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Original Article

Pathogenesis of aortic wall complications in Marfan syndrome

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ABSTRACT

Background: Patients with Marfan (MFS) syndrome and patients with a bicuspid aortic valve (BAV) are more prone to develop aortic dilation and dissection compared to persons with a tricuspid aortic valve (TAV). To elucidate potential common as well as distinct pathways of clinical relevance, we compared the histopathological substrates of aortic pathology.
Patient and Methods: Ascending aortic wall specimen were divided in five groups: BAV (n=36) and TAV (n=23) without and with dilation and non-dilated MFS (n=8). We performed routine histology to study aortic wall features based on the aortic consensus statement. Immunohistological markers for vascular smooth muscle cell (VSMC) maturation, and expression of fibrillin-1 were additionally investigated for the underlying pathogenesis.
Results: On basis of the routine histology the aorta in MFS was similar to the aorta in dilated TAVs (overall medial degeneration, elastic fiber fragmentation, loss and disorganization, , and VSMC nuclei loss). The other markers aided in clustering the MFS and BAV patients with a significantly lower fibrillin-1 expression as compared to the TAVs ($p<0.05$), a lower level of differentiated VSMC markers ($p<0.05$) and elastic fiber thinning.
Conclusions: Pathogenesis of aortopathy in MFS overlaps with mechanisms seen in BAV and TAV, leading to a so called double hit hypothesis for aortic complications in MFS. The ascending aortic wall in MFS is immature with undifferentiated VSMCs and low levels of fibrillin-1. The immature media becomes even more vulnerable for aortopathy due to other degenerative features which develop probably as a direct consequence of the *fibrillin-1* mutation.

1. Introduction

Marfan syndrome (MFS) is an autosomal dominant connective tissue disorder with multiple organ manifestations. The genetic cause of this syndrome is a mutation in the *fibrillin*-1 (*FBN1*) gene, encoding for the extracellular matrix protein fibrillin-1 [1]. Fibrillins are large glycoproteins that form complex extracellular structures called microfibrils [2]. These molecules provide elasticity and structural support to tissues modulating elastic fiber biogenesis and homeostatis, and regulating the bioavailability and activity of different growth factors like transforming growth factor beta [3]. Mutations in the *FBN1* gene lead to impaired fibrillin-1 protein synthesis, secretion and/ or incorporation in extracellular matrix (ECM) [4–6], which determines the degeneration of the elastic microfibrillar architecture [7], loss of tissue homeostasis and subsequent destruction of the ECM integrity [8,9]. In this disorder an array of cardiac, skeletal and ocular symptoms are seen, but the prognosis in MFS is dominated by cardiovascular life threatening complications of the aorta. The wall of the ascending aorta consists of three basic layers: the internal layer, (tunica intima); the middle layer, (tunica media), and the outer layer, (tunica adventitia). Vascular smooth muscle cells (VSMCs) are the major cell type in the aorta and their main function is to regulate blood flow and pressure through vessel wall contraction and relaxation [10]. VSMCs have the ability to undergo a phenotypic switch, from a quiescent contractile state to an immature, proliferative synthetic state. Disability of this phenotypic switch has been shown to play a critical role in a variety of cardiovascular diseases [10,11]. We have recently shown that the VSMC in the ascending aortic wall in MFS patients are less differentiated leading to an immature vessel wall [12]. The question however remains whether the immaturity of the ascending aorta is sufficient to explain the characteristic histopathologic degenerative features of the aorta seen in MFS as cytolytic necrosis, (also termed medial degeneration), defined as VSMC dropout, apoptosis of VSMCs and elastic fiber degeneration [7]. It is highly suggestive that an additional pathologic mechanism is responsible for the observed histopathologic features in MFS besides the immaturity of the aortic wall.

Bicuspid aortic valve (BAV), the most common congenital cardiac malformation [13], is another condition which, like MFS, is associated with an increased risk for aortic dilation and dissection [14,15]. Although both patients with MFS and BAV show aortic dilation, the

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anatomic site of vulnerability is distinct in both conditions. While maximal aortic dilation is observed above the sinotubular junction in BAV, in the MFS population it is mainly found at the level of the sinuses of Valsalva, also referred to as aortic root [16]. In bicuspidy all patients have an immature ascending aortic wall too, characterized by less differentiated VSMCs [17]. However, as 'only' 60-80% of the BAV patients exhibit aortic pathology, immaturity of the aortic wall per se was also not sufficient to explain the aortic complications in BAV. Recently, a pathway of activated pc-Kit was described which distinguishes BAV patients with an increased susceptibility for future aortic wall complications [18].

The aim of our current study is to understand the pathogenesis of aortic wall complications in MFS which seems to overlap with the immaturity seen in bicuspidy and degenerative features seen in pathogenesis of the aortic wall in patients with tricuspid aortic valve (TAV) [17].

To study this we compared the aortic wall between MFS, BAV and TAV, starting with the investigation of the differentiation of VSMCs in the ascending aortic wall. Smooth muscle 22 alpha (SM22α) and smoothelin were used as markers of fully differentiated contractile VSMCs [10,17] and alpha smooth muscle actin (αSMA) and Lamin A/C were used as a marker for differentiation of VSMCs and myoblasts respectively [17,19,20]. Progerin, a splice variant of lamin A/C, and a marker of cardiovascular aging was studied to further elucidate differences in the pathogenesis of aortopathy between the patient groups [17,21–23]. We also studied and compared general histopathological features,VSMC apoptosis and the expression of fibrillin-1 protein in MFS, BAV and TAV.

2. Material and Methods

2.1. Ethical approval

Approval of this study was obtained from the institutional ethics committee at the Leiden University Medical Centre (LUMC), Leiden. Six non-dilated BAV aortic wall specimen were provided by the Heart Valve Bank, Thoraxcenter, Erasmus Medical Center (EMC), Rotterdam, these specimen were not suitable for transplantation. Inclusion of this specimen in our study was approved by their Scientific Advisory Board. Eight MFS aortic wall specimen were provided by the Academic Medical Center (AMC), Amsterdam also with approval of the Medical Ethical Committee. Written informed consent was obtained.

2.2. Patients and tissue samples

Ascending aortic wall specimens were obtained from the aortotomy site in patients with MFS with a TAV and non-MFS individuals with TAV or BAV. The specimens were divided into five groups: MFS patients (MFS), TAV patients non- and dilated (TA and TAD respectively), BAV patients non- and dilated (BA and BAD respectively). Aortic dilation was clinically defined by surpassing an ascending aortic wall diameter of 45 mm [24]. MFS n=8, mean age 34.1 ± 11.8 years, 62.5% males; TA n=11, mean age 64.5 ± 9.0 years, 54.5% males; TAD n=12, mean age 72.3 ± 11.2, 33.3% males, BA n=17, mean age 55.8 ± 9.8 years, 70.1% males; BAD n=19, mean age 60.7 ± 7.8 years, 84.2% males. The non-dilated BAV specimen were obtained from the non-suitable transplantation hearts and during surgery from the aortotomy site when the preferred stentless aortic root replacement was performed [17]. Information on aortic dimensions and aortic valve pathology is listed in Table 1.

2.3. Sample processing and routine histology

The sectioning and staining protocols have been described previously [17,18]. In short, after excision of the specimen in the operating room all specimen were fixed in formalin, decalcified, embedded in paraffin and subsequently sectioned (5 μm). To study the morphology of the vessel wall the sections were stained with hematoxylin-eosin (HE) and resorcin fuchsin (RF) and Movat pentachrome staining. To describe the aortic wall in a standardized way we used terms from the grading system described in the recently published aortic consensus paper statement on surgical pathology of the aorta [25]. Terms which we used are: overall medial degeneration (EMD), elastic fiber fragmentation and loss (EFF/L), elastic fiber thinning (EFT), elastic fiber disorganization (EFD), mucoid extra cellular matrix accumulation (MEMA) and smooth muscle cell nuclei loss (SMCNL). In HE stained sections the aortic adventitial inflammation was further quantified, indexed from zero (no inflammatory cells) to 6 (large clusters of cells). In RF stained sections the maximum intimal thickness was quantified in μm.

Table 1
Clinical characteristics of all patients

Characteristics	TA	TAD	BA	BAD	MFS
Ascending aorta diameter (mean)	*	55.0 ± 10.7	36.5 ± 7.4†	52.7 ± 6.2	28.4 ± 12.8
Aortic root diameter (mean)	‡	‡	‡	‡	48.1 ± 3.0
Aortic valve pathology					
- No valve pathology	N=11	N=6	N=6	N=3	N=7
- Aortic stenosis	N=0	N=1	N=4	N=8	N=0
- Aortic regurgitation	N=0	N=5	N=1	N=5	N=1
- Aortic stenosis and regurgitation	N=0	N=0	N=5	N=3	N=0

* data unavailable, clinically defined as non-dilated by pathologist. † data unavailable for 5 patients, clinically defined as non-dilated by pathologist. ‡ aortic root diameters unavailable.

2.4. Immunohistochemistry

The staining protocols which we used in this study have previously been described [12,17,18]. The primary antibodies were used against αSMA 1/5000 (A2547, Sigma-Aldrich Chemie), cleaved caspase-3 1/250 (9661, Cell Signaling), SM22α 1/100 (AB10135, Abcam), smoothelin 1/200 (16101, Progen Biotechnik), lamin A/C 1/100 (MAB3211, Millipore), progerin 1/50 (SC-81611, Bio-Connect), fibrillin-1 1/100 (MAB1919, Millipore). The secondary antibodies used were peroxidase-conjugated rabbit antimouse 1/250 (DAKO p0260) for alpha smooth muscle actin (αSMA), goat anti-rabbit biotin 1/200 (Vector Laboratories, USA, BA-1000) and goat serum 1/66 (Vector Laboratories, USA, S1000) for cleaved caspase-3, smooth muscle 22 alpha (SM22α) and progerin and horse anti-mouse biotin 1/200 (Santa Cruz Biotechnology, Inc., CA, USA, SC-9996-FITC) in horse serum 1/66 (Brunschwig Chemie, Switzerland, S-2000) for smoothelin, lamin A/C and fibrillin-1.

2.5. Histologic parameters, immunohistochemical analyses and morphometry

Sections were studied with a Leica BM500 microscope equipped with plan achromatic objectives (Leica Microsystems, Wetzlar, Germany). EMD, EFF/L, EFT, EFD, MEMA and SMCNL were graded semi-quantitatively in HE, αSMA,RF and MOVAT stained sections in the aortic media. All features were indexed from 0 (none), 2 (mild), 4 (moderate) to 6 (severe) on three predetermined locations (left, middle and right) of every section, that we refer to as 'microscopic fields' maintained in evaluation of all stainings on sister sections.

The cytoplasmatic level of expression of αSMA, SM22α and smoothelin, intra- and extracellular expression of fibrillin-1, nuclear expression of lamin A/C, progerin and cleaved-caspase-3, were analyzed in an identical way. In each microscopic field the level of expression was indexed for αSMA, SM22α, smoothelin and fibrillin-1 . We only graded the aortic media for the VSMC markers and fibrillin as 0 (no expression in the media layer), 2 (expression in less than one third of the medial layer), 4 (expression in two thirds of the medial layer) and 6 (expression in the whole medial layer). To determine the

level of lamin A/C, progerin and caspase-3 expression, the number of positively stained nuclei was counted and analysed using ImageJ in the three fields for each stained section. A threshold was applied to filter background noise. The total number of cells (positively and negatively stained nuclei and cytoplasm) was not different between specimens. Therefore, in each microscopic field the number of lamin A/C, progerin and caspase-3 positive cells was normalized to the total number of cells per $10^5 \mu m^2$. Finally, the number of normalized positive cells for each staining was averaged between the three microscopic fields. All specimens were re-evaluated by an independent, experienced histopathologist who was blinded to the clinical data.

2.6. Statistical analyses

All numerical data are presented as mean ±SD of three microscopic fields on each stained slide. For comparison between the groups statistical differences were evaluated with the Mann-Whitney U-test. One, two and three way ANCOVA tests were performed to correct for age and gender. Significance was assumed when $p<0.05$ using SPSS 20.0 software program (SPSS Inc. Chicago, USA). Graphpad software was used to create graphics of statistical analysis.

3. Results

3.1. Patient characteristics

Statistically MFS patients were the youngest patients in the study population, followed by the BAV patients. Male and females were equally affected in the MFS group, however the BAVs showed a marked male predominance in both the non- and dilated groups. Despite the noticeable variance in age and gender distribution, statistically both age and gender were not found confounding in the study.

Typical root dilation was seen in all MFS patients, (diameter 48.1 ± 3.0 mm), with a non-dilated ascending aorta (diameter 28.4 ± 12.8 mm). All other groups (TA, BA, TAD, BAD) did not show marked root dilation.

3.2. Histologic features of the ascending aorta

The normal ascending aortic wall, defined as a non-dilated aorta in individuals with a tricuspid aortic valve (TA), consists of three layers (Fig. 1A). The inner most layer, the tunica intima, contains a single layer of endothelial cells (Fig. 1B, arrows) and a subendothelial layer of loosely organized elastic fibers and vascular smooth muscle cells (VSMCs) (Fig. 1B, arrow heads); the middle layer, tunica media, mainly contains VSMCs (Fig. 1C), elastic fibers (Fig. 1D) and collagen and the outer layer, tunica adventitia, predominantly consists of loose fibrous tissue containing nerve fibers, fibroblasts, adipocytes and vasa vasorum lined by endothelium and VSMCs (Fig. 1E).

3.3. Aortic VSMCs in Marfan syndrome

In MFS specimen the level of expression of fibrillin-1 was significantly lower as compared to all TAV specimen (TA, TAD) ($p<0.05$) (Fig. 2A,B). In BAV specimen (BA, BAD) the expression of fibrillin-1 was also significantly lower as compared to the TAV (TA, TAD) ($p<0.05$) (Fig. 2C). In BAV and MFS the distribution and localization of fibrillin-1 was intracellular, whereas is was mostly seen extracellular in the TAVs .

In MFS specimen the level of expression of differentiated VSMC markers was significantly lower as compared to the TAV (TA, TAD): αSMA ($p<0.05$) (Fig. 2 D-F), SM22α ($p<0.01$) (Fig. 2G-I) and smoothelin ($p<0.001$) (Fig. 2J-L). In BAV (BA, BAD) the expression of the above mentioned markers was also significantly lower as compared to the TAVs (TA, TAD) (Fig. 2C, F, I). The expression of Lamin A/C and the cardiovascular aging marker progerin was also significantly lower in MFS ($p<0.05$ and $p<0.05$ respectively) (Fig. 3A, B) and BAV (BA, BAD) ($p<0.01$ and $p<0.05$ respectively) as compared to the TAVs (TA, TAD) (Fig. 3A, B).

The apoptosis marker cleaved-caspase-3 in the VSMCs was also significantly higher expressed in the MFS and dilated TAV (TAD) group as compared to the dilated BAV (BAD) group ($p=0.033$ and $p=0.0286$ respectively).

3.4. General histopathologic features in the MFS aorta

The total aortic wall thickness (excluding the highly variable adventitia thickness) was not found significantly different between the studied MFS, TAV and BAV groups. However the intima was significantly thinner in the MFS specimen as compared to the TAVs (TA, TAD) ($p<0.001$) (Fig. 4A, B). The phenomenon of a thinner aortic tunica intima has earlier been described in BAV patients [17].

The histopathologic features, graded in the aortic media comparing MFS, TAV and BAV, are summarized in Table 2. The graded pathologic features EMD (Fig. 4B), EFF/L (Fig. 4B,H), EFD (Fig. 4H) and SMCNL

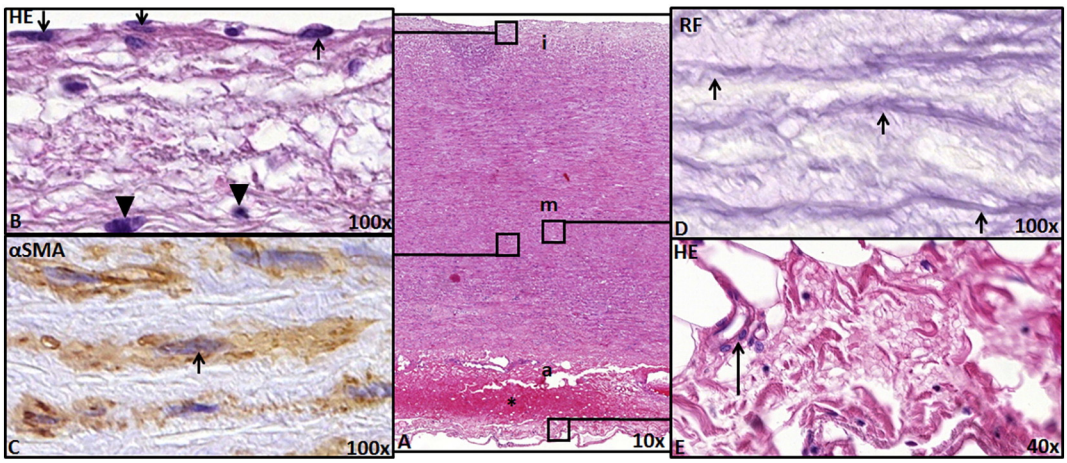

Fig. 1. The normal ascending aortic wall Transverse histologic sections (5μm) stained with Hematoxylin Eosin (HE), alpha Smooth Muscle Actin (αSMA) and Resorcin Fuchsin (RF), and of a non-dilated ascending aortic specimen in a tricuspid aortic valve patient (TA group). HE stained overview section (A) shows the tunica intima (i), tunica media (m) and tunica adventitia (a), with some hemorrhage (asterisk) red blood cells seen in the adventitial layer. 1B: The intima, is lined by a single layer of endothelial cells (arrows) and a subendothelial layer of connective tissue and vascular smooth muscle cells (VSMCs) (arrow heads) which is shown in this HE stained section. 1C: The aortic media shows predominantly VSMCs, shown in the αSMA stained section with a nucleus indicated with an arrow. 1D: Elastic lamellae (arrows), are shown in the RF stained section. 1E: The adventitia, shown in the HE stained section, predominantly consists of loose fibrous tissue containing nerve fibers, fibroblasts, adipocytes and vasa vasorum lined by endothelium and VSMCs (arrow). . Magnification: A 10x; B-D 100x, E 40x Abbreviations: HE: Hematoxylin Eosin, αSMA: alpha smooth muscle actin, RF: Resorcin Fuchsin

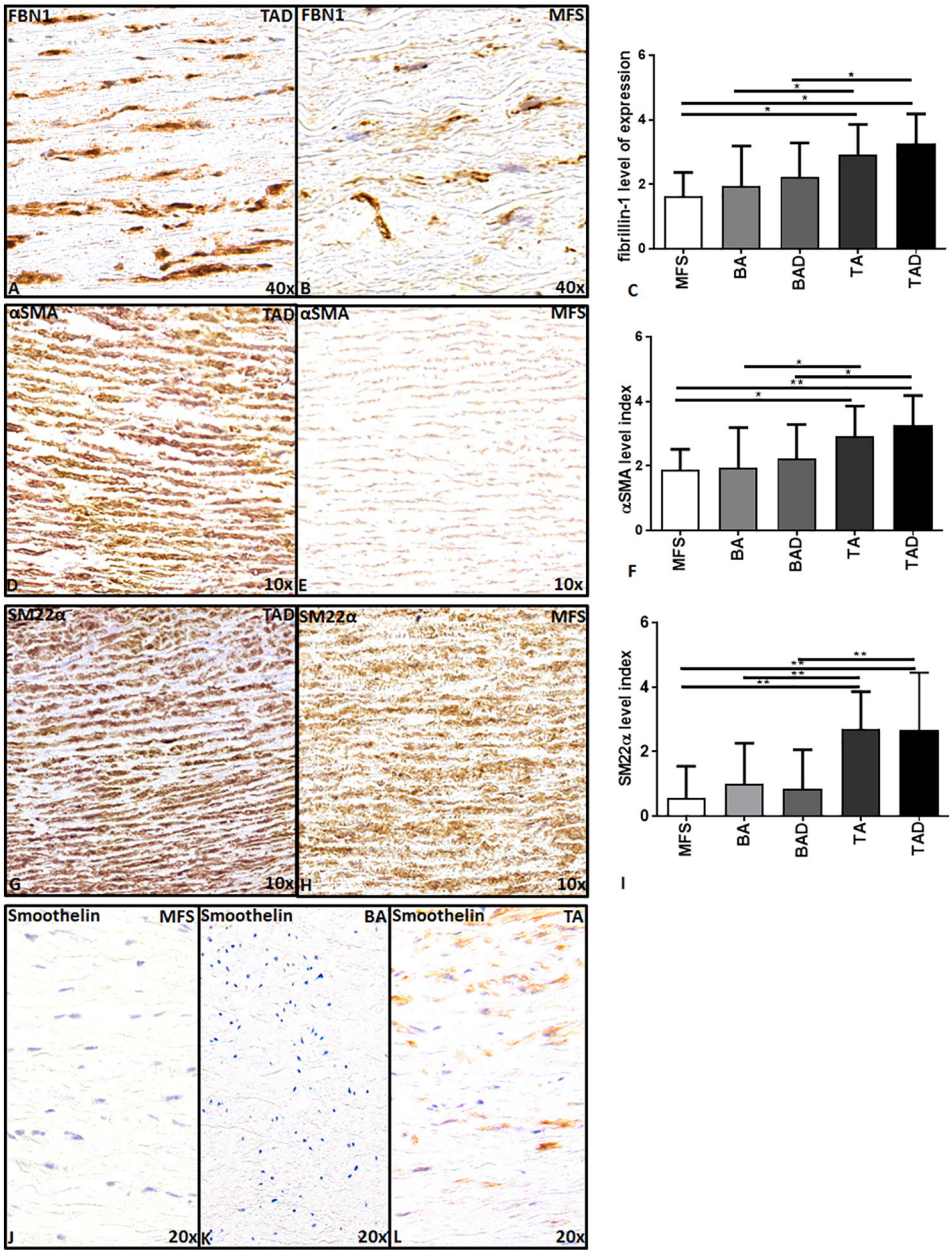

Fig. 2. Aortic VSMCs in Marfan syndrome Transverse histologic sections (5μm) stained with fibrillin-1 (FBN1) (A, B). 2A: Expression of FBN1 is shown in the TAV with dilation (TAD) and 2B: Marfan syndrome without dilation (MFS). Graph C: The level of expression was significantly lower in all patients with MFS, BA and BAD as compared to TA and TAD. Transverse histologic sections (5μm) stained with αSMA (D,E). 2D: Expression of αSMA is shown in the aortic media of a patient with a TAV with dilation (TAD) and 2E: Marfan syndrome without dilation (MFS). Graph F: The level of expression was significantly lower in all patients with MFS, BA and BAD as compared to TA and TAD. Transverse histologic sections (5μm) stained with SM22α (G,H). 2G: Expression of SM22α is shown in the aortic media of a patient with a TAV with dilation (TAD) and 2H: Marfan syndrome without dilation (MFS). Graph I: The level of expression was significantly lower in all patients with MFS, BA and BAD as compared to TA and TAD.. Fig. 2J-L show a transverse histologic sections (5μm) stained with smoothelin in the aortic media of patients with MFS(2J), BAV without dilation (BA)(2K) and TAV without dilation (TA) (2L). Expression was only observed in the TA group. Maginification: A,B: 40x; D,E,G,H: 10x;J-L: 20x * = $p<0.05$ ** = $p<0.01$ Error bar: standard deviation Abbreviations: MFS: Marfan syndrome, BA: non-dilated BAV, BAD: dilated BAV, TA: non-dilated TAV, TAD: dilated TAV, αSMA: alpha smooth muscle actin, SM22α: smooth musclce 22 alpha.

(Fig. 4C-F) were similar between the MFS and dilated TAV groups. These features (EMD, EFF/L, EFD and SMCNL) were significantly less in all non-dilated TAV and BAV patients and dilated BAV patients. EFT (Fig. 4H) was the only feature which was similar between the MFS and BAV patients. The elastic fibers were significantly thicker in all TAV patients. The feature MEMA was significantly highest in the MFS group as compared to all other groups (Table 2).

Adventitial inflammatory cells were most outspoken in the dilated TAV (TAD) as compared to all other groups (MFS $p<0.0001$, BAD $p<0.001$) (Fig. 4G).

4. Discussion

Marfan syndrome is a connective tissue disorder characterized by cardiovascular, skeletal and ocular manifestations. The progressive dilation of the aortic root culminating in dissection and rupture is a major cause of morbidity and mortality in MFS patients. Due to the high prevalence of this syndrome and associated life threatening cardiovascular complications the aim of our study was to further unravel the pathogenesis of aortopathy in MFS by comparing MFS to other common causes of aortic complications namely bicuspidy and cardiovascular aging.

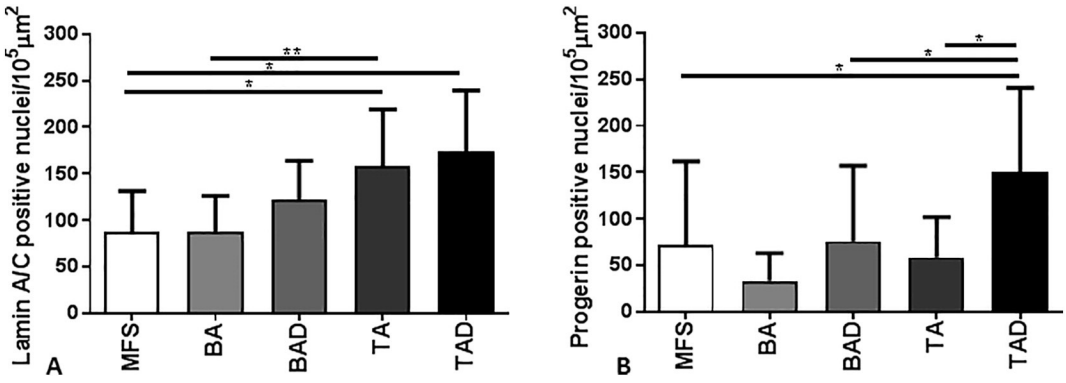

Fig. 3. Expression of Lamin A/C and progerin Graph A and graph B show the expression of lamin A/C and the cardiovascular aging marker progerin marker respectively. Expression of lamin A/C and progerin was similar in MFS and BAV and was significantly lower than in dilated TAV (TAD). * = p<0.05 ** = p<0.01 *** = p<0.001 Error bar: standard deviation Abbreviations: MFS: Marfan syndrome, BA: non-dilated BAV, BAD: dilated BAV, TA: non-dilated TAV, TAD: dilated TAV

We used terms from the grading system described in the recently published Cardiovascular Pathology/ Association for European Cardiovascular Pathology consensus paper statement on surgical pathology of the aorta to describe the aortic wall in a standardized way [25]. Recently, Waters et al [26] performed a study on these histopathologic features in syndromic and non-syndromic cases with aortopathy. Based on the abovementioned consensus statement they graded the aortic specimen and compared that between the MFS, BAV, TAV and patients with Loeys Dietz syndrome. In part their findings support our data, while other observations were in contrast. In our study we found the histopathologic features, enhanced overall medial degeneration, elastic fiber fragmentation and loss, , elastic fiber disorganization and smooth muscle cell nuclei loss both in MFS and TAV patients, comparable to Waters et al. We also found that extra cellular matrix accumulation was highest in the MFS group.

We could however not differentiate the pathogenesis underlying these features in both patient groups on bases of the routine histopathological stainings as it did not explain our data on VSMC differentiation defects of the aortic wall in BAV and TAV [17,27]. Therefore, we performed additional immunohistochemical stainings to achieve more insight into the mechanism leading to the aortopathy in MFS and TAV. In TAVs we found an increased expression of the cardiovascular aging protein progerin [17] and the apoptosis marker cleaved caspase-3. Progerin expression has been reported to correlate with increased apoptosis [17] In MFS however the expression of progerin was significantly lower, as compared to TAV. We postulate that the observed

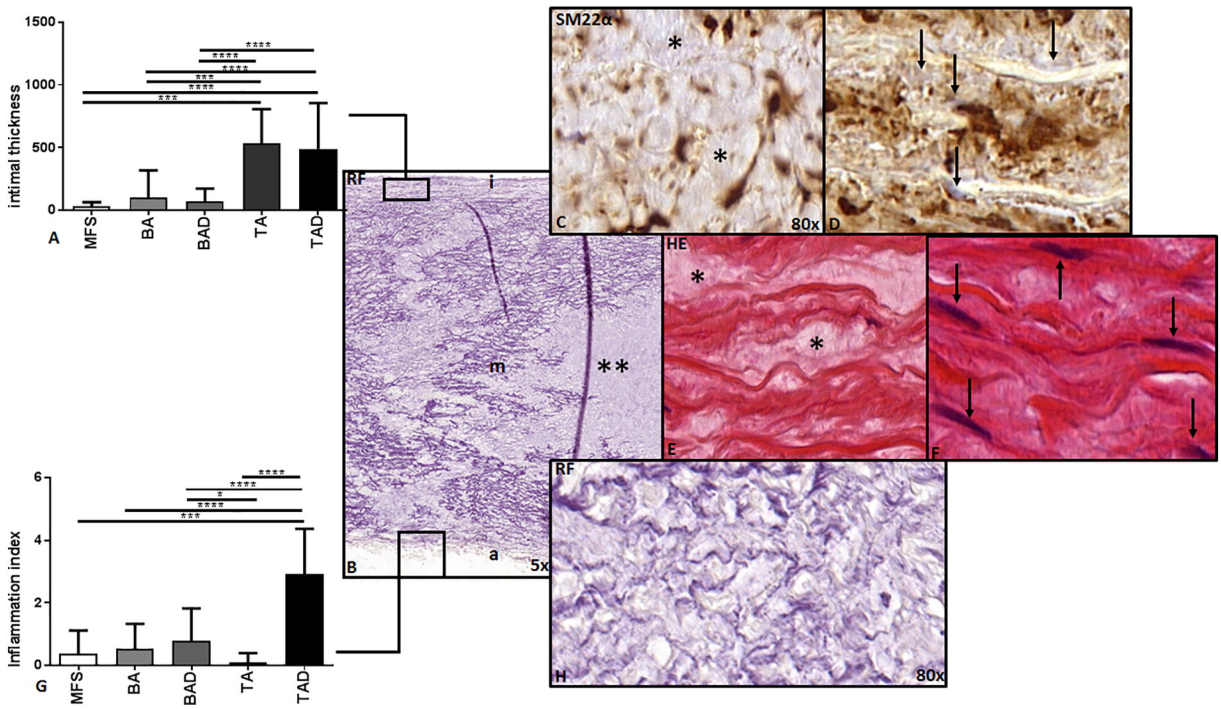

Fig. 4. General histopathologic features in the Marfan syndrome aorta Transverse histologic sections (5µm) stained with Resorcin Fuchsin (RF), Hematoxylin Eosin (HE) and alpha Smooth Muscle Actin (αSMA) in the ascending aortic wall in Marfan syndrome (MFS). Graph A: The intimal layer was significantly thinner in the MFS specimen as compared to the non- and dilated TAVs (TA, TAD respectively). BAV intima was also significantly thinner as compared to the TAVs. 4B: The aortic media in MFS showed significant pathology with overall medial degeneration and elastic fiber loss (asterisks). 4C shows smooth muscle cell nuclei loss in the aortic media and loss of αSMA indicated with an asterisk, 4D compares a section without smooth muscle cell nuclei and αSMA loss, nuclei indicated with an arrow. 4E shows smooth muscle cell nuclei loss in an HE stained section indicated with an asterisk, 4F compares a HE stained section without smooth muscle cell nuclei loss, nuclei indicated with an arrow. 4H: shows elastic fiber fragmentation (H), elastic fiber disorganization and elastic fiber thinning with enlarged inter-lamellar distance. Adventitial inflammatory cells were most outspoken in the dilated TAV (TAD) as compared to all other groups (MFS p<0.0001, BAD p<0.001) (Fig. 4E). Magnification: B5x, C-E: 80x * = p<0.05 *** = p<0.001 **** = p<0.0001 Error bar: standard deviation Abbreviations: MFS: Marfan syndrome, BA: non-dilated BAV, BAD: dilated BAV, TA: non-dilated TAV, TAD: dilated TAV, αSMA: alpha smooth muscle actin, HE: Hematoxylin Eosin, RF: Resorcin Fuchsin, m: media

Table 2
histopathologic grading of medial degeneration (EMD), elastic fiber fragmentation and loss (EFF/L), elastic fiber thinning (EFT), elastic fiber degeneration (EFD), extra cellular matrix accumulation (MEMA), smooth muscle cell nuclear loss (SMCNL)

Feature	Score	MFS N (%)	TA N (%)	TAD N (%)	BA N (%)	BAD N (%)	P-value
EMD	0 (none)	0 (0)	8 (73)	0 (0)	14 (82)	16 (84)	MFS vs TA, MFS vs BA, MFS vs BAD, TA vs TAD, BAD vs TAD p<0.0001
	2 (mild)	0 (0)	3 (27)	3 (25)	3 (18)	2 (11)	
	4 (moderate)	6 (75)	0 (0)	4 (33)	0 (0)	1 (5)	
	6 (severe)	2 (25)	0 (0)	5 (42)	0 (0)	0 (0)	
EFF/L	0 (none)	0 (0)	8 (73)	0 (0)	14 (82)	16 (84)	MFS vs TA, MFS vs BA, MFS vs BAD, TA vs TAD, BAD vs TAD p<0.0001
	2 (mild)	0 (0)	3 (27)	3 (25)	3 (18)	2 (11)	
	4 (moderate)	6 (75)	0 (0)	4 (33)	0 (0)	1 (5)	
	6 (severe)	2 (25)	0 (0)	5 (42)	0 (0)	0 (0)	
EFT	0 (none)	0 (0)	8 (73)	6 (50)	1 (6)	0 (0)	MFS vs TA p=0.003, MFS vs TAD p=0.0010, TA vs BA p=0.0002, TA vs BAD p<0.0001, TAD vs BA p=0.0015, TAD vs BAD p=0.0001
	2 (mild)	3 (38)	3 (27)	6 (50)	10 (59)	11 (58)	
	4 (moderate)	5 (62)	0 (0)	0 (0)	6 (35)	8 (42)	
	6 (severe)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
EFD	0 (none)	0 (0)	8 (73)	6 (50)	14 (82)	16 (84)	MFS vs BA p<0.0001, MFS vs BAD P<0.0001, MFS vs TA p=0,0023, MFS vs TAD p=0.027, TA vs BA p 0.043, TAD vs BA p=0.0069, TAD vs BAD p=0.0149
	2 (mild)	0 (0)	3 (27)	6 (50)	3 (18)	2 (11)	
	4 (moderate)	7 (88)	0 (0)	0 (0)	0 (0)	1 (5)	
	6 (severe)	1 (12)	0 (0)	0 (0)	0 (0)	0 (0)	
MEMA	0 (none)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	MFS vs TA p=0.0015, MFS vs TAD p=0.0006, MFS vs BA p<0.0001, MFS vs BAD p<0.0001
	2 (mild)	1 (13)	9 (82)	10 (83)	15 (88)	16 (84)	
	4 (moderate)	2 (25)	2 (18)	2 (17)	2 (12)	3 (16)	
	6 (severe)	5 (62)	0 (0)	0 (0)	0 (0)	0 (0)	
SMCNL	0 (none)	0 (0)	9 (82)	1 (8)	14 (82)	16 (84)	MFS vs TA, MFS vs BA, MFS vs BAD, TA vs TAD, TAD vs BA, TAD vs BAD p<0.0001
	2 (mild)	0 (0)	2 (18)	2 (17)	3 (18)	2 (11)	
	4 (moderate)	6 (75)	0 (0)	3 (25)	0 (0)	1 (5)	
	6 (severe)	2 (25)	0 (0)	6 (50)	0 (0)	0 (0)	

apoptosis in MFS which leads to VSMC nuclei loss and overall medial degeneration might be a direct consequence of the *FBN1* mutation in MFS patients. As the *FBN1* mutation leads to an increased Angiotensin II (AT2) receptor signaling and subsequently induction of TGFβ signaling [28–30], the latter leading to increased apoptosis and thus smooth muscle cell nuclei loss. The MFS and TAV thus showed similar degenerative features of the aorta, but with a distinct pathogenetic mechanism (Fig. 5).

Besides similarities with the TAV patients, the ascending aortic wall in MFS patients showed resemblance with the BAVs as well. The aortic media in both MFS and BAV was characterized by less differentiated VSMCs as compared to the aortic specimen in TAV patients [17,27]. Especially smoothelin, a specific marker of highly differentiated VSMCs, was nearly absent in the MFS and BAV groups. Furthermore, in line with the immaturity of the VSMCs, a significantly lower expression of lamin A/C was found in MFS and BAV. Marked elastic fiber thinning was also a feature linking MFS and BAV. It could be postulated that the thinning is perhaps the result of less production by the immature VSMCs. It could at least contribute to the aortic wall weakness observed in both MFS and BAV. The immaturity could also explain the significant lower FBN1 expression that was comparable between BAV and MFS. This finding is additional to the *FBN1* mutation in MFS (Fig. 5). Besides a decreased amount of fibrillin-1 in the aortic wall, in line with earlier research, we found that the distribution and localization of fibrillin-1 was different in the MFS and BAV, with more accumulation within the VSMCs [7,31,32], whereas it was mostly seen extracellular in the TAVs. As earlier described by Nataatmadja et al., the few extracellular fibers observed in BAV and MFS were thick and short [7]. Hollister et al. also found a deficiency in the amount of microfibrillar fibers, analyzed immunohistochemically [32], comparable with our results.This shows that the consensus statement based grading system can be used to uniformly describe a vascular wall, however it is not sufficient to elucidate pathogenesis of certain disease processes.

VSMCs have the ability to undergo a phenotypic switch from a quiescent contractile state to an immature, proliferative synthetic state. Disability of this switch has been shown to play a critical role in a variety of cardiovascular diseases, such as hypertension and atherosclerosis [10,11]. During development the, neural crest cells (NCC) and second heart field progenitor cells (SHF) contribute to the VSMCs in the ascending aorta and the wall of the aortic root. These progenitor cells however not only contribute to the aortic VSMCs but also to the development of semilunar valves which is a complex process with a plethora of involved genes

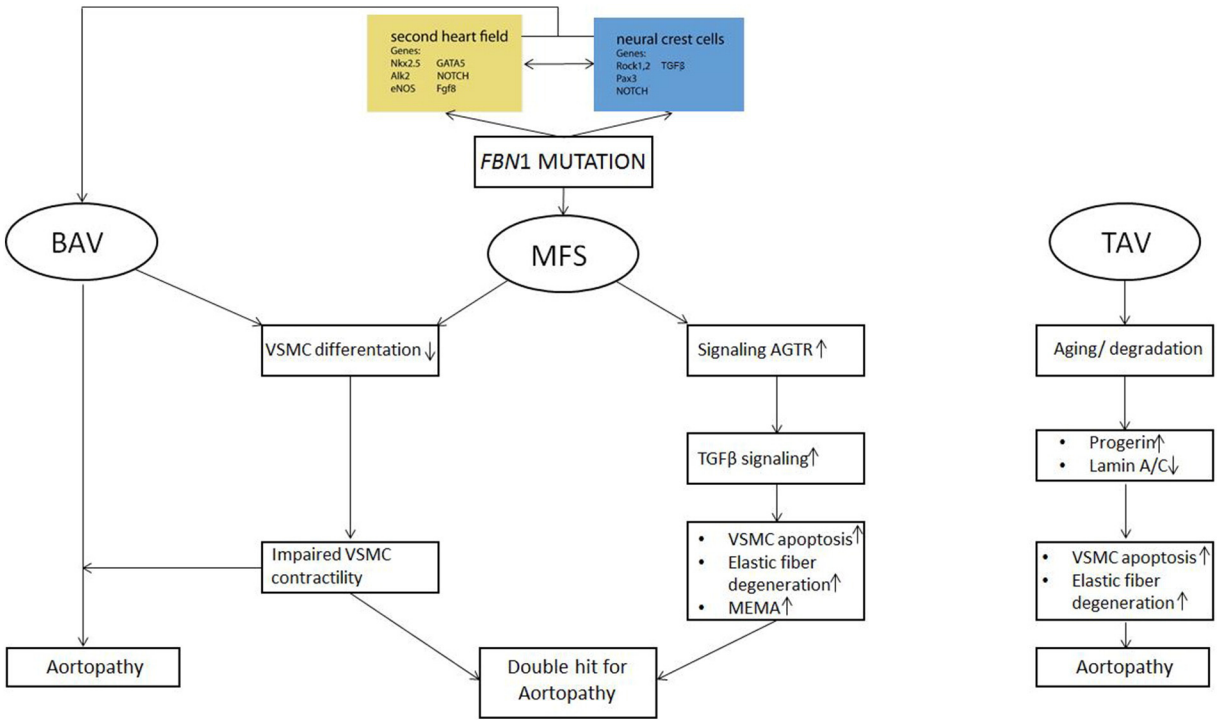

Fig. 5. Schematic overview of our hypothesis regarding similarities and differences in aortic wall pathology between Marfan syndrome (MFS), bicuspid aortic valve (BAV) and tricuspid aortic valve (TAV). The ascending aortic wall in Marfan syndrome (MFS) is characterized by less mature vascular smooth muscle cells (VSMCs), which leads to an impaired contractility of the vascular wall. In patients with a bicuspid aortic valve (BAV) the ascending aortic wall is also populated by immature VSMCs. Both show less fibrillin-1 (FBN1) expression Deficiency of progenitor cells (either neural crest cells (NCC) or second heart field cells (SHF cells) and their interaction can lead to aortic wall as well as semilunar valve abnormalities. Likewise, in the MFS patients a defect in progenitor cells NCC and SHF can be responsible for the immature VSMCs. The aortic media in MFS further shows degenerative features such as, overall medial degeneration and elastic fiber fragmentation and loss. These features were also apparent in the TAVs, however the mechanisms leading to the pathologic features might be distinct in MFS and TAVs. In TAV cardiovascular aging (increased progerin) is responsible for the degenerative features, whereas in MFS cardiovascular aging (increase in progerin expression) is not seen. In MFS the *FBN1* (fibrillin-1) mutation leads to VSMC apoptosis through an increased signaling of Angiotensin II receptors (AT2 receptor). In MFS patients thus a double hit for aortopathy is possible caused by a combination of an immature state and severe degenerative features of the aortic media, which is distinct from the pathogenesis of aortopathy in BAV and TAV.

(Fig. 5) [33]. Deficiency of either NCC or SHF cells and their interaction can lead to aortic wall as well as semilunar valve abnormalities (Fig. 5) [33].

In MFS the *FBN1* mutation leads to an increased Angiotensin II (AT2) receptors signaling and subsequently induction of transforming growth factor β (TGFβ) signaling [30,34,35]. TGFβ is involved in the differentiation of NCC into VSMCs [36,37]. Inamoto has also shown that mutations in the TGFβ receptor alter the VSMC phenotype [38]. In MFS aortic dilation occurs predominantly at the level of the sinus of Valsalva, in which the expansion of the aorta includes the wall of the sinuses as well as the interleaflet triangles [39]. In these regions both SHF as well as NCC populations are found during development. Dedifferentiation of VSMCs as seen in MFS could be a developmental defect in the NCC and SHF progenitor cells caused by the *FBN1* mutation. It would be interesting to perform lineage tracing studies for NCC and SHF cells in a Marfan mouse model to understand to which extent the NCC and SHF cells are involved in the dilation at the level of the aortic root.

The hypothesis and the underlying observations as described in Fig. 5 could explain why angiotensin-receptor-blockers (ARBs), as Losartan, have been identified as a potentially therapeutic agent to prevent progressive dilation of the ascending aorta in MFS. ARBs reduce the signaling that occurs through both AT receptors, (AT1 and AT2 receptor) [40–46]. A similar positive effect has not been reported in a population of BAV patients that were treated with an angiotensin inhibitor (ACE inhibitor) [47], which can be understood on the basis of our current results. In BAV medical treatment might not effective because the histopathology and weakness of the aortic wall in BAVs does not seem to be based on a similar process leading to apoptosis and medial degeneration.

In this histopathologic study, we thus found that the aorta in MFS shows similarities with both BAV (immaturity of the aortic media, EFT, a thin tunica intima and decreased level of fibrillin-1 expression) and TAV (aortic media degenerative features, due to increased VSMC apoptosis, related in MFS to increased AT2 receptor signaling and in TAV to vascular ageing). Comparison of the aortic wall samples in MFS, BAV and TAV demonstrates that aortopathy in MFS has a different pathogenesis as compared to the BAV and TAV, explaining the higher incidence and the younger age of occurrence. These findings could be of therapeutic relevance.

4.1. Study Limitations

Limitations of our study were the relative low number of control TAV samples that were obtained post-mortem. Also the MFS group is relatively low in number but statistical analysis showed that the power was adequate to support our conclusions. We did not analyze 6 subsequent sections for each different staining but performed measurements at 3 different sites (microscopic fields) in one section. The use of different stainings in subsequent sister sections also allowed to confirm certain features. For example when MEMA was suspected in the HE section it could then be confirmed in the subsequent Movat staining for site and extension. In an earlier study [48] we have shown that the histopathologic features measured in the aortic media were not different between the convex and the concave site. In the current study we therefore decided to keep sampling as standard as possible, taking the convex side in all groups studied.

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Conflict of interest

The authors declare that they have no conflict of interest.

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