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ORIGINAL ARTICLE

Correlation between large *FBN1* deletions and severe cardiovascular phenotype in Marfan syndrome: Analysis of two novel cases and analytical review of the literature

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Abstract

Background: Marfan syndrome (MFS) is a clinically heterogeneous hereditary connective tissue disorder. Severe cardiovascular manifestations (i.e., aortic aneurysm and dissection) are the most life-threatening complications. Most of the cases are caused by mutations, a minor group of which are copy number variations (CNV), in the *FBN1* gene.

Methods: Multiplex ligation-dependent probe amplification test was performed to detect CNVs in 41 MFS patients not carrying disease-causing mutations in *FBN1* gene. Moreover, the association was analyzed between the localization of CNVs, the affected regulatory elements and the cardiovascular phenotypes among all cases known from the literature.

Results: A large two-exon deletion (exon 46 and 47) was identified in two related patients, which was associated with a mild form of cardiovascular phenotype. Severe cardiovascular symptoms were found significantly more frequent in patients with *FBN1* large deletion compared to our patients with intragenic small scale *FBN1* mutation. Bioinformatic data analyses of regulatory elements located within the *FBN1* gene revealed an association between the deletion of STAT3 transcription factor-binding site and cardiovascular symptoms in five out of 25 patients.

Conclusion: Our study demonstrated that large CNVs are often associated with severe cardiovascular manifestations in MFS and the localization of these CNVs affect the phenotype severity.

KEYWORDS

cardiovascular manifestation, *FBN1*, large deletion, Marfan syndrome, regulatory DNA

Tibor Rauch and Judit Bene equally contributed.

1 | INTRODUCTION

Marfan syndrome (MFS; OMIM #154700) is an autosomal-dominant disorder of the connective tissue with high clinical heterogeneity. The malfunction of this tissue manifests in a multi-systemic disorder, where the ocular, skeletal, and cardiovascular systems are mostly affected. Expression of the disease can vary in a spectrum from mild isolated features to severe and progressive multiorgan disease (Judge & Dietz, 2005). The most life-threatening complications in MFS are related to the cardiovascular system, including dilation of the aortic root and ascending aorta, which can result in aortic dissection and sudden death (Adams & Trent, 1998; Canadas et al., 2010). Although characteristic inter- and intrafamilial variability have been found in the clinical presentation, prognosis, and the disease progression, MFS shows complete penetrance (Scriver, 2001). The estimated prevalence of MFS is about 1/5000–1/10000 and it is caused by mutations in the fibrillin 1 (*FBN1*) gene (OMIM *134797) (Judge & Dietz, 2005). The *FBN1* gene is located on the long arm of chromosome 15 (15q21.1), it consists of 66 exons, 65 of which is a coding one and encodes a protein called fibrillin-1, which is a major component of microfibrils in the extracellular matrix. There are a number of MFS-related disorders such as homocystinuria, Loeys–Dietz syndrome, Ehlers–Danlos syndrome vascular types, congenital contractural arachnodactyly, stiff skin syndrome, familial thoracic aortic aneurysm and dissection (familial TAAD) or MASS syndrome (mitral valve prolapse, aortic enlargement, skin, and skeletal findings), which have overlapping phenotypic features with MFS, therefore differential diagnostics and/or genetic testing is essential in the establishment of the precise diagnosis (Hoffjan, 2012; Ramachandra et al., 2015). Pathogenic variations in the genes encoding transforming growth factor β receptors 1 and 2 (*TGFBR1* and *TGFBR2*, respectively) have been previously associated with type 2 Marfan syndrome (OMIM 154705). However, this phenotype was merged into Loeys–Dietz Syndrome 2 (OMIM 610168), therefore mutations in the *TGFBR1* (OMIM *190181) and *TGFBR2* (OMIM *190182) genes are considered as the cause for Loeys–Dietz syndrome 1 and 2, respectively.

According to *FBN1* Universal Mutation Database (UMD, http://www.umd.be/FBN1/- last updated August 28, 2014) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), currently more than 3000 variations are known in the *FBN1* gene, almost half of them are disease-causing pathogenic or likely pathogenic mutations. Missense mutations are the most prevalent ones (Collod-Beroud et al., 2003), but several frameshift, splice-site or nonsense mutations and in-frame deletions and insertions have also been identified. In addition, the number of known large genomic rearrangements are increasing as well. So far, only large deletions involving single or multiple exons of the *FBN1* gene as well as whole *FBN1* deletions have been reported (Tables 1–3) (Aalberts et al., 2014; Apitz et al., 2010; Benke et al., 2018; Blyth et al., 2008; Breckpot et al., 2010; Campbell et al., 2011; Colovati et al., 2012; Dordoni et al., 2017; Faivre et al., 2010; Fang et al., 2017; Franken et al., 2016; Furtado et al., 2011; Hilhorst-Hofstee et al., 2011; Hung et al., 2009; Kainulainen et al., 1992; Lerner-Ellis et al., 2014; Li et al., 2017; Liu et al., 2001; Loeys et al., 2001; Lu Xin-xin et al., 2015; Mannucci et al., 2020; Matyas et al., 2007; McInerney-Leo et al., 2013; Nayak et al., 2021; Nazarali et al., 2017; Ogawa et al., 2011; Pees et al., 2014; Proost et al., 2015; Raghunath et al., 1994; Singh et al., 2007; Spitalieri et al., 2017; Takeda et al., 2021; Voermans et al., 2009; Weidenbach et al., 1999; Wooderchak-Donahue et al., 2015; Yang et al., 2018; Yoo et al., 2010). However, duplications involving one single exon or only a set of exons have not been found yet. Moreover, until now, no genomic rearrangements were detected in either *TGFBR1* or *TGFBR2*. Copy number changes of entire *TGFBR1* or *TGFBR2* were identified as part of microdeletion or microduplication involving several other genes (Breckpot et al., 2010; Campbell et al., 2011). In the case of *FBN1*, 2–7% of MFS patients have been reported to carry a copy number variation (CNV) (Lerner-Ellis et al., 2014; Yang et al., 2018).

The structure and the components of the human genome is yet to be fully understood. However, our knowledge is rapidly growing in the field of functional non-coding elements, due to large-scale collaborative efforts, such as ENCODE (Consortium, 2012), FANTOM (Lizio et al., 2017), and Roadmap Epigenomics Project (Roadmap Epigenomics et al., 2015). Moreover, several GWAS studies demonstrate that SNPs localized to certain non-coding regions exert their effects through regulatory DNA elements (Harismendy et al., 2011). There is a growing evidence that gene regulatory elements are not only involved in the pathogenesis of common and complex diseases, but also may have a contribution to the development of Mendelian diseases as well (Vockley et al., 2017). Structural variants (SV) potentially separate regulatory elements from their target gene, thereby SV can indirectly affect the expression level of a gene by altering the spatial relationship between a regulatory element and a gene (Hollox et al., 2021). Several sophisticated technologies have been developed recently to identify regulatory elements. ChIP-seq, DNase-seq, ATAC-seq, and FAIRE-seq are useful tools for analyzing among others transcription factor-binding sites (TFBSs) even in a genome-wide manner (Meyer & Liu, 2014; Takata, 2019).

Cardiovascular manifestation is a known phenomenon among patients with MFS. Several research groups

have performed genotype–phenotype studies in order to find an association between cardiovascular disease severity and *FBN1* mutations (Arnaud et al., 2021; Baudhuin et al., 2015; Detaint et al., 2010; Du et al., 2021; Franken et al., 2017; Milleron et al., 2020; Sakai et al., 2016; Takeda, Hara, et al., 2018; Takeda, Inuzuka, et al., 2018). However, these studies focused on intragenic *FBN1* mutations, and until now no study was performed to explore the connection between CNVs in the *FBN1* gene and their effects on cardiovascular phenotype.

In this study, we performed multiplex ligation-dependent probe amplification (MLPA) assays to search for CNVs of *FBN1* and *TGFBR2* genes in our MFS patients whose disease-causing mutations have not been identified previously. In addition, the aim of our study was to reveal association between the detected large *FBN1* deletions so far and the severity of the cardiovascular manifestations. In order to find a link between severe cardiovascular symptoms and the large deletions of the *FBN1* gene we have analyzed the possible regulatory elements located within the gene especially focusing on TFBSs.

2 | MATERIALS AND METHODS

2.1 | Participants

Patients with suspected MFS or a related connective tissue disorder were referred for genetic testing to our institute. Of these, 41 patients were enrolled into this study for *FBN1* and *TGFBR2* large del/dup screening. These patients originated from 38 unrelated families (28 males, 13 females; mean age: 23 years [age range:1–47 years]) and no casual mutations have been identified in their samples after systematic sequencing of *FBN1*, *TGFBR1*, and *TGFBR2* genes. As a control, 17 patients [7 females, 10 males, mean age at the time of examination: 27 years, (age range: 0.5–59)] with intragenic *FBN1* mutations were enrolled into the study as well. In the control group 15 various disease-causing mutations were detected: 11 missense, (G214S, C494Y, C570S, R627C, C763Y, C1068Y, D1238N, D1487G, R1530H, G2536R, and C2459G), three nonsense (R429*, R1125*, and R1644*), and one splicing (c.5788+5G>A) mutations.

All the patients fulfilled the revised Ghent criteria (B. L. Loeys et al., 2010). Main clinical characteristics of our patient cohort and the control group are summarized in Table 4. Intragenic small scale mutations detected in the control cohort are presented in Table 5 along with the corresponding cardiovascular manifestations.

2.2 | MLPA analysis

Genomic DNA was isolated from peripheral blood leukocytes using E.Z.N.A.® Blood DNA Maxi kit (Omega BIO-TEK, Norcross, USA).

MLPA assays were performed for screening large deletions or duplications in *FBN1* and *TGFBR2* genes using the commercially available SALSA MLPA kits P065 and P066 (MRC-Holland, Amsterdam, The Netherlands), which contained probes for all exons of *FBN1* and *TGFBR2*. According to the manufacturer's instructions, a total of 100–200 ng of genomic DNA of each patient and the same amount of three control genomic DNA was used for hybridization. Amplification products from each MLPA assay were separated by capillary electrophoresis on an ABI 3130 Genetic Analyzer (Life Technologies, USA) and the results were analyzed using Coffalyser software (MRC-Holland, Amsterdam, The Netherlands). Each MLPA signal was normalized and compared to the corresponding peak area obtained from the three control samples. Deletions and duplications of the targeted regions were suspected when the signal ratio exceeded 30% deviation. Positive results were verified by repeated MLPA experiments.

2.3 | Analyses of regulatory elements within *FBN1* gene

In silico analysis was performed for identifying regulatory elements within the whole *FBN1* gene on the genomic data of our patients as well as on previously published data. For this purpose, mainly the USCS genome browser was used with GRCh37/hg19 assembly. The used tracks were ORegAnno, TFBS Conserved, Vista Enhancers, ENCODE Transcription Binding Factors, base positions, and UCSC genes. UCSC genome browser (https://genome.ucsc.edu/) provide data for the transcription factors (TF) through their ORegAnno identifier. These data are originated from the JASPAR and PAZAR datasets, and include the names and genomic positions of the given TF. The genomic positions of the TFBSs were correlated to the *FBN1* deletions with known genomic positions. The breakpoints were converted into hg19 genome build where it was necessary. Based on Chip-seq analyses described previously by other research groups, several various TFBSs have been mapped to *FBN1* gene. These data were exported for further association analysis.

Genomic localization of all published large *FBN1* deletions encompassing single or a few exon deletions has been harmonized with and has been given according to GRCh37 in order to make the data comparable.

TABLE 1 Summary of MFS patients with single-exon deletion in *FBN1* gene.

Original exon numbering	Harmonized exon numbering considering 66 exons	IF/OF	Affected domains[a]	Technique
Ex1 and promoter region	Ex1-2 and promoter region	OF	–	MLPA
Ex1 and promoter region	Ex1 and promoter region	OF	–	MLPA
Ex1	Ex2	OF	–	MLPA
Ex2	Ex3	OF	–	PCR then SSCP and CSGE
Ex 3	Ex3	OF	1st EGF-like	NGS
Ex3	Ex4	IF	1st EGF-like, 2nd EGF-like	High-Throughput Microarray and MLPA
Ex6	Ex6	IF	3rd EGF-like	MLPA
Ex18	Ex19	IF	11th cbEGF-like	N/A
Ex 20	Ex20	IF	12th cbEGF-like, 13th cbEGF-like	NGS
Ex29	Ex30	IF	19th cbEGF-like, 20th cbEG-like	SSCP
Ex30	Ex30	IF	19th cbEGF-like, 20th cbEG-like	aCGH
Ex33 (no probes for exon 32)	Ex34 (no probes for ex 33)	IF	(22nd-)23rd cbEGF-like, 24th cbEG-like	DHPLC/MLPA
Ex 35	Ex35	IF	24th cbEGF-like, 25th cbEGF-like	NGS
Ex36	Ex37	IF	26th cbEGF-like	PCR-DHPLC/MLPA
Ex43	Ex43	IF	7th TB, 29th cbEGF-like	MLPA
Ex 49	Ex49	IF	34th cbEGF-like, 35th cbEGF-like	MLPA
Ex50	Ex50	IF	35th cbEGF-like	MLPA
Ex52	Ex53	IF	36th cbEGF-like, 37th cbEGF-like	PCR then SSCP and CSGE
Ex54	Ex54	IF	37th cbEGF-like, 38th cbEGF-like	MLPA
Ex56	Ex56	IF	39th cbEGF-like, 40th cbEGF-like	MLPA

Note: Exon numbering: original numbering shows the affected exons as it was reported in the referred article.

Cardiovascular symptoms: major: aortic ascendens dilation with or without aortic regurgitation and involving the sinuses of Valsalva, aorta ascendens dissection; minor: Mitral valve prolapse, mitral annulus calcification (age of onset, <40 years), pulmonary artery dilation, descending aorta or abdominal aorta dilation or dissection (age of onset, <50 years).

Abbreviations: #, no explicit clinical information; IF, in frame; N/A, not available; OF, out of frame.

[a]Determination of the domains were based on the first and last amino acids of the deleted exons obtained from the 66 exon numbering harmonized with the determined domain boundaries by Uniprot.

2.4 | External sources of data

The Open Regulatory Annotation (ORegAnno) displays experimentally proven, literature-curated regulatory regions, TFBSs, and regulatory polymorphisms. JASPAR database (http://jaspar.genereg.net/) includes curated and non-redundant, experimentally determined TFBSs in different eukaryote organisms. PAZAR database (http://www.pazar.info) is a public database of transcription factor and regulatory sequence annotations.

The following keywords and their combinations were applied in the PubMed search engine to find the relevant publications related to CNVs in MFS: *FBN1*, large deletion, gross deletion, CNV, MFS, copy number variation.

2.5 | Statistical analysis

All statistical analyses were performed with SPSS version 27 (SPSS Inc.). Two-tailed Fisher's exact test was used to assess whether there is a difference in the frequency of severe cardiovascular manifestations between patients with *FBN1* large deletion and patients with intragenic *FBN1* mutations. A difference with $p < 0.05$ was considered as significant.

Confirmation/breakpoints determination	Phenotype in paper	Cardiovascular symptoms		Ref
		Major	Minor	
Affymetrix Human Mapping 500 K Array Set	severe MFS	X	X	Matyas et al. (2007)
Sequencing	Classic MFS	N/A	N/A	McInerney-Leo et al. (2013)
N/A	Classic MFS	N/A	N/A	Lerner-Ellis et al. (2014)
N/A	Classic MFS	X	-	Loeys et al. (2001)
Agilent custom-designed oligonucleotide array-CGH	MFS	X	N/A	Takeda et al. (2021)
N/A	MFS	N/A	N/A	Ogawa et al. (2011)
gap PCR and sequencing	Potential MFS	–	–	Li et al. (2017)
N/A	Potential MFS	–	–	Xin-xin et al. (2015)
Agilent custom-designed oligonucleotide array-CGH	MFS	X	N/A	Takeda et al. (2021)
N/A	Neonatal MFS	X	–	Weidenbach et al. (1999)
MLPA	Neonatal MFS	X	–	Wooderchak-Donahue et al. (2015)
N/A	Neonatal MFS	X	–	Blyth et al. (2008)
Agilent custom-designed oligonucleotide array-CGH	MFS	X	N/A	Takeda et al. (2021)
N/A	Classic MFS	#	#	Hung et al. (2009)
qPCR/long range PCR then Sanger sequencing	Classic MFS	X	-	Yang et al. (2018)
Sanger sequencing	MFS	N/A	N/A	Proost et al. (2015)
qPCR/long range PCR then Sanger sequencing	Suspected MFS	X	–	Yang et al. (2018)
N/A	Classic MFS	X	-	Loeys et al. (2001)
qPCR/long range PCR then Sanger sequencing	Suspected MFS	X	–	Yang et al. (2018)
qPCR/long range PCR then Sanger sequencing	Classic MFS	X	–	Yang et al. (2018)

3 | RESULTS

3.1 | CNV analysis

In a total of 41 patients screened for large *FBN1* rearrangements by MLPA, one large deletion was identified in two patients from the same family. The deletion was found in a 22-year-old female and her 1-year-old son. Her clinical examination started when she was 12 months old. A suspicion for MFS emerged based on arachnodactyly, long arms, pectus excavatum, myopia, and lens subluxation. However, at the age of 4 her symptoms did not fulfill the Ghent criteria. Since elevated homocysteine level was measured in her urine and her vague initial symptoms shared common features with homocystinuria, therefore homocystinuria emerged as a clinical diagnosis. For differential diagnostic purposes, the mutation analysis of *CBS* gene (cystathionine beta synthase) was performed with a negative result. Later, between the ages of 6 and 8, new symptoms (mitral valve prolapse, skin striae, pectus carinatum, scoliosis, and joint hypermobility) appeared, and the patient finally fulfilled the diagnostic criteria of Ghent nosology for MFS as a sporadic case with major criteria in two organ

TABLE 2 Summary of MFS patients with multiple exon deletions in *FBN1* gene.

Original exon numbering	Harmonized exon numbering considering 66 exons	IF/OF	Affected domains[a]	Technique
Ex1–4	Ex1–4	OF	1st EGF-like	MLPA
Ex1–5	Ex1–5	OF	1–2nd EGF-like, 3rd EGF-like	MLPA/Array-CGH
Ex1–5	Ex2–6	OF	1–3rd EGF-like	MLPA
Upstream of ex1–15	Ex1–15	OF	1–3rd EGF-like, 1–2nd TB, 4–9th cbEGF-like, 10th cbEGF-like	MLPA
Ex1–16	Ex1–17 and promoter region	OF	1–3rd EGF-like, 1–2nd TB, 4–10th cbEGF-like, 3rd TB	MLPA
Ex1–36	Ex1–36	OF	1–3rd EGF-like, 4–25th cbEGF-like, 1–5th TB, 26th cbEGF-like	MLPA
Ex2–4	Ex3–5	OF	1–2nd EGF-like, 3rd EGF-like	NGS (panel then WGS)
Ex4–53	Ex4–53	IF	1-3rd EGF-like, 4–36th cbEGF-like, 1–8th TB, 37th cbEGF-like	NGS
Ex5–54	Ex5–54	IF	2-3rd EGF-like, 4–37th cbEGF-like, 1–8th TB, 38th cbEGF-like	NGS
Ex6–65	Ex7–66	OF	4–47th cbEGF-like, 1–9th TB	MLPA
Ex13–15	Ex13–15	IF	7–9th cbEGF-like, 10th cbEGF-like	MLPA
Ex13–49 mosaic	Ex14–50 mosaic	IF	8–35th cbEGF-like, 3–7th TB	DHPLC/MLPA
Ex17–21	Ex18–22	IF	3–4th TB, 11-13th cbEGF-like	MLPA
Ex23–25	Ex23–25	IF	14th cbEGF-like, 4–5th TB, 15th cbEGF-like	NGS
Ex24–26	Ex25–27	IF	5th TB, 15-16th cbEGF-like	N/A
Ex32–37	Ex33–38	IF	22-26th cbEGF-like, 6th TB	MLPA
Ex33–38	Ex34–39	IF	23-26th cbEGF-like, 6th TB, 27th cbEGF-like	MLPA
Ex34–43	Exon 35–44	IF	24–29th cbEGF-like, 6-7th TB, 30th cbEGF-like	MLPA
Ex37–65	Exon 38–66	OF	27–47th cbEGF-like, 6–9th TB	MLPA
Ex39-40	Exon 39–40	IF	6th TB, 27th cbEGF-like	NGS
Ex42–43	Exon 43–44	IF	7th TB, 29th cbEGF-like	sequencing and RT-PCR
Ex44–46	Exon 45–47	IF	30–32th cbEGF-like, 33th cbEGF-like	sequencing and RT-PCR
Ex44–66	Exon 44–66	OF	30–47th cbEGF-like, 7–9th TB	MLPA
Ex46-47	**Exon 46–47**	**IF**	**31–32th cbEGF-like**	**MLPA**
Ex48–53	Exon 48–53	IF	33–36th cbEGF-like, 8th TB, 37th cbEGF-like	MLPA
Ex49–50	Exon 49–50	IF	34–35th cbEGF-like	MLPA
Ex50–63	Exon 51–64	OF	36–46th cbEGF-like, 8-9th TB, 47th cbEGF-like	DHPLC
Ex50–63	Exon 51–64	OF	36–46th cbEGF-like, 8-9th TB, 47th cbEGF-like	MLPA
Ex51–63	Exon 51–63	IF	36–45th cbEGF-like, 8-9th TB, 46th cbEGF-like	NGS
Ex58–63	Exon 59–64	OF	41–46th cbEGF-like, 47th cbEGF-like	FISH, Southern blot, sequencing, Western blot
Ex59–63	Exon 59–63	IF	41–45th cbEGF-like, 46th cbEGF-like	MLPA
Ex60–62	Exon 61–63	IF	43–45th cbEGF-like, 46th cbEGF-like	SSCP/Southern-blot
Ex60–65	Exon 61–66	OF	43–47th cbEGF-like	MLPA
Ex61–66	Exon 61–66	OF	43–47th cbEGF-like	MLPA

Note: Exon numbering: original numbering shows the affected exons as it was reported in the referred article.

Cardiovascular symptoms: major: ascending aorta dilation with or without aortic regurgitation and involving the sinuses of Valsalva, ascending aorta dissection; minor: Mitral valve prolapse, mitral annulus calcification (age of onset, <40 years), pulmonary artery dilation, descending aorta or abdominal aorta dilation or dissection (age of onset, <50 years).

Abbreviations: IF, in frame; N/A, not available; OF, out of frame.

[a]Determination of the domains were based on the first and last amino acids of the deleted exons obtained from the 66 exon numbering harmonized with the determined domain boundaries by Uniprot.

Confirmation/breakpoints determination	Phenotype in paper	Cardiovascular symptoms		Ref
		Major	Minor	
SNP microarray analysis	MFS	N/A	N/A	Proost et al. (2015)
N/A	Classic MFS	X	X	Furtado et al. (2011)
N/A	MFS	N/A	N/A	Franken et al. (2016)
N/A	MFS	N/A	N/A	Nayak et al. (2021)
Affymetrix Array	Classic MFS	X	X	Matyas et al. (2007)
gap PCR and sequencing	Classic MFS	X	X	Li et al. (2017)
MLPA and PCR with sanger sequencing	Classic MFS	X	X	Benke et al. (2018)
N/A	Aortopathy	X	X	Fang et al. (2017)
N/A	Classic MFS	X	-	Fang et al. (2017)
N/A	Classic MFS	N/A	N/A	Lerner-Ellis et al. (2014)
N/A	MFS	N/A	N/A	Nayak et al. (2021)
N/A	MFS	X	X	Blyth et al. (2008)
N/A	MFS	X	N/A	Mannucci et al. (2020)
Agilent custom-designed oligonucleotide array-CGH	MFS	X	N/A	Takeda et al. (2021)
N/A	Neonatal MFS	X	X	Apitz et al. (2010)
N/A	Neonatal MFS	X	X	Nazarali et al. (2017)
N/A	Neonatal MFS	X	X	Pees et al. (2014)
N/A	Classic MFS	X	X	Yoo et al. (2010)
N/A	Classic MFS	N/A	N/A	Lerner-Ellis et al. (2014)
Agilent custom-designed oligonucleotide array-CGH	MFS	X	N/A	Takeda et al. (2021)
N/A	Classic MFS	X	-	Liu et al. (2001)
N/A	Neonatal MFS	X	X	Liu et al. (2001)
qPCR/long range PCR then sequencing	Classic MFS	X	–	Yang et al. (2018)
long range PCR then Sanger sequencing	**Juvenile onset MFS**	**–**	**X**	**Current**
gap PCR and sequencing	Neonatal MFS	X	X	Li et al. (2017)
gap PCR and sequencing	Neonatal MFS	X	X	Li et al. (2017)
N/A	MFS	X	–	Voermans et al. (2009)
N/A	MFS	N/A	N/A	Franken et al. (2016)
Agilent custom-designed oligonucleotide array-CGH	MFS	X	N/A	Takeda et al. (2021)
N/A	Juvenile onset classic MFS	X	–	Singh et al. (2007)
N/A	MFS	N/A	N/A	Nayak et al. (2021)
N/A	Classic MFS	X	–	Kainulainen et al. (1992); Raghunath et al. (1994)
N/A	MFS	N/A	N/A	Franken et al. (2016)
SNP microarray analysis	MFS	N/A	N/A	Proost et al. (2015)

TABLE 3 Summary of MFS patients with whole *FBN1* gene deletion.

Original exon numbering	IF/OF	Affected domains	Technique	Confirmation/ breakpoints determination	Phenotype in paper	Cardiovascular symptoms		Ref
						Major	Minor	
FBN1:Ex1–66	–	Full gene	MLPA	FISH/aCGH	Incomplete MFS	–	X	Faivre et al. (2010)
FBN1:Ex1–66	–	Full gene	MLPA	SNP array	(Potential) MFS	#	#	Hilhorst-Hofstee et al. (2011)
FBN1:Ex1–66	–	Full gene	MLPA	SNP array	MFS	N/A	N/A	Proost et al. (2015)
FBN1:Ex1–66**	–	Full gene	MLPA	N/A	MFS	N/A	N/A	Franken et al. (2016)
FBN1:Ex1–66**	–	Full gene	MLPA	N/A	MFS	X	X	(Spitalieri et al., 2017)
FBN1:Ex1–66**	–	Full gene	N/A	N/A	N/A	N/A	N/A	Aalberts et al. (2014)
FBN1:Ex1–66	–	Full gene	Conventional chromosome analysis/ Affymetrix Cytogenetics Array 2.7	FISH	Classic MFS	X	X	Colovati et al. (2012)
FBN1:Ex1–66	–	Full gene	MLPA	aCGH	MFS	X	X	Dordoni et al. (2017)

Note: Cardiovascular symptoms: major: ascending aorta dilation with or without aortic regurgitation and involving the sinuses of Valsalva, aorta ascendens dissection; minor: Mitral valve prolapse, mitral annulus calcification (age of onset, <40 years), pulmonary artery dilation, descending aorta or abdominal aorta dilation or dissection (age of onset, <50 years).
#, the referred paper discusses 10 patients belonging to six families. Six of them had major cardiovascular symptoms, three of this six patients had minor symptoms also. One patient had only minor symptoms and one patient had no cardiovascular symptoms.

systems (skeletal: pectus carinatum, reduced upper to lower segment ratio, positive wrist and thumb signs, scoliosis and joint hypermobility and ocular: ectopia lentis), and involvement of one additional organ system (skin and/or cardiovascular). She has no severe cardiovascular manifestation. At the age of 22, the size of the aortic root and aortic ascendens were in the normal range, 23 and 31 mm, respectively. The 1-year-old male patient presented several symptoms of the affected skeletal system, namely pectus excavatum, positive wrist and thumb sign, scoliosis, arachnodactyly and tendency towards tall stature. He had mild myopia and no abnormality was detected in his cardiovascular system.

The detected large deletion encompassing exons 46–47 (Figure 1) results in the deletion of the 31st and 32nd calcium-binding EGF-like domains of the fibrillin-1 protein. The breakpoint analyses (described previously) revealed a 4916-bp long deletion along with a TG insertion (Buki et al., 2022). The female patient had no positive family history and the molecular genetic testing of her parents have confirmed the de novo origin of the deletion. Mosaicism was ruled out in the proband and her parents based on the MLPA signal ratios, which were 0.54 and 0.53 for exon 46 and exon 47, respectively in the proband and it was normal for the parents.

3.2 | Investigation of the association between CNVs and severity of cardiovascular manifestations

In order to compare the severity of cardiovascular manifestations between patients carrying large deletion in the *FBN1* gene and patients with intragenic *FBN1* mutations cardiovascular symptoms (CV) were classified into two groups. Mitral valve prolapse, mitral annulus calcification (age of onset, <40 years), pulmonary artery dilation, descending aorta or abdominal aorta dilation or dissection (age of onset, <50 years) were considered as minor CV symptoms, while ascending aorta dilation with or without aortic regurgitation and involving the sinuses of Valsalva, ascending aorta dissection were considered as major CV symptoms (Loeys et al., 2010). CV symptoms of our patient along with the patients published so far in the literature carrying large deletion in the *FBN1* gene are summarized in Tables 1–3. Among patients with single-exon deletion 13 out of 20 (65%) displayed major CV symptoms, moreover, one patient had minor symptoms as well. Two patients had no CV manifestations, in four patients clinical data were not available and in one patient the clinical information was not clear. The rate of major CV symptoms was a little bit higher in patients carrying multiple exon deletions (23 out of 34; 68%). In addition, in 13 patients

TABLE 4 Clinical characteristics of our patients involved in the CNV analyses.

System involvement	Patient cohort (41)	Patient #7	Patient #41	Control group (17)
Mean age (range) [years]	23 (1–47)	22	1	27 (0.5–59)
Gender (males/females)	28/13	Female	Male	7/10
Cardiovascular system				
Dilation of the aortic root	7	–	–	7
Dissection of the ascending aorta	4	–	–	2
Mitral valve prolapse	12	X	–	8
Mitral annular calcification, below age 40	0	–	–	–
Skeletal system				
Pectus carinatum	7	X	–	4
Pectus excavatum or chest asymmetry	8	X	X	4
Wrist AND thumb sign	10	X	X	2
Wrist OR thumb sign	5	–	–	3
Pes planus	9	–	–	6
Acetabular protrusion	1	–	–	–
Hindfoot deformity	1	X	–	–
Scoliosis or thoracolumbar kyphosis	27	X	X	9
Arachnodactyly	21	X	X	9
Joint hypermobility	11	X	–	6
Typical facial features[a]	2	–	–	1
Decreased upper to lower segment ratio <0.85	0	–	–	–
Span-height ratio >1.05	7	–	X	–
Reduced elbow extension (<170)	1	X	–	–
Dental crowding, High palate	17	X	–	2
Tendency towards tall stature	28	–	X	6
Muscle hypotonia	2	–	–	3
Uveitis	1	–	–	–
Spondylolisthesis	0	–	–	–
Skin integument				
Skin striae	10	X	–	5
Recurrent or incisional hernia	4	–	–	–
Ocular system				
Flat cornea	0	–	–	–
Myopia	17	X	X	4
Lens subluxation	4	X	–	5
Pulmonary				
Pneumothorax	10	–	–	–
Apical blebs	0	–	–	–
Dura				
Lumbosacral dural ectasia	1	–	–	–
Other				
Occasional features[b]	0	–	–	2
Positive family history		–	+	

[a]Dolichocephaly, enophthalmos, downward slanting palpebral fissures, malar hypoplasia, retrognathia.

[b]Large ears, cataracts, retinal detachment, glaucoma, strabismus, refractive errors, diaphragmatic hernia, hemivertebrae, colobomata of iris, cleft palate, incomplete rotation of the colon, ventricular dysrhythmias, cardiomyopathy, intracranial aneurysms, sleep apnea, neuropsychologic impairment including learning disabilities and attention deficit.

TABLE 5 Different types of *FBN1* gene mutation detected in the control group with cardiovascular symptoms.

Nucleotide change	Amino acid alteration	Effect of mutation	Cardiovascular symptoms	
			Major	Minor
c.640G>A	p.Gly214Ser	DN	–	–
c.1481G>A	p.Cys494Tyr	DN	–	–
c.1709G>C	p.Cys570Ser	DN	–	–
c.4589G>A	p.Arg1530His	DN	–	–
c.4930C>T	p.Arg1644*	HI	–	–
c.5788+5G>A	splicing	DN	–	–
c.1481G>A	p.Cys494Tyr	DN	–	X
c.1879C>T	p.Arg627Cys	DN	–	X
c.2288G>A	p.Cys763Tyr	DN	–	X
c.3712G>A	p.Asp1238Asn	DN	–	X
c.1481G>A	p.Cys494Tyr	DN	X	–
c.1285C>T	p.Arg429*	HI	X	–
c.3373C>T	p.Arg1125*	HI	X	–
c.3203G>A	p.Cys1068Tyr	DN	X	X
c.4460A>G	p.Asp1487Gly	DN	X	X
c.7375T>C	p.Cys2459Gly	DN	X	X
c.7606G>A	p.Gly2536Arg	DN	X	X

Abbreviations: DN, dominant-negative; HI, haploinsufficiency.

minor CV symptoms were noted along with the major CV symptoms. Six patients did not display minor CV symptoms, one patient (demonstrated in this study) had minor CV symptoms only and in 10 patients no clinical information was available. In the whole *FBN1* gene deletion cohort nine out of 17 (53%) showed major CV symptoms, in three patients clinical data were not available.

In our control patient cohort with *FBN1* intragenic mutation six patients did not have any cardiovascular manifestation, four patients displayed mitral valve prolapse only (minor CV symptom, 23%) and seven patients showed major CV symptoms (41%), four of them had mitral valve prolapse as well.

A significant difference was observed in the frequencies of the major CV symptoms between patients with large deletion in the *FBN1* gene carrying single exon or multiple exons deletion and our patients with intragenic *FBN1* mutation (66 vs. 41%, respectively; $p = 0.042$). However, no statistically significant difference was observed comparing the frequency of major CV symptoms between patients with full *FBN1* gene deletion and our patients with intragenic *FBN1* mutation.

3.3 | Analyses of regulatory elements within *FBN1* gene focusing on transcription factor-binding sites

Preliminary in silico analysis of the entire *FBN1* gene displayed by UCSC genome browser revealed several tissue-specific enhancer regions in the intronic regions of the *FBN1* gene. For profound in silico analysis of our patient, patients carrying single exon deletion with known genomic localization or few exon deletions were selected from the literature. In order to reveal possible associations between cardiovascular manifestations and the abolishment of TFBS due to large CNV, major CV symptoms were further classified (namely ascending aorta dissection was further indicated). Results of the in silico analysis are listed in Table 6.

A number of various TFBSs have been found in the region of *FBN1* gene affected by different CNVs known from the literature. However, the CNV detected in our patient harbors only a few TFBSs (Figure 2) The following TFBSs were affected more frequently by a number of CNVs: FOXA1, FOS, CEBPB, DUX4, and STAT3, however, no experimentally based literature data have been found for the association of FOXA1, CEBPB or DUX4 genes and cardiovascular manifestations. TFBS of FOS gene, which belongs to the Activator Protein 1 (AP-1) transcription factor family, was found in five patients. Tobin et al (Tobin et al., 2019) investigated the role of AP-1 complexes in aortic aneurysm, however, they found that the expression of AP-1 factors in Marfan aneurysmal tissue was not significantly different from normal aorta, therefore the possible role of the deletion of this transcription factor-binding site on cardiovascular manifestation was ruled out. Thus, after a comprehensive evaluation only STAT3 showed any correlation with

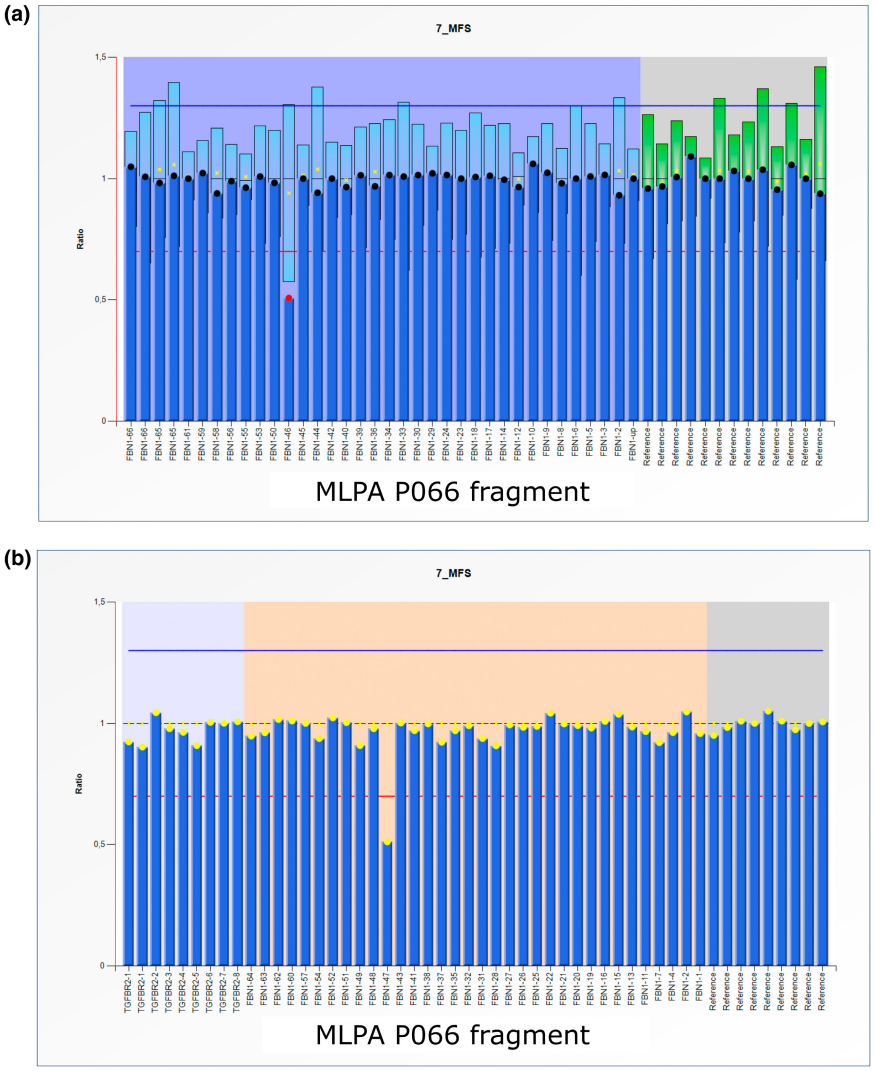

FIGURE 1 (a, b) Results of semiquantitative MLPA analyses. Normalized relative peak areas measured with P065 and P066 kits. (a) Reduced relative peak areas of *FBN1* exon 46. (b) Reduced relative peak areas of *FBN1* exon 47. Combined results from P065 and P066 MLPA kit indicate the heterozygous deletion of exons 46–47.

cardiovascular symptoms among TFBSs listed in Table 6. Five patients carry such a deletion involving STAT3-binding site. Among these patients, four developed aortic dilations and one patient suffered from an acute dissection of the ascending aorta and right coronary artery as well. Therefore, deletion of STAT3-binding site may play a role in the development of cardiovascular manifestations. Moreover, several regulatory elements (promoters and/or enhancers) known to be active in the aorta have been found in the region of *FBN1* gene affected by different CNVs.

4 | DISCUSSION

CNV is one of the major sources of genetic diversity in humans (Zhang et al., 2009), however, the roles of CNVs in disease pathogenesis have increasingly emerged nowadays thanks to the sophisticated molecular laboratory techniques capable to detect various CNVs. Moreover, there is a growing evidence recently that CNVs are not only responsible for the development of genomic disorders, but also may cause Mendelian diseases or sporadic traits as well. In MFS ca 2%–7% of the disease-causing mutations belong to this category (Lerner-Ellis et al., 2014; Yang et al., 2018). There are various molecular mechanisms by which CNVs can convey abnormal phenotypes, including dosage sensitivity of a gene within the CNV; gene fusion or gene interruption at the breakpoint junctions; deletion of a regulatory element; or unmasking of recessive alleles or functional polymorphism. Moreover, CNVs can affect noncoding regulatory elements such as promoters or enhancers as well (Harel & Lupski, 2018).

Several laboratory techniques have been developed for the detection of copy number, which can be either genome-wide or locus specific. While comparative genomic hybridization (CGH), SNP genotyping arrays, and various next-generation sequencing (NGS) technologies are the tools for genome-wide analysis, quantitative real-time PCR is usually used for single locus association analysis and MLPA assays are applied for multiple loci analysis as well (Hu et al., 2018).

TABLE 6 Summary of transcription factor-binding site analyses of *FBN1* gene. TFBS analyses was performed in our patient and published CNV patients carrying single exon deletion with known genomic localization or few exon deletions.

Genomic position (GRCh37)	Affected region considering 66 exons	Transcription factors	Active regulatory elements in aorta	Patient age (years)	Cardiovascular symptoms	
					Major	Minor
Single exon deletion						
g.48,941,579–48,914,693	Ex1–2 and promoter	MAFK (x2), RBL2 (x2), ZNF263, ZBTB33, E2F4, EBF1 (x2), E2F1, CTCF, PRDM1, SMARCA4, GATA3, CEBPB, USF1, BATF::JUN, FOXA1, **STAT3**, STAT1	+	25	X	X
g.48,907,500–48,904,685	Exon 3	–	+	54	X	N/A
g.48,890,441–48,873,891	Exon 6	SMARCA4 (x4), CEBPB (x2), EBF1 (x3), DUX4, TP63, ZNF263	+	49	–	–
g.48,789,354–48,788,226	Exon 20	–	–	34	X	N/A
~g.48,777,400 to 48,778,050	Exon 30	–	–	<1	X	–
g.48,765,384–48,764,320	Exon 35	DUX4	+	3	X	N/A
g.48,753,819–48,749,026	Exon 43	FOXA1, JUN (var.2), JUNB, FOS, STAT1, GATA3, RBL2 (x2), MAX	–	24	XX	–
g.48,734,801–48,730,690	Exon 50	PRDM1	+	14	X	–
g.48,727,672–48,726,338	Exon 54	–	+	5	X	–
g.48,724,560–48,722,281	Exon 56	–	–	38	X	–
Few exon deletion						
N/A	Exon 1–5	E2F4, EBF1 (x2+x1), E2F1, CTCF, PRDM1, SMARCA4 (x2), GATA3, CEBPB (x2+x1), USF1, BATF::JUN (x2), FOXA1 (x2), MAFK, **STAT3**, STAT1, DUX4 (x2), MAX, JUND (var.2)	+	27	X	X
g.48,922,918–48,890,962	Exon 3–5	EBF1, FOXA1 (x2), MAFK, **STAT3**, STAT1, BATF::JUN, DUX4 (x2), MAX, JUND (var.2), CEBPB	+	32	X	X
N/A	Exon 18–22	JUND (var.2)	+	37	X	N/A
g.48,786,684–48,781,065	Exon 23–25	AR, JUNB, MAFF, MAFK	+	12	X	N/A
N/A	Exon 25–27	AR	–	After birth	XX	X
N/A	Exon 33–38	IGF1R (x2), CEBPB, FOXA1, DUX4, E2F1, AR, SMARCA4	+	5	X	X
N/A	Exon 34–39	AR, DUX4, FOXA1, CEBPB, IGF1R (x2), E2F1, SMARCA4	+	1	X	X
g.48,758,142–48,756,692	Exon 39–40	–	–	15	X	N/A
g.48,754,954–48,748,519	Exon 43–44	JUN, JUND (var.2), FOXA1, JUN (var.2), JUNB, FOS, STAT1, GATA3, RBL2 (x2), MAX, **STAT3**	–	>46	X	–
g.48,745,163–48,738,026	Exon 45–47	GATA2, FOS (x2), JUN (var.2)	–	>6	X	X
g.48,743,774–48,738,856[a]	*Exon 46–47*	*GATA2, FOS (x2), JUN (var.2)*	–	*22*	–	X
g.48,738,131–48,727,786	Exon 48–53	PRDM1	+	15	X	X
g.48,737,397–48,732,835	Exon 49–50	–	+	3	X	X
g.48,718,149–48,706,671	Exon 59–64	FOS, PRDM1, TFAP2A, JUN, JUND (var.2), FOXA1 (x5), MAFF, MAFK, ELF1 (x2), GATA2, **STAT3** (x2), JUND	+	17	XX	–
N/A	Exon 61–63	FOS, PRDM1, TFAP2A	+	48	X	–

Note: Major cardiovascular symptoms: X: patients with ascending aorta dilation and XX: patients with ascending aorta dilation along with dissection.

Abbreviation: N/A, not available.

[a]Indicates our patient.

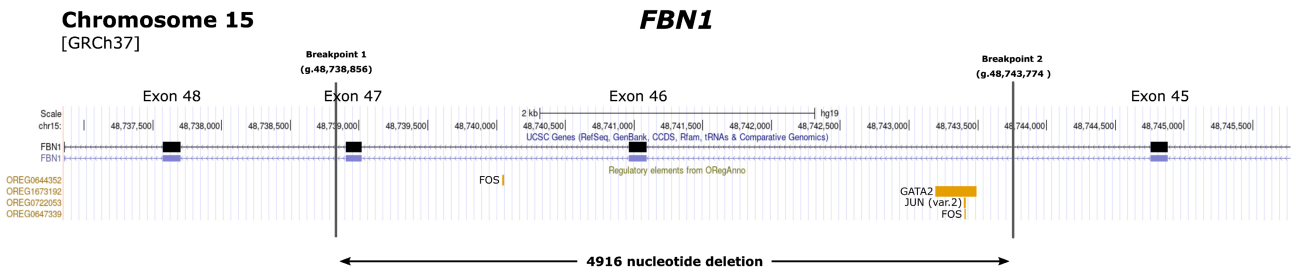

FIGURE 2 Localizations of TFBSs within the deleted region of *FBN1* gene as found in our patient. Data illustrated in GRCh37 reference genome. Black rectangles represent exons of the *FBN1* gene, amber markings represent regulatory elements.

In our patient cohort a large CNV encompassing exon 46 and 47 was identified in the *FBN1* gene with the aid of MLPA assay. Two out of 41 patients (4.8%) with diagnosed or suspected MFS but with no casual mutations in *FBN1*, *TGFBR1*, and *TGFBR2* genes had large *FBN1* deletions. Molecular genetic testing of our primary patient and her parents revealed the de novo origin of the deletion. The detection rate of CNVs in our patient cohort is similar to previously published data, therefore MLPA is capable to detect large CNVs in a cost-effective manner in MFS patients with negative genetic result.

In MFS the most serious clinical manifestation is thoracic aortic aneurysm (TAA) and dissection (TAAD). The highly dynamic aortic wall is subject to strong hemodynamic changes. With the help of refined biomechanical functions, it is able to give appropriate response to these stimuli. Dysregulation and destruction of the cellular and extracellular components of the aortic wall result in dilation, dissection, and potential rupture of the aorta (Shen & LeMaire, 2017). Fibrillin-1 containing microfibrils in association with essential elastin contribute significantly to the stability and elasticity of the aorta (Sakai et al., 1986). Adult patients with MFS can develop various cardiovascular manifestations comprising dilation of aortic root, proximal ascending aorta and pulmonary artery, calcification of mitral and aortic valves, dilated cardiomyopathy and arrhythmia with dissection or rupture of thoracic aortic aneurysm, which is the leading cause of sudden death in the natural history of MFS (Judge & Dietz, 2005; Keane & Pyeritz, 2008). In children cardiovascular manifestations, specifically severe mitral valve prolapse, valvular regurgitation and aortic root dilation with congestive heart failure are less frequent (Morse et al., 1990; Ramachandra et al., 2015). Fibrillin-1 encoded by *FBN1* gene is a major component of microfibrils in the extracellular matrix of elastic and non-elastic tissues (Sakai et al., 1986). This multi-domain glycoprotein consists of 47 EGF (epidermal growth factor)-like domains and 9 TB (TGF-ß1-binding protein) domains, two of the latter domains (namely TB1 and TB4) are part of the two hybrid domains. Most EGF-like domains contain also a calcium-binding sequence (cb-EGF[calcium-binding EGF domains]) (Corson et al., 1993). Calcium binding to fibrillin-1 has a crucial role in the structure and function of the protein. It stabilizes the microfibril architecture (Handford, 2000; Jensen & Handford, 2016; Werner et al., 2000), provides protection of the protein against proteolysis (Reinhardt et al., 1997) and controls interactions with various extracellular matrix components (Rock et al., 2004; Tiedemann et al., 2001). *FBN1* mutations generally disrupt microfibril formation, thereby result in the degeneration of microfibril architecture and loss of extracellular matrix integrity and weaken the connective tissue, which in turn leads to a final instability of the aortic wall.

Several genotype–phenotype studies were published so far to reveal association between various *FBN1* mutations and clinical features as well as disease severity. Faivre et al (Faivre et al., 2007) found that premature termination codon mutations (PTC) are associated with more severe skin and skeletal phenotype as compared to in-frame mutations. PTC mutations, such as frame-shifts, stop codons, and out-of-frame splice mutations lead to no or a truncated form of fibrillin-1. In-frame mutations comprise missense mutations, in-frame deletions, duplications and splice site mutations. In addition, in another study MFS patients with mutations leading to in-frame exon skipping were reported to have a severe phenotype (Jensen & Handford, 2016; Liu et al., 1996). Moreover, an association was found between the type of *FBN1* mutations (haploinsufficient vs dominant negative) and the aortic phenotype severity in a number of studies. Patients with haploinsufficient-type *FBN1* variants, such as nonsense and out-of-frame variants that presumably result in nonsense-mediated mRNA decay, have developed more severe aortic phenotypes than those with dominant-negative-type mutations, such as missense and in-frame variants that are expected to exert loss-of-function effects (Baudhuin et al., 2015;Franken et al., 2017; Takeda, Inuzuka, et al., 2018). In addition, in a retrospective study Takeda et al. revealed that a group of dominant-negative mutation patients had a 6.3-fold higher risk for aortic events than other dominant-negative mutation patients.

This specific group had mutations affecting or creating cysteine residues and in-frame deletion variants in the cb-EGF domains of exons 25–36 and 43–49 (Takeda, Hara, et al., 2018; Takeda, Inuzuka, et al., 2018). These studies do not focus on CNVs, probably because they comprise only a small portion of *FBN1* mutations. Copy number variations of *FBN1* gene including single or multiple exon deletions can generate in-frame (IF) or out-of-frame (OF) variations as well. These types of mutations are associated with classic and neonatal form of MFS regardless of their reading frame alterations. (Tables 1–3).

There are 50 various CNVs beyond full *FBN1* gene deletion detected to date (Tables 1 and 2). Detailed clinical evaluation of these patients revealed severe cardiovascular manifestations (dilation and/or dissection of the thoracic aorta) in the majority (36 of 54) of the patients. The rare feature of our primary patient is the observed mild form of the cardiovascular symptom (i.e., mitral valve prolapse). In her 1-year old infant, bearing the same CNV, no abnormality was detected in his cardiovascular system. Therefore, in addition to the CNV in *FBN1*, other factors are also thought to play a role in the development of severe cardiovascular manifestations. These factors per se or superimposed to the *FBN1* mutation result in the severe cardiovascular phenotype. In 15 patients no clinical data or no clear clinical information was available. In two patients published in the literature the deletion was not associated with any cardiovascular symptoms. However, these patients differ from our ones, since they carry single exon deletion (exon 6 and exon 18), whereas a two-exon deletion (exon 46–47) can be found in our patients. Interestingly, contrarily to previous studies (Franken et al., 2017; Takeda, Inuzuka, et al., 2018) in the CNV patient cohort the severity of the cardiovascular manifestations did not seem to depend on the mutation being of the IF (dominant-negative type) or OF (haploinsufficient-type) *FBN1* variants. Moreover, we found that large CNVs were associated with more severe and more frequent cardiovascular manifestations than intragenic *FBN1* gene mutations.

Accumulating evidence suggest that beside the structural weakness of connective tissue, other factors contribute to the complicated pathogenesis of TAA and TAAD in MFS (Shen & LeMaire, 2017). Recently it became clear that microfibrils have an important role in regulating the bioavailability of the transforming growth factor beta (TGF-β). Since fibrillin-1 binds to latent TGF-β-binding protein (LTBP) and sequesters TGF-β in the ECM (extracellular matrix), thus inhibiting TGF-β signaling (Dallas et al., 1995; Isogai et al., 2003). Mutations in *FBN1* affects the matrix sequestration of the latent TGF-β complex, resulting in the uncontrolled release of TGF-β and leading to the overactivation of TGF-β signaling (Habashi et al., 2006; Neptune et al., 2003). In MFS mice the lack of LTBP have reduced aortic destruction and improved survival, suggesting the contribution of TGF-β signaling to aortic disease progression (Zilberberg et al., 2015). Among CNV patients carrying single exon deletion or few exon deletions published so far, the deletion resulted in the removal of TB-binding domains in nine patients (exon 43, exon 18–22, exon 23–25, exon 25–27 exon 33–38, exon 34–39, exon 39–40, exon 43–44 or exon 48–53, respectively). Moreover, in all these patients the deletion generates an in-frame mutation leading to a defective fibrillin-1 protein that results in the degeneration of microfibril architecture and loss of extracellular matrix integrity. In our hypothesis the deletion of TB domains (namely TB3-4, TB4-5, TB5, TB6, TB7, and TB8) in these patients causes the release of active TGF-β into ECM in the aortic wall which in turn overactivates the canonical TGF-β signaling pathway. This effect then may superimpose to the microfibril degeneration and finally together lead to severe cardiovascular manifestations (i.e., aortic dilation and aortic dissection) in these patients. Seven patients displayed dilated aorta and two patients, a neonate and a 24-year-old patient, suffered from aortic dissection, too. It is a well-known phenomenon that cardiovascular manifestations are age dependent (Shen & LeMaire, 2017). Therefore, it is supposed that five of the seven patients did not develop aortic dissection along with their aortic dilation because of their young age.

Involvement of gene regulatory elements in the pathogenesis of common and complex diseases are well known. However, genetic variations affecting gene regulation can also lead to the development of Mendelian diseases. Structural variants such as large deletions or chromosomal translocations belong to this group that uncouple regulatory elements from their target genes (Vockley et al., 2017). Recently it was established that non-coding genetic variants have an effect on gene regulation, moreover, this effect is exerted in a tissue-specific manner (Maurano et al., 2012; Vockley et al., 2017). Gomez et al. investigated the epigenetic control of vascular smooth muscle cells in Marfan and non-Marfan thoracic aortic aneurysms. They demonstrated that epigenetic mechanisms play a role in the development of the pathological phenotype of the vascular SMCs in human TAA regardless of etiology (Gomez et al., 2011). This supports that in MFS, besides *FBN1* mutations, cell type-specific epigenetic predisposition may also be involved in the development of TAA.

In our in silico analysis of *FBN1* gene based on Chip-seq data from various databases (JASPAR, PAZAR, UCSC, and Ensembl) revealed several tissue-specific enhancer regions in the intronic regions of this gene. A number of potential

transcription factor-binding sites can be found within the deleted region in CNV patients carrying single or a few exon deletions. However, after comprehensive evaluation, many have been shown to play no role in the development of cardiovascular symptoms (Table 6). Our data analyses on previously published CNVs demonstrated the presence of potential transcription-binding site for STAT3 in five out of 25 patients. In STAT3 deficient adult patient, Chandesris et al found that aneurysm formation was one of the most frequent vascular abnormalities. They supposed that the observed vascular abnormalities are the consequence of a systemic connective tissue disorder that includes arterial fragility (Chandesris et al., 2012). Moreover, they observed a greater susceptibility to vascular aneurysm after inhibition of STAT3-dependent signaling in mouse models. We suppose that in CNV patients carrying a deletion involving STAT3-binding site, the deletion itself has an effect on STAT3 signaling pathways that may superimpose to the *FBN1* gene defect and together they lead to a severe cardiovascular manifestation in these patients.

Interestingly in our patient cohort there were a number of patients who presented severe cardiovascular manifestations along with other features characteristic for MFS, however, no mutation in the *FBN1*, *TGFBR1*, or *TGFBR2* genes was identified in these patients. The limitation of our study is that the disease-causing defect may be a deep intronic alteration, however, the applied methods, such as Sanger sequencing and MLPA are not capable to detect this kind of genetic defect.

5 | CONCLUSION

Cardiovascular manifestation is a known phenomenon among Marfan patients. Several phenotype–genotype studies have been performed to find association between cardiovascular phenotype and *FBN1* gene mutation, however, these studies focused on intragenic small-scale mutations only. In this study, we investigated the effect of various CNVs in the *FBN1* gene on the cardiovascular symptoms and found that large CNVs are often associated with severe cardiovascular manifestations in MFS. Moreover, we investigated the effect of factors on CV symptoms beyond the *FBN1* gene mutation and we found that the abolishment of regulatory elements by a deletion (like lack of transcription-binding site for STAT3) may lead to more severe manifestations and seem to play a role in the development of cardiovascular phenotype in this monogenic disorder. However, additional research is required to elucidate the role of these regulatory elements in the development of severe cardiovascular manifestations that may serve as novel therapeutic targets in the management of MFS.

AUTHOR CONTRIBUTIONS

These authors contributed equally: Tibor Rauch and Judit Bene. Tibor Rauch and Judit Bene conceived and designed research; Gergely Buki and Renata Szalai performed the genetic investigations, Adrienn Pinter and Kinga Hadzsiev performed patient examinations, Gergely Buki prepared figures; Gergely Buki and Judit Bene drafted manuscript; Bela Melegh, Kinga Hadzsiev and Judit Bene revised manuscript; Tibor Rauch and Judit Bene approved final version of manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

On behalf of all authors, the corresponding author states that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was approved by the Ethics Committee of the University of Pecs (Protocol 8581–7/2017/EUIG). Written informed consent was obtained from all patients or their legal guardians. All experiments were performed in accordance with the Helsinki Declaration of 1975 and with the Hungarian legal requirements of genetic examination, research and biobanking.

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