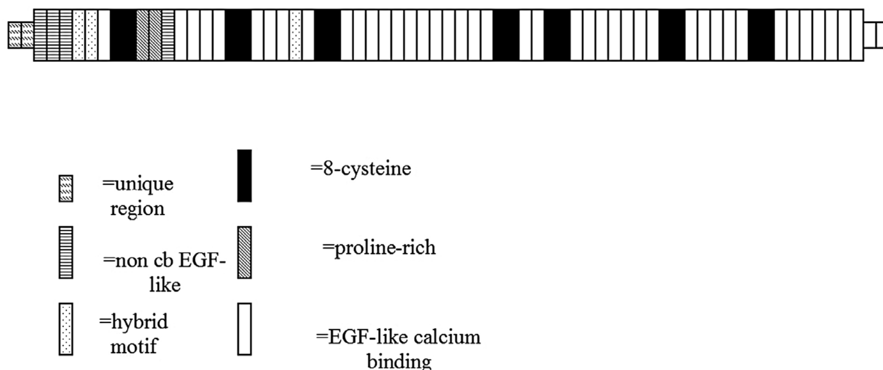

Figure 1. Schematic representation of the domain structure of Fibrillin-1[1]
cb=calcium-binding EGF= epidermal-like growth factor

Methods

Patient selection

Patient selection has been reported previously and is shown in Figure 2.[11] Briefly, 529 consecutive patients, identified in four specialised university hospital MFS out-patient clinics, fulfilled the diagnostic (Ghent) criteria for MFS.[3] In 295 patients previous aortic surgery or significant valvular abnormalities noted on echocardiogram (aortic and/or mitral regurgitation ≥grade 2) precluded participation in this study. Of the remaining 234 MFS patients, DNA data were available for 184 patients. Two more patients were excluded because their DNA results did not allow for a single interpretation (see also Results/Patients section). We thus had 182 patients in our study group (Figure 2).

Echocardiography

Echocardiograms were performed in accordance with standard techniques and as reported previously.[11,13] Briefly, LVEDD was derived from two-dimensional echocardiograms. Only echocardiograms of sufficient quality were accepted. For each patient the predicted normal value for LVEDD was calculated according to their age and body surface area using the regression equations proposed by Henry.[14] The LVEDD was expressed as a percentage of the predicted value: observed dimension/predicted normal value x 100. In accordance with the guidelines, 112% (mean + 2 SD) was used as the cut-off value for increased LV dimension.[15] As part of the patients' routine follow-up, echocardiograms are made on a regular basis, but for the purpose of the present study only the most recent echocardiogram was used.

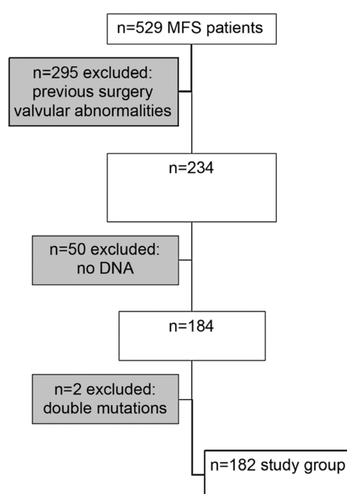

Figure 2. Flow chart of the patients' selection; the gray boxes give the numbers of excluded patients and the reasons for exclusion.

DNA analysis

From each patient either a blood sample or a skin biopsy specimen for fibroblast culture was available. Genomic DNA was extracted from peripheral blood leukocytes or skin fibroblasts. Mutational analysis of the *FBN1* gene was performed by Denaturing High Performance Liquid Chromatography (DHPLC) with subsequent sequencing of fragments with aberrant patterns.[16] The effect of potential splice-site mutations was confirmed using reverse-transcription PCR. If DHPLC analysis was negative, additional investigations were performed to elucidate a potential pathogenic role of the *FBN1* gene, namely: (a) Multiplex Ligation-dependent Probe Amplification (MLPA) analyses to assess large genomic duplications or deletions, and (b) determination of potential mono-allelic expression on messenger RNA of heterozygous polymorphisms representative for a null-allele.

If the *FBN1* studies were negative, we screened the *TGFBR1* and -2 genes by direct sequencing (the primers and conditions for all the analyses described are available upon request).

If an *FBN1* mutation was present, we defined its characteristics according to the predicted effect on the protein and the type of mutation, as reported previously.[4] Besides the location within the gene, and the affected module of the *FBN1* protein were defined:

Predicted effect on protein: mutations within this category were divided into:

(a) missense mutations

(b) mutations most likely leading to haploinsufficiency (large deletions/null-alleles, frameshift mutations, nonsense mutations and mutations affecting splicing).

Type of mutation: Mutations were categorised in the following groups: cysteine missense, non-cysteine missense, nonsense, splice site- and frameshift mutations, small deletions/insertions, large deletions, and null-alleles.

Location of mutation: Three parts of the *FBN1* gene were considered, namely exons 1-15, exons 16-49 and exons 50-65. In the case of missense mutations affecting a cysteine residue, the distance of the mutation to one end of the gene is important, since mutations in exons 1-15 or 50-65 have been shown to have less impact.[17] For mutations leading to a premature termination codon, the location of the affected exon might also be of importance, since the potential (remaining) function of the protein may be determined by the residual length of the truncated protein.

Affected module: Three groups were considered, namely the calcium-

binding epidermal growth factor-like module (cbEGF), the latent transforming growth factor-binding protein 1 module (LTBP), and the remaining modules (non-cbEGF, FIB, the proline rich sequence, the NH_2 unique region and the C-terminal module) (see also Figure 1). A mutation affecting cbEGF will probably have a negative effect on the stability of the protein whereas a mutation in LTBP will probably affect the docking of transforming growth factor-β to *FBN1*.[18]

Statistics

Data are presented as a mean with SD or proportions/percentages. Differences between groups were evaluated using the Student's t-test for continuous variables, the Mann-Whitney U test was used for non-parametric variables and the Chi-square test for proportions/percentages. A p-value <0.05 was considered to indicate statistical significance. Analyses were performed using SPSS for Windows, version 14.0.

Results

Patients

Data on DNA analyses were available for 184 patients. Two of these patients carried two *FBN1* mutations that may both influence the phenotype. They were therefore excluded from further analysis, leaving 182 patients in the final study group (Figure 2). Their mean age was 33.5 ± 11.8 years and 92 (50.5%) were male (Table 1). The mean body surface area was 2.0 ± 0.2 m^2 and the mean aortic root diameter was 41 ± 5.8 (25-57) mm. Two-thirds of patients (122/182) were on beta-blocker therapy (Table 1).

Echocardiography

The mean LVEDD in the group of 182 patients was 50.7 ± 5.7mm, which corresponded with 103.3 ± 9.8% of the predicted value. In 29 of these 182 patients, the LVEDD exceeded the upper limit of normal (112%) (Table 1). The records of these 29 patients with LV dilatation were reviewed for factors other than MFS that might explain the dilatation (e.g. hypertension, signs of coronary disease, sustained supraventricular arrhythmias), but none of these factors could be identified. Twenty-two of the 29 patients with LVEDD >112% were male (76%), which is significantly more than in the group of MFS patients with LVEDD≤112% (p<0.01) (Table 1).

Table 1: Characteristics of the total study group and the subgroups +/- FBN1 mutation, ≤ />LVEDD112% and combinations thereof.

	Subgroup	Male Sex (%)	Age in years (SD)	On Beta-blockade
Total group (n=182)		92 (51%)	33.5 (11.8)	122 (67%)
Total group (n=182)	FBN+ (n=151)	68 (45%)*	33.6 (11.9)	101 (67%)
	FBN- (n=31)	24 (77%)*	32.8 (11.5)	21 (68%)
Total group (n=182)	LVEDD≤112 (n=153)	70 (46%)*	34.2 (12.1)	101 (66%)
	LVEDD>112 (n=29)	22 (76%)*	29.5 (8.8)	21 (72%)
FBN1 + group (n=151)	FBN+/LVEDD≤112 (n=132)	55 (42%)	33.9 (12.2)	86 (65%)
	FBN+/LVEDD>112 (n=19)	13 (68%)	31.7 (9.1)	15 (79%)
FBN1 – group (n=31)	FBN-/LVEDD≤112 (n=21)	15 (71%)	36.2 (11.7) †	15 (71%)
	FBN-/LVEDD>112 (n=10)	9 (90%)	25.5 (7.0) †	6 (60%)

* = p<0.01, †=p<0.05 all other differences are non-significant

DNA analysis

In 151/182 (82.9%) patients a putative pathogenic mutation in the *FBN1* gene or a null-allele was identified, whereas no mutation (n=27) or a silent mutation (n=4) in *FBN1* was found in 31 (17.1%) patients. The groups of patients with or without an *FBN1* mutation did not differ in terms of mean body surface area, mean aortic root diameter (data not shown) or beta-blocker use, but more males (24/31) without an *FBN1* mutation were identified (Table 1).

In total, 84 different mutations were identified, 56 of which were unique

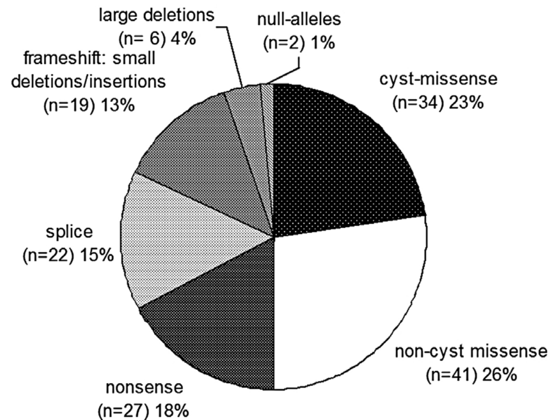

Figure 3. Distribution of the different types of *FBN1* mutations identified in 151 patients with Marfan syndrome.

Table 2: Distribution of mutations according to localisation within the *FBN1* gene and the module involved.

	LVEDD>112%	No. of patients
Localisation within the gene:		
Exon 1-15	2	25
Exon 16-49	4	42
Exon 50-65	7	60
Large deletions, splice site mutations, null-alleles	6	24
Total	19	151
Module involved:		
NH-unique	1	3
Non cb EGF		5
Cb EGF	8	78
Fib	1	6
LTBP	1	23
Proline rich sequence		3
C-terminus		2
Large deletion/insertion/null-allele/splice-site mutation	8	31
Total	19	151

and 28 of which were found more often. The distribution of the different types of mutations is presented in Figure 3. The distribution of MFS patients with respect to the exon-groups involved and the affected modules is shown in Table 2.

In addition the *TGFBR1* and *TGFBR2* genes were screened in all *FBN1*-negative patients which led to the identification of one *TGFBR2* variant (c.1274T>A; p.Met425Lys) in a patient who also demonstrated LVEDD >112% (patient 1; Table 3). Although a mutation affecting the identical amino-acid residue has been described before, its pathogenic character remains to

Table 3: Clinical criteria of *FBN1*-negative MFS patients fulfilling Ghent criteria with LVEDD>112%.

	Cardio vascular	Ocular	Skeletal	Skin	Pulmonary	Dural	Family	Remarks
1	involved		involved			major	major	*TGFBR2* variant
2	major	involved	involved				major	
3	major		involved	involved			major	
4	major		involved	involved			major	
5	major	major	major	involved				
6	major		involved	involved			major	
7	major	major	major					
8		involved	major				major	
9	major		involved	involved			major	
10	major	major	involved	involved				

be established as this mutation could not be found in the patient's brother (patient 6; Table 3) who showed a similar clinical picture.[19]

Genotype - phenotype relationship

Figure 4 and Table 4 show the relationship between LV dilatation (LVEDD >112%) and the categories: missense mutations, mutations most likely leading to haploinsufficiency (subdivided in large deletions/null-alleles, nonsense, frameshift and splice mutations) and absence of an *FBN1* mutation.

Patients carrying missense mutations less frequently (5/75; 6.7%) showed LV dilatation compared to patients having a mutation most likely leading to haploinsufficiency (14/75; 18.7%) (p<0.05) (Table 4). This effect was mainly caused by patients carrying large deletions/null-alleles and frameshift mutations (Table 4). We neither found no relationship between LV dilatation and the location of the mutation, including the "neonatal region" (data not shown), nor with the affected module (Table 2).

LV dilatation was present in 10/31 patients without an *FBN1* mutation or silent mutation carriers (32.7%) versus 19/151 patients carrying an *FBN1* mutation (12.6%); the difference is significant (p<0.05) (Table 4). Patients in the *FBN1*-negative group with LVEDD >112% were younger than those with normal LVEDDs (25.5 vs. 36.2 years, p<0.05) (Table 1).

The organ systems involved according to the Ghent criteria of all the

Table 4. Distribution of number of patients according to type of *FBN1* mutation in the two LV dilatation categories.

	Mutation according to groups	Subtype of mutation	LVEDD ≤112%	LVEDD >112%		
FBN1+	Missense (n=75)	non-cysteine missense	38	3 (7.3%)	5 (6.7%)*	19 (12.6%)†
		cysteine-missense	32	2 (6.3%)		
	Haplo insufficiency (n=75)	large deletions, null-alleles (n=6, 2)	4	4 (44.4%)	14 (18.7%)*	
		nonsense	24	2 (13.3%)		
		frameshift	15	4 (21.1%)		
		Splice mutations	18	4 (18.8%)		
	Rest (n=1)	6bp insertion	1			
FBN1-			21	10	10 (32.7%)	10 (32.7%)†
Total			153	29		

*=p<0.05, †=p<0.05

FBN1+ = with *FBN1* mutation; FBN1- =without *FBN1* mutation; bp=basepair

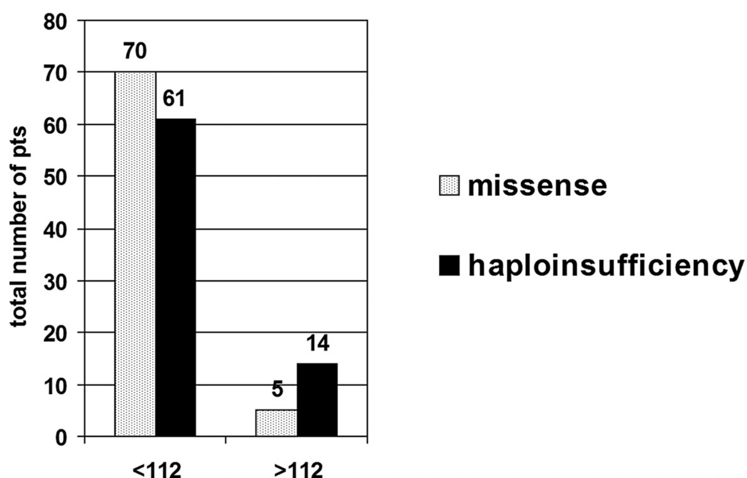

Figure 4: Distribution of number of patients with *FBN1* missense mutations (dotted bar) or with mutations most likely leading to haploinsufficiency (black bar) in the LVEDD ≤112% and >112% categories.

patients with LVEDD >112% in whom no *FBN1* mutation or a silent mutation was identified, are presented in Table 3.

Discussion

We found an important subset of patients with MFS characterised by LV dilatation, even in the absence of predisposing factors like valvular insufficiency, and as described by others.[11,12] Interestingly, our study resulted in the novel observation that carriers of mutations most likely leading in haploinsufficiency (in particular large deletions/null-alleles and frameshift mutations) significantly more often demonstrate LV dilatation than carriers of missense mutations ($p<0.05$). The location of a mutation within the gene or the affected module was not associated with LV dilatation. In addition, MFS patients without an *FBN1* mutation demonstrated LV dilatation significantly more often than those with a mutation.

Genotype-phenotype relationships in MFS

Many attempts have been made to identify genotype-phenotype relationships in MFS, most focusing on the type of *FBN1* mutation and the location within the gene. In general, no major genotype-phenotype relationships have been identified, although a few exceptions have been recognised such as a severe prognosis and neonatal MFS, which is associated with the

clustering of *FBN1* mutations in and around exons 26-28, severe skeletal and skin phenotypes in patients with truncating mutations and the occurrence of ectopia lentis in the presence of a mutation affecting a cysteine residue.[2,4] For the cardiovascular system, an association was found between forms of MFS characterised by a lack of significant aortic pathology and mutations within the 3' region of *FBN1* (exons 59-65).[17]

Dilated cardiomyopathy, characterised by both marked LV dilatation (LVEDD >117% [mean + 2 SD + 5%] and systolic dysfunction [fractional shortening <25%]), in the absence of significant valvular pathology is very rare in MFS.[15] However, isolated mild LV dilatation (LVEDD >112%) is relatively common, occurring in about one in six patients with MFS.[10,11] This is not an entirely unexpected finding given the fact that fibrillin-1 is a component of the myocardium. In the present study, we investigated the genotype-phenotype relationship regarding LV dilatation in a large group of patients with MFS described before.[11] All patients fulfilled the Ghent criteria for MFS, but none had significant valvular pathology or previous aortic root surgery. No relationship between the presence or absence of LV dilatation and the location of mutation or affected module could be demonstrated in the group of patients with an *FBN1* mutation. As patients with LV dilatation, regardless of their mutation carrier status, were younger than those with normal LV dimensions, it is unlikely that the larger LV dimension is caused by ageing.

However, our data show that patients carrying mutations most likely leading to haploinsufficiency more often had LV dilatation than patients carrying other types of mutations. We speculate that missense mutations might be less disturbing, with a milder effect on myocardial function than other mutations. Although a dominant negative effect of premature stop-codon-introducing mutations, leading to production of shortened proteins, can be expected, this is probably largely eliminated due to preferential degradation of transcripts (nonsense mediated mRNA decay), which is believed to be common in *FBN1* premature termination mutations.[20,21] For in-frame deletions, it has been established that the mutant protein product interferes with microfibril assembly by a dominant negative mechanism, which might also underlie the more severe ventricular phenotype.[22] However, the explanations for these differences are highly speculative, in particular given the complex interactions of fibrillin with other extracellular matrix proteins and growth factors.[23-25]

Our data show that it is important whether an *FBN1* mutation is present or not, because in those patients without such a mutation the prevalence of LV

dilatation was significantly higher than in those in whom an *FBN1* mutation was identified. In fact, in as many as one-third of all patients without an *FBN1* mutation, the phenotype was characterised by LV dilatation. However, there appeared to be a possible gender effect, since males more often than females did not harbour an *FBN1* mutation and they also showed LV-dilatation more often (Table 1). A possible explanation for this phenomenon might be that aortic root size in males is on average 2.4 mm larger than females, even after correction for larger body size and thus may lead to more MFS diagnoses in males, as enlarged aortic root size is a major criterion in the diagnostics of MFS.[3,26,27] As this can be considered a phenocopy, this subsequently may lead to a lower yield in *FBN1* analysis. Another explanation might be the presence of a yet unrecognised phenocopy with MFS (-like) features and enlarged LV diameters, which is more predominant in males. Although we can not provide a conclusive explanation for the observed male predominance, this may have led to a potential confounder effect in the analyses.

Other possible genetic mechanisms

Recently, mutations in *TGFBR2*, on chromosome 3p24.2-p25, encoding transforming growth factor β (TGFβ) receptor type 2 were identified in MFS patients.[5,28] TGFβ is a member of a family of dimeric polypeptide growth factors, and it regulates proliferation and differentiation of cells.[29] After secretion TGFβ is stored in the extracellular matrix, and functional relationships between TGFβ and fibrillin-1 have been demonstrated.[25] Moreover, several lines of evidence have implicated TGFβ signalling in the pathogenesis of connective tissue disorders, including MFS. *TGFBR2* missense mutations found in patients with MFS have been shown to cause decreased TGFβ signaling in an *in vitro* assay suggesting that these mutations lead to a loss of function.[5] Based on the above considerations, it can be speculated that the present study should have revealed *TGFBR1* or *TGFBR2* mutations in the subset of patients without a mutation in *FBN1* and that these mutations could play a causative role in the process of LV dilatation. However, we only identified one *TGFBR2* variant in this subset which is in line with other studies that identified *TGFBR1* or -2 mutations only in small subsets of MFS criteria-positive, *FBN1*-negative patients.[5-9] We can therefore exclude overrepresentation of *TGFBR1* or -2 in LV dilatation in MFS criteria-positive patients who do not carry an *FBN1* mutation.

Strengths and weaknesses of this study

This is the first study on the relationship between genotype and LV function in MFS patients and is unique given the size of the population studied.

Nonetheless, the sizes of different subgroups that were compared were sometimes still relatively small. As a result, certain trends may not have reached significance. In addition, not all of the initial 234 MFS patients had undergone *FBN1* mutation analysis. It can therefore not be excluded that this has given rise to a certain bias. Finally, we did not study the potential influence of concomitant medication such as ACE-inhibitors or angiotensin II receptor antagonists on LVEDD. At the time of this study however, these medications were infrequently prescribed in patients with MFS.

Because an *FBN1* mutation was identified in 82.9% of the patients studied, which is in accordance with figures from the literature, we feel that the clinical diagnostics used in this group is appropriate.[4] A definite strength of this study lies in the extensive and complete genetic analyses we performed, including MLPA to exclude large rearrangements, the demonstration of bi-allelic *FBN1* expression, and complete *TGFBR1* and *-2* analyses in patients in whom no *FBN1* mutation was identified.

Conclusion and clinical implications

Isolated dilatation of the LV is not uncommon in patients with MFS, but we found no genotype-phenotype relationship of this phenomenon with regards to the localisation within *FBN1* or an affected module involved. Importantly however, we have shown for the first time that this LV dilatation may occur particularly in MFS patients carrying mutations most likely leading to haploinsufficiency, in particular large deletions/null-alleles and to a lesser extent frameshift mutations, and in those in whom no *FBN1* mutation is demonstrable. Therefore, clinicians should be aware of the possibility of LV dilatation developing in these patients even in the absence of predisposing factors like valvular pathology.

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