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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.

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.

2.3. 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.

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,