Proteomic Analysis in Aortic Media of Patients With Marfan Syndrome Reveals Increased Activity of Calpain 2 in Aortic Aneurysms

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Background—Marfan syndrome (MFS) is a heritable disorder of connective tissue, affecting principally skeletal, ocular, and cardiovascular systems. The most life-threatening manifestations are aortic aneurysm and dissection. We investigated changes in the proteome of aortic media in patients with and without MFS to gain insight into molecular mechanisms leading to aortic dilatation.

Methods and Results—Aortic samples were collected from 46 patients. Twenty-two patients suffered from MFS, 9 patients had bicuspid aortic valve, and 15 patients without connective tissue disorder served as controls. Aortic media was isolated and its proteome was analyzed in 12 patients with the use of 2-dimensional difference gel electrophoresis and mass spectrometry. We found higher amounts of filamin A C-terminal fragment, calponin 1, vinculin, microfibril-associated glycoprotein 4, and myosin-10 heavy chain in aortic media of MFS aneurysm samples than in controls. Regulation of filamin A C-terminal fragmentation was validated in all patient samples by immunoblotting. Cleavage of filamin A and the calpain substrate spectrin was increased in the MFS and bicuspid aortic valve groups. Extent of cleavage correlated positively with calpain 2 expression and negatively with the expression of its endogenous inhibitor calpastatin.

Conclusions—Our observation demonstrates for the first time upregulation of the C-terminal fragment of filamin A in dilated aortic media of MFS and bicuspid aortic valve patients. In addition, our results present evidence that the cleavage of filamin A is highly likely the result of the protease calpain. Increased calpain activity might explain, at least in part, histological alterations in dilated aorta. (Circulation. 2009;120:983-991.)

Key Words: aneurysm ■ aorta ■ calpain ■ Marfan syndrome ■ proteomics

Marfan syndrome (MFS) is a disorder of connective tissue caused by mutations in the fibrillin-1 gene (FBN-1) that may lead to abnormal regulation of transforming growth factor-β (TGF-β) signaling. Clinical manifestation implies principally the cardiovascular, skeletal, and ocular systems, and other systems such as skin, lung, and dura may also be affected. Cardiovascular defects involve dilatation of the ascending aortic root leading to aortic aneurysm and dissection, which are the main cause of morbidity and mortality in MFS, and mitral valve prolapse with or without regurgitation.

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Molecular mechanisms of aneurysm formation and its treatment are the subject of intensive research. Three key proteins seem to directly contribute to aneurysm formation. First, defective fibrillin-1 is thought to alter targeting and sequestration of TGF-β, leading to increased TGF-β activity. Blocking TGF-β activity with antagonists such as TGF-β-neutralizing antibodies prevents aneurysm formation in a mouse model of MFS. Second, angiotensin II (Ang II) is upregulated in MFS. Treatment of patients with Ang II type 1 (AT₁) receptor (AT₁R) blocker or angiotensin-converting enzyme (ACE) inhibitors reduces progression of aortic dilation in MFS. Attenuation of aortic remodeling by lowering Ang II levels can be mediated by TGF-β-dependent and –independent mechanisms. Third, proteases play a pivotal role in the pathology of aortic dilatation. We have profiled the activity of matrix metalloproteinases (MMPs) in MFS aortic aneurysms. Studies...
in the MFS mouse model revealed the upregulation of MMP-2 and -9.10

Calpains are another class of proteases not yet associated with MFS aortic aneurysms. Calpains are calcium-dependent cysteine proteases that cleave many intracellular signaling and structural proteins. The 2 major isoforms, calpain 1 and calpain 2, are ubiquitously expressed and differ in their tertiary structure and Ca\(^{2+}\) requirements.11 Activation of calpain is regulated by its endogenous inhibitor calpastatin, intracellular localization of the protease, and local Ca\(^{2+}\) concentrations. Recent studies have implicated the calpain/calpastatin system in Ang II–induced cardiovascular remodeling.12 Evidence exists that Ang II induces calpain expression and activity and further leads to upregulation of MMP-2.12,13 Because elevated Ang II levels and increased MMP-2 activity are elements in the initiation and progression of aortic aneurysms, the calpain/calpastatin system might play an important role in MFS aortic remodeling and dilatation.

The aim of the present study was to investigate differences in the proteome of aortic media in patients with and without MFS to gain new insights into the pathological mechanisms of aortic dilatation. Increased filamin A fragmentation in dilated aortic media of MFS and bicuspid aortic valve (BAV) patients led us to further investigate the proteolytic calpain/calpastatin system.

**Methods**

**Source of Tissue**

Aneurysm tissue from ascending aorta was obtained from 22 MFS patients at the time of aortic root replacement and from 9 BAV patients. Ascending aorta of 15 non-MFS and non-BAV patients without aortic dilation served as a control group. Heart transplant patients and patients suffering from tricuspid aortic valve stenosis, undergoing elective aortic valve replacement, were primarily included. MFS diagnosis was based on Ghent criteria and in 5 patients was confirmed by genetic mutation analysis (for results, see the online-only Data Supplement). All MFS patients had a tricuspid aortic valve. Written informed consent was obtained from all patients, according to local ethics committee approval of the University Hospital of Bern and the University of Pennsylvania. Aortic tissue was dissected into adventitia, media, and intima. From each tissue, 1 section was immediately snap-frozen in liquid nitrogen and stored at −70°C until protein analysis. Another section was fixed with 4% paraformaldehyde in phosphate-buffered saline for histological examination.

**Histology**

Fixed tissue was dehydrated in an automatic tissue processor, embedded in paraffin, and cut into 4-μm sections for histochemical evaluation. Sections were stained with Verhoeff–van Gieson protocol for evaluation of tissue separation.

**Two-Dimensional Differential Gel Electrophoresis**

Samples from 12 patients were used for proteomic analysis. For high resolution of proteins and minimal gel-to-gel variation, 2-dimensional differential gel electrophoresis (2D-DIGE) was performed. Therefore, proteins from aortic aneurysms were labeled with different fluorophores than nonaneurysm proteins (Cy3 and Cy5). For details, please refer to the online-only Data Supplement.

**Image Analysis**

Labeled proteins were visualized with the use of a Typhoon 9400 imager (GE Healthcare). Spot matching, quantification, and statistical analyses were performed with the use of Proteomweaver software (Definiens AG, Munich, Germany). Scanned images were uploaded into the software, and automated spot detection and quantification were performed. The software identified spot candidates by detecting local maxima in the gray-scale gel image, and the outline of those was calculated with the use of a downhill gradient flood-filling algorithm. The same spots in different gels were matched with the use of a pattern-matching algorithm that depends on spot features and the geometry and features of its neighbors. The spot quantification calculated the spot volume with the use of the integral of a Gaussian approximation corrected by a factor that depends on the local background level. Normalized protein spots in the Cy3 and Cy5 images were compared with the internal standard (Cy2) to generate ratios of relative amounts. Protein spots were considered to be significantly differentially regulated when the t test value was \(P<0.05\) and the regulation factor \(>2\).

**Mass Spectrometry**

For identification of differentially regulated protein spots, spot candidates were picked from the gel and trypsin digested. Eluted peptides were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and liquid chromatography mass spectrometry. For details, please refer to the online-only Data Supplement.

**Immunoblotting**

Protein cleavage and relative abundance of differentially expressed proteins were examined with the use of Western blot technology. Briefly, 5 μg of aortic media extracted in DIGE buffer was loaded onto a 10% polyacrylamide/sodium dodecyl sulfate gel and subjected to separation by electrophoresis. Proteins were electroblotted onto polyvinylidene fluoride membrane (Hybond, GE Healthcare). Antibodies against the C-terminus of filamin A, calpastatin, α-2 spectrin, β-actin (Santa Cruz Biotechnology, Santa Cruz, Calif), and calpain 2 (Cell Signaling Technology, Danvers, Mass) were used. Positive immunoreactivity was detected with chemiluminescence substrate (ECL detection reagent, GE Healthcare) and exposure of the blots to film (Hyperfilm, GE Healthcare). After development, films were digitized, and quantitative densitometric image analysis was performed (ImageJ 1.39C; http://rsweb.nih.gov/ij/). Protein cleavage was assessed as the amount of cleavage fragment relative to the amount of full-length protein. Cleavage of α-2 spectrin was used as a measure for calpain activity.14

**Statistical Analysis**

For statistical comparisons, parametric (t test, Pearson correlation coefficient) and nonparametric (Mann-Whitney test, Spearman correlation coefficient) tests were used. If \(>2\) groups were compared, ANOVA followed by Bonferroni post hoc test for multiple comparisons and Kruskal-Wallis followed by Dunn post hoc test for multiple comparisons were applied. Pearson χ² test was used for categorical data. Distributional normality was tested with the use of D’Agostino and Pearson omnibus normality test. Values are given as mean±SEM.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Patient Characteristics**

There were no differences in sex (controls, 67±13% male; MFS, 83±8% male; BAV, 67±17% male; \(P=0.50\)). Mean age differed between MFS and control groups (controls, 60±3 years; MFS, 38±4 years; \(P<0.001\)) but not between BAV and control groups (BAV, 57±3 years; \(P=0.70\)). Mean aortic diameters were similar in MFS and BAV groups (MFS, 4.99±0.12 cm; BAV, 4.84±0.19 cm; \(P=0.29\)) and different from controls (controls, <3.5 cm; \(P<0.001\)). Arterial hypertension was present in 14 patients but was independent of
MFS status (controls, 27%; MFS, 36%; BAV, 22%; \( P = 0.69 \)). Most patients with hypertension received AT1R blockers or ACE inhibitors (controls, 27%; MFS, 14%; BAV, 11%; \( P = 0.51 \)). Treatment with \( \beta \)-blockers was significantly higher in the MFS group (controls, 33%; MFS, 73%; BAV, 33%; \( P = 0.03 \)).

Histology
Histology and quality of separation technique were investigated in aortic samples for proteomic analysis with the use of van Gieson elastic stain (Figure 1). The tissue separation technology revealed well-dissected media in control, MFS, and BAV samples. Fragmentation of elastic fibers was present in media of MFS patients and to a lesser extent in BAV patients.

Differentially Expressed Proteins in MFS Aortic Media
Protein expressions of aortic media from 6 randomly selected MFS aneurysm patients and 6 randomly selected controls were compared with the use of 2D-DIGE. Each gel contained the aortic media proteome of MFS patients, control patients, and an internal standard. Between 997 and 1247 protein spots were resolved according to the spot detection tool of the image analysis software. Six spots were found to be significantly upregulated in MFS aortic media, whereas 20 protein spots were downregulated >2-fold \( (P < 0.05) \) (Figure 2). From the colloidal Coomassie-stained gels, 17 spots could be picked and subjected to mass spectrometry. The Table lists the identified protein spots as outlined in Figure 2. Details of mass spectrometric identification are summarized in Table I of the online-only Data Supplement. Upregulated proteins were myosin-10 heavy chain, vinculin, filamin A, microfibril-associated glycoprotein 4 (MFAP 4), and calponin 1. Downregulated proteins were vitamin-D binding protein, fructose-bisphosphate aldolase C, apolipoprotein A-I, serum amyloid P, transgelin, and hemoglobin \( \beta \) and \( \alpha \) chains. Comparison of the molecular weight of separated proteins with theoretical values and analysis of the sequence coverage achieved by mass spectrometry revealed that filamin A was not upregulated as an entire protein but as C-terminal fragment. Proteolysis was also observed for calponin 1. The entire protein with a molecular weight of 33 kDa was upregulated as well as its fragment of 20 kDa. The occurrence of fragmentation of these 2 proteins attracted our interest in relation to proteolytic activity of MFS media from aortic aneurysms.

Filamin A C-Terminal Fragment
We confirmed the upregulation of filamin A C-terminal fragment from proteomic analysis in the entire study population using Western blot. Use of a specific antibody against the C-terminus of filamin A revealed bands at 280 kDa, representing the full-length protein, and bands at 90 and 110 kDa, representing the fragments detected in our proteomic analysis (Figure 3). Comparison of the ratio of cleavage products to substrate revealed a significant upregulation of filamin A cleavage in the MFS and BAV groups compared with controls (controls, 2.04 \( \pm \) 0.21; MFS, 2.64 \( \pm \) 0.17; \( P = 0.02 \) from controls; BAV, 3.04 \( \pm \) 0.21; \( P = 0.004 \) from controls).

Calpain 2
Calpain 2 activity was assessed by measuring the cleavage fragments of \( \alpha \)-2 spectrin at 145/150 kDa.\(^{14} \) We found a significantly upregulated cleavage of \( \alpha \)-2 spectrin in aneurysmal aortic media compared with controls (controls, 0.70 \( \pm \) 0.08; MFS, 1.05 \( \pm \) 0.09; \( P = 0.007 \) from controls; BAV, 1.15 \( \pm \) 0.10; \( P = 0.006 \) from controls). Subgroup analysis in all patients revealed that hypertension did not result in elevated spectrin cleavage (see online-only Data Supplement). Furthermore, there was a strong correlation between filamin A and \( \alpha \)-2 spectrin cleavage \( (r = 0.92, P < 0.001; n = 46) \), confirming that calpain 2 is involved in filamin A cleavage (Figure 4). Quantification of calpain 2 expression and its inhibitor calpastatin revealed a positive correlation of calpain 2 with filamin A \( (r = 0.33, P = 0.03; n = 46) \) and spectrin cleavage \( (r = 0.41, P = 0.005; n = 46) \) and a negative correlation of calpastatin with filamin A \( (r = -0.31, P = 0.04; n = 46) \).
and spectrin cleavage ($r = -0.43$, $P=0.003$; $n=46$) (Figure 5). Interestingly, the ratio of calpain 2 and its inhibitor calpastatin highly correlated with filamin A ($r=0.47$, $P<0.001$; $n=46$) as well as with spectrin cleavage ($r=0.60$, $P<0.001$; $n=46$).

Discussion
Thoracic aortic aneurysms and aortic dissection are the major cause of premature death in MFS. Until recently, the common treatment methods for MFS aneurysms were lifelong medication with $\beta$-blockers and repair of dilated aorta. New insights into molecular mechanisms of aortic dilatation in MFS have revealed that TGF-$\beta$, Ang II, and MMPs are upregulated, thus leading to new treatment options. Antagonism of TGF-$\beta$ with neutralizing antibodies, blockage of AT$_1$R with losartan, and inhibition of ACE by perindopril and of MMP-2 and -9 by doxycycline showed attenuation of aortic root dilation and reduced TGF-$\beta$ activity in aortic media of mice and humans.

In the present study, we analyzed aortic media to explore new molecular alterations associated with thoracic aortic aneurysms in MFS. We used 2D-DIGE to detect differences in protein abundance and mass spectrometry for subsequent protein identification. To minimize contamination of the aortic proteome by blood cells or plasma proteins and to investigate the aortic tunica that is primarily involved in the pathogenesis of aneurysm formation, we dissected the aorta and only analyzed aortic media for differential regulation.

Most interesting was the finding that the filamin A C-terminal fragmentation is upregulated in aortic media of MFS and BAV patients. Intact filamin A is a high-molecular-weight protein that cross-links and organizes actin filaments into parallel arrays of 3-dimensional webs. In addition, filamin A–interacting proteins such as TGF-$\beta$ receptor–activated Smads, which bind to the C-terminus, are important signaling molecules.

Mutations in filamin A are responsible for familial cardiac valvular dystrophy and aortic aneurysms in periventricular nodular heterotopia. Furthermore, filamin gene expression in mitral valves in a mouse model of MFS is significantly increased in homozygote animals. The C-terminal region of filamin A, which we found to be upregulated in MFS and BAV aneurysm media, reveals functions other than the intact protein. The fragment itself has the capacity to translocate to the nucleus together with the androgen receptor, resulting in the repression of androgen receptor transcriptional activity. Moreover, filamin A C-terminal fragments as well as the spectrin fragment have recently been proposed as parts of biomarker panels. The filamin A C-terminal fragment was secreted from aorta that underwent arterial wall remodeling. Increased spectrin fragments were found in cerebrospinal fluid of patients with mild central nervous system injury.

Filamin A is cleaved by the proteases calpain, caspase, and granzyme B. Evaluating caspase 3 and granzyme B activity in a subset of patients showed no difference between normal aorta and MFS aneurysm (data not shown), whereas calpain did. Calpain activity was assessed by measuring cleavage of its endogenous substrate spectrin. We found increased cleavage of spectrin in MFS and BAV aortic aneurysm that highly correlated with the increase of filamin A cleavage. Furthermore, increase of spectrin and filamin A proteolysis correlated with increasing calpain expression and decreasing expression of its inhibitor calpastatin. Because of
the high correlation between spectrin and filamin A cleavage in the absence of increased activity of caspase 3 and granzyme B, we conclude that both proteolytic digestions result from the action of calpain, which is attributable to higher protease expression as well as lowered expression of its inhibitor.

Increased activity of calpain is known from different pathological states such as from cardiovascular remodeling after Ang II–induced hypertension and from aging. Recently, Letavernier et al demonstrated that infusion of Ang II in mice resulted in increased calpain and decreased calpastatin expression. The group further showed that overexpression of the inhibitor calpastatin decreased MMP-2 and -9 activity and attenuated Ang II–induced vascular remodeling with focus on hypertrophy, perivascular inflammation, and tissue fibrosis. Interestingly, administration of losartan restored basal calpain levels. Losartan therefore offers a pharmacological approach to reduce excessive activity of calpain. Furthermore, TGF-β-induced apoptosis in rat hepatocytes was greatly attenuated by inhibiting calpain 1 and more efficiently by inhibiting calpain 2. Thus, calpain inhibition might potentially also attenuate TGF-β activity.

Ang II mRNA expression in MFS aortic aneurysm and Ang II concentrations measured by enzyme-linked immunosorbent assay were elevated compared with controls. Confirming this, antagonism of Ang II with the AT1 receptor blocker losartan or ACE

**Table. Regulation and Function of Identified Proteins**

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein</th>
<th>Accession No.</th>
<th>Regulation</th>
<th>Theoretical Molecular Weight/Isoelectric Point</th>
<th>Main Protein Function*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Myosin-10</td>
<td>P35580</td>
<td>(+)2.03</td>
<td>228.9/5.4</td>
<td>Appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping</td>
</tr>
<tr>
<td>2</td>
<td>Vinculin</td>
<td>P18206</td>
<td>(+)2.11</td>
<td>123.6/5.5</td>
<td>Involved in cell adhesion, may be involved in attachment of actin-based microfilaments to plasma membrane, may play role in cell morphology and locomotion</td>
</tr>
<tr>
<td>3</td>
<td>Filamin A</td>
<td>P21333</td>
<td>(+)2.18</td>
<td>280.7/5.7</td>
<td>Promotes orthogonal branching of actin filaments, links actin filaments to membrane glycoproteins, anchors transmembrane proteins to actin cytoskeleton, serves as scaffold for wide range of cytoplasmic signaling proteins</td>
</tr>
<tr>
<td></td>
<td>FLNA protein</td>
<td>Q96C61</td>
<td></td>
<td>88.5/5.9</td>
<td>Localizes to nucleus to regulate androgen receptor and coactivator functions</td>
</tr>
<tr>
<td>4</td>
<td>MFAP 4</td>
<td>P55083</td>
<td>(+)2.08</td>
<td>28.6/5.2</td>
<td>Could be involved in calcium-dependent cell adhesion or intercellular interactions</td>
</tr>
<tr>
<td>5</td>
<td>Calponin 1 (33 kDa)</td>
<td>P51911</td>
<td>(+)3.03</td>
<td>33.2/9.1</td>
<td>Thin filament-associated protein, implicated in regulation and modulation of smooth muscle contraction; binds to actin, calmodulin, troponin C, and tropomyosin</td>
</tr>
<tr>
<td>6</td>
<td>Calponin 1 (20 kDa)</td>
<td>P51911</td>
<td>(+)3.88</td>
<td>33.2/9.1</td>
<td>Thin filament-associated protein, implicated in regulation and modulation of smooth muscle contraction; binds to actin, calmodulin, troponin C, and tropomyosin</td>
</tr>
<tr>
<td>7</td>
<td>Vitamin-D binding protein</td>
<td>P02774</td>
<td>(-)2.56</td>
<td>53.0/5.2</td>
<td>Multifunctional plasma protein and on surface of many cell types; carries vitamin D sterols, prevents polymerization of actin by binding its monomers</td>
</tr>
<tr>
<td>8</td>
<td>Fructose-bisphosphate aldolase C</td>
<td>P09972</td>
<td>(-)3.85</td>
<td>39.5/6.5</td>
<td>Involved in glycolysis, catalytic activity</td>
</tr>
<tr>
<td>9</td>
<td>Apolipoprotein A-I</td>
<td>P02647</td>
<td>(-)10.00</td>
<td>30.5/5.3</td>
<td>Reverse transport of cholesterol from tissues to the liver</td>
</tr>
<tr>
<td>10</td>
<td>Serum amyloid P</td>
<td>P02743</td>
<td>(-)3.57</td>
<td>25.4/6.1</td>
<td>Can interact with DNA and histones, may scavenge nuclear material released from damaged circulating cells, may function as a calcium-dependent lectin</td>
</tr>
<tr>
<td>11</td>
<td>Transgelin</td>
<td>Q5UOD2 Q01995</td>
<td>(-)2.56</td>
<td>22.6/8.9</td>
<td>Actin cross-linking/gelling protein, involved in calcium interactions and contractile properties of the cell that may contribute to replicative senescence</td>
</tr>
<tr>
<td>12</td>
<td>Hemoglobin β chain</td>
<td>P68871</td>
<td>(-)7.14</td>
<td>16.0/6.8</td>
<td>Involved in oxygen transport from the lung to the various peripheral tissues</td>
</tr>
<tr>
<td>13</td>
<td>Hemoglobin α chain</td>
<td>P69905</td>
<td>(-)5.88</td>
<td>15.3/8.7</td>
<td>Involved in oxygen transport from the lung to the various peripheral tissues</td>
</tr>
</tbody>
</table>


**Figure 3.** Filamin A cleavage analysis. Filamin A cleavage was increased in aortic specimens from MFS and BAV patients relative to controls (CTRL). Representative Western blot shows full-length filamin A at 280 kDa and C-terminal fragments at 110 and 90 kDa (right). Values are mean±SEM. *P<0.05, **P<0.01.
inhibitors blunted aortic dilatation and decreased TGF-β activation.3,4,6 Ang II also stimulated Smad 2–dependent signaling and fibrosis in vascular smooth muscle cells (VSMCs) in a TGF-β-independent manner, an effect prevented by selective AT1R blockade.8 Thus, we speculate that calpain plays a role in TGF-β–dependent and –independent Smad activation in the setting of increased Ang II concentrations.

Calpain can be activated in VSMCs by mechanical stress. Mechanical stress led to an increase in VSMC Ang II by elevation of ACE expression, and it increased calpain activation, seeming to counteract VSMC apoptosis by degradation of p53.25,26 We observed a correlation between aortic diameter and α-2 spectrin cleavage (see the online-only Data Supplement). Because mechanical stress increases with aortic

Figure 4. Spectrin cleavage analysis. Cleavage of α-2 spectrin was significantly increased in MFS and BAV patients compared with controls (CTRL) (A) and correlated with cleavage of filamin A (B). Representative Western blot shows full-length protein at 240 kDa and cleavage fragments at 145/150 kDa (C). MFS (●), BAV (+), and control patients (○) are shown. Values are mean±SEM. **P<0.01.

Figure 5. Calpain 2 and calpastatin analysis. Increasing cleavage of filamin A correlates with increasing calpain 2 expression (A) and decreasing calpastatin expression (D). Cleavage of spectrin and therewith calpain activity correlates in a similar manner (B and E). The ratio between calpain 2 and calpastatin strongly correlates with filamin A (G) and α-2 spectrin cleavage (H). Representative Western blots show calpain 2 (C) and calpastatin 2 (F). MFS (●), BAV (+), and control patients (CTRL) (○) are shown. Dashed lines indicate the 95% confidence interval.
diameter, elevated calpain activity may be the result of augmented wall stress.

Nearly 50% of patients with BAV develop a dilatation of the ascending aorta or aortic sinus. The mechanism remains controversial; genetic predisposition seems to play an important role in the initiation of aortic dilation rather than aberrant postvalvular hemodynamics. Histological changes in dilated aortic media share similarities with those seen in MFS, including increased VSMC apoptosis, cystic medial necrosis, elastin fragmentation, and upregulation of MMP-2. Histological parallels, together with presumed genetic defects of the extracellular matrix, lead us to assume that calpain is also activated in BAV. Indeed, our results present evidence that filamin A and spectrin cleavage increase to a similar extent in MFS compared with controls. Therefore, increased calpain activity is a feature of both syndromes and suggests that BAV-associated aortic dilation is pathogenetically closer to that in MFS than assumed previously. To analyze whether the same holds true for conditions such as age- and hypertension-associated ectasia with tricuspid valves, we examined calpain activity in this additional group and observed further increased spectrin cleavage (see the online-only Data Supplement). Because hypertension is another cause of wall stress, we achieved further evidence that wall stress may increase calpain activity by possible elevation of tissue Ang II and may further lead to abnormal vascular remodeling.

Using 2D-DIGE, we observed upregulation of 4 additional proteins in MFS aneurysm. The actin-binding molecules vinculin and calponin were more highly expressed in the MFS group compared with controls. Vinculin is a membrane-anchored protein involved in the actin cytoskeleton and plays a role in the formation of filopodia, which are important for cell migration. It is therefore involved in regulation and modulation of smooth muscle cell contraction and act as a signal for cell differentiation. The MFS aneurysm is associated with increased levels of TGF-β, and, interestingly, stimulation of VSMCs with TGF-β increases calpain 1. Therefore, upregulation of calpain 1 could be an indirect marker of increased TGF-β signaling. We observed 2 different types of calpain-1, accounting for the full-length protein (33 kDa) and a fragment thereof (20 kDa). In analogy to filamin A, calponins can be cleaved by calpain.

Another observation was the upregulation of MFAP 4 in aortic dilation. MFAP 4 is a 29-kDa elastin-binding protein and plays a role in elastogenesis. Furthermore, MFAP 4 has high sequence homology to the aortic aneurysm–associated protein-40, which has been found in human abdominal aortic aneurysm. Although not much is known about MFAP 4, there are indications that it might play an important role in aneurysm formation.

Furthermore, we found upregulation of cellular myosin-10. It has been reported that myosin-10 heavy chain creates links between microtubules and the actin cytoskeleton as well as between integrins and the cytoskeleton. Myosin-10 heavy chain overexpression is associated with an increased number of filopodia, which are important for cell migration. It is difficult to attribute a function to myosin-10 heavy chain in the context of MFS. Because aneurysm formation is in part an inflammatory process, it possibly leads to increased migration of VSMCs.

Limitations of the Study
First, the proteome of aortic media consists of an enormous number of different proteins, and 2 subsequent protein separation techniques are not feasible to separate all of them in a satisfactory manner. Additionally, the dynamic range of protein expression spans 7 or 8 orders of magnitude. Furthermore, proteins within a given proteome are structurally diverse and present various physicochemical characteristics. Some of these proteins are alkaline or hydrophobic, are of poor solubility, or have different staining characteristics. Therefore, the present study in the proteome of aortic aneurysm in MFS revealed interesting protein expression changes, but it could never detect them all. Second, results of proteomic studies have to be validated with an alternative method as we did using Western blot technology. Because our interest was the filamin A C-terminal fragment and its proteolysis, we did not validate the other proteins discussed here. Third, enzyme-linked immunosorbent assay and zymography are 2 additional methods of confirming calpain upregulation. Because samples of human aortic media are rare and quantity is limited, further calpain confirmation could not be performed. Fourth, control subjects were in general older than MFS patients, and calpain is known to be upregulated with aging. However, in the present study, calpain was upregulated in younger MFS patients, and we therefore did not consider this finding to be biased by differences in age.

Conclusions
Using proteomic techniques, we identified upregulation of the filamin A C-terminal fragment in aortic media of MFS patients suffering from aneurysm of the ascending aorta. We further observed indicators of increased TGF-β signaling and enhanced mechanical stress in MFS aortic aneurysm. Elevated filamin A cleavage was a result of higher calpain activity in MFS as well as in BAV patients. Increased filamin A fragmentation and elevated calpain activity have been shown to play a role in Ang II–induced vascular remodeling. Increased calpain activity in aortic aneurysms may be the result of increased aortic wall stress. Pharmacological inhibition of calpain might prevent uncontrolled proteolysis and further signaling. Because the development of calpain inhibitors has been complicated, AT1R blockers are the preferred approach for normalizing calpain activity in aortic media of MFS and BAV patients. Further research in the field of serum filamin A and spectrin fragmentation in MFS and BAV patients might present evidence for them to serve as potential serum biomarkers.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Thoracic aortic aneurysms are a life-threatening risk in patients with Marfan syndrome, bicuspid aortic valve, and severe hypertension. With progressive dilation, thoracic aneurysms may rupture and lead to severe internal bleeding and sudden death. To date, aortic aneurysms are treated with β-blocker therapy and surgery. Recent evidence suggests delayed dilatation of thoracic aorta in Marfan syndrome patients treated with inhibitors of the renin-angiotensin system. Neither the mechanisms leading to aneurysm formation nor the mechanisms of reduced aortic dilatation seen under treatment with antagonists of the renin-angiotensin system are as yet clearly elucidated. Proteomic technology enables large-scale screening of protein expression in tissues to be performed to unravel pathological mechanisms leading to aneurysm formation. In this study, we present the first time protein expression analysis comparing aneurysmal aorta of Marfan patients with nonaneurysmal aorta of controls. Thirteen proteins or fragments thereof were differentially expressed in aortic media of Marfan patients. Among these, a fragment of filamin A was investigated further. Analysis of proteolytic activity in aneurysmal aorta revealed increased calpain 2 activity, thus leading to fragmentation of filamin A. Similar activation of calpain 2 was found in bicuspid valve– and hypertension-associated aneurysm. Because calpain activity depends on angiotensin II levels, our findings present further evidence for the positive effect of targeting the renin-angiotensin system on aneurysm progression in Marfan syndrome. They further suggest that inhibiting the renin-angiotensin system in patients with thoracic aortic aneurysms not associated with Marfan syndrome might also be beneficial.
Proteomic Analysis in Aortic Media of Patients With Marfan Syndrome Reveals Increased Activity of Calpain 2 in Aortic Aneurysms

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/content/121/6/e38.full.pdf

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2009/08/31/CIRCULATIONAHA.108.843516.DC1

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In the article “Proteomic Analysis in Aortic Media of Patients With Marfan Syndrome Reveals Increased Activity of Calpain 2 in Aortic Aneurysms” by Pilop et al, which appeared in the September 15, 2009 issue of the journal (Circulation. 2009;120;983–991), the Sources of Funding on page 990 was incorrect and should read:

“This work was supported by the Swiss National Foundation for Scientific Research grants 3100A0-102153 and 320000-122135/1 to Dr Frey and grant 3100A0-120504 to Dr Matyas. Drs J.H. Gorman and R.C. Gorman were supported by individual Established Investigator Awards from the American Heart Association (Dallas, Tex).”

The online version of the article has been corrected.

DOI: 10.1161/CIR.0b013e3181d41311
2D - Gel Electrophoresis. Aortic media was powderized and dissolved in differential gel electrophoresis buffer (DIGE) according to the manufacturer’s instructions (GE Healthcare, Buckinghamshire, UK). DNA was sheared using sonification. Equal amounts of aortic samples (50µg) from MFS patients and controls were labeled with Cy3 dye and Cy5 dye respectively. To guarantee uniform labeling, Cy3 and Cy5 were inverted in half of the patients. The internal standard containing equal amounts of protein of each sample was tagged with Cy2 dye. Labeled protein samples were pooled (MFS media, control media and internal standard) and 150µg loaded onto an immobilized pH gradient (IPG) dry strip with a non-linear pH range of pH 3-11 (GE Healthcare). After isoelectric focusing (total 73kVh), the IPG strips were equilibrated with dithiothreitol and iodoacetamide equilibration buffer sequentially according to the manufacturers instructions for 15min. The second dimension was conducted with a 12,5% SDS-polyacrylamide gel electrophoresis at 0.03 W/cm for 6 hours. 6 gels were run simultaneously.

Mass Spectrometry. Colloidal Coomassie staining was used to visualize protein spots for spot picking. Individual and visible spots were cut from the gel and in-gel digested overnight with 10ng/µl trypsin in 50mM NH₄HCO₃ (Promega, Madison, USA). Extracted peptides were desalted using Zip Tips (Millipore, Billerica, USA) and analyzed by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS) and MALDI-MS/MS using an Ultraflex TOF/TOF II
equipped with the control and analysis software Compass v.1.1 (Bruker Daltonics, Bremen, Germany). Combined MS and MS/MS spectra were used to search the Uniref100 database (release 6.0) using MASCOT software v.2.0 (Matrix Science, London, UK). Probability-based MASCOT scores greater than 62 were considered to be significant and not a random event (p<0.05). Samples which could not be identified by MALDI-MS/MS were subjected to liquid chromatography (LC) – LTQ Orbitrap™ (Thermo Scientific, Bremen, Germany). MS/MS peak lists were generated using Mascot Distiller (Matrix Science, London, UK) and used to search the Uniref100 database (release 6.0) using MASCOT software v. 2.0.

**Supplemental Results**

**Subgroup analysis.** Calpain 2 activity assessed as spectrin cleavage was not higher in hypertensive (N=14) compared to normotensive (N=32) patients (hypertensive: \(0.82\pm0.12\), normotensive: \(1.02\pm0.07\), p=0.13). The same result was found when the subpopulations in the control, MFS and BAV group were analysed separately (data not shown).
### Supplemental Table I. Heterozygous sequence variants identified in this study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Year of birth</th>
<th>Phenotype</th>
<th>Gene</th>
<th>Location</th>
<th>Nucleotide change</th>
<th>AA change</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGD/91</td>
<td>1993</td>
<td>(+)</td>
<td>FBN1</td>
<td>Exon 3</td>
<td>c.266G&gt;A</td>
<td>p.Cys89Tyr</td>
<td>This study</td>
</tr>
<tr>
<td>GA/30291</td>
<td>1991</td>
<td>+</td>
<td>FBN1</td>
<td>Exon 13</td>
<td>c.1693C&gt;T</td>
<td>p.Arg565*</td>
<td>Quan et al.</td>
</tr>
<tr>
<td>SP/410</td>
<td>1990</td>
<td>(+)</td>
<td>FBN1</td>
<td>Intron 49</td>
<td>c.6164-1G&gt;A</td>
<td>putative aberrant splicing</td>
<td>This study</td>
</tr>
</tbody>
</table>
| WP/58B  | 1987         | + (+)     | FBN1 | Exon 51  | c.6354C>T         | p.Glu2105_Val2126del\(\)
| BJM/330 | 1958         | (+)       | FBN1 | Exon 60  | c.7540G>C         | p.Gly2514Arg| This study|

* Stop codon  
† Coded designations (letter code and numbers).  
‡ SS, skeletal system; OS, ocular system; CS, cardiovascular system; FH, family history; +, affected; (+), slightly affected; -, negative; ?, lack of information.  
§ Mutation analysis was performed using total DNA derived from EDTA blood samples (all patients) and total RNA derived from fibroblasts (Patients WP/58B and SB/374) according to our previously described mutation screening strategy, which has recently been extended by MLPA. Sequence variants are described in relation to the translation initiation site of the FBN1 mRNA reference sequence NM_000138.3. Mutation numbering is after HGVS standards (http://www.hgvs.org/mutnomen), with +1 as the A of the ATG initiation codon.  
\(\) RT-PCR analysis of the silent mutation c.6354C>T (p.Ile2118Ile) revealed the skipping of exon 51 (c.6314_6379del), leading to the in-frame deletion of 22 amino acids (p.Glu2105_Val2126del).
### Supplemental Table II. Identification of protein spots by mass spectrometry after in-gel digestion.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein</th>
<th>Accession no.</th>
<th>MALDI-TOF-MS/MS</th>
<th>LC-LTQ-Orbitrap</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MASCOT score</td>
<td>Unique peptides</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sequence coverage (%)</td>
<td>No. of matching peptides/MS-MS</td>
</tr>
<tr>
<td>1</td>
<td>Myosin-10</td>
<td>P35580</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>Vinculin</td>
<td>P18206</td>
<td>117</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>FLNA protein/Filamin A</td>
<td>Q96C61 P21333</td>
<td>113</td>
<td>84</td>
</tr>
<tr>
<td>4</td>
<td>Microfibril associated glycoprotein</td>
<td>P55083</td>
<td>94</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>Calponin 1</td>
<td>P51911</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Calponin 1</td>
<td>P51911</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Vitamin-D binding protein</td>
<td>UPI000013D9E0</td>
<td>62</td>
<td>22</td>
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<tr>
<td></td>
<td>Protein Name</td>
<td>Accession Number</td>
<td>Score1</td>
<td>Score2</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------</td>
<td>------------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>8</td>
<td>Fructose-bisphosphate aldolase C</td>
<td>P09972</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Apolipoprotein A1</td>
<td>P02647</td>
<td>66</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>Serum amyloid P</td>
<td>P02743</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Transgelin</td>
<td>Q5U0D2</td>
<td>67</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q01995</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Hemoglobin beta chain</td>
<td>UPI0000110332</td>
<td>117</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P68871</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Hemoglobin alpha chain</td>
<td>P68871</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Supplemental Table III:** α-2 Spectrin and Filamin A cleavage in ascending aortic aneurysms.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MFS</th>
<th>BAV</th>
<th>TAV*</th>
<th>P (TAV vs controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>15</td>
<td>22</td>
<td>9</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>α-2 Spectrin cleavage</td>
<td>0.70±0.08</td>
<td>1.05±0.09</td>
<td>1.15±0.10</td>
<td>1.56±0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Filamin A cleavage</td>
<td>2.04±0.21</td>
<td>2.64±0.17</td>
<td>3.04±0.21</td>
<td>5.98±0.49</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*TAV: Aortic aneurysm from patients with tricuspid aortic valve and without Marfan syndrome (N=17, gender: 71±11% male, mean age: 65±3 years, mean aortic diameter: 5.1±0.2cm, AT1 receptor blockers or ACE inhibitors: 53%, beta-blockers: 76%).

**Supplemental Figures**

![Graph showing correlation between aortic diameter and α-2 Spectrin cleavage](image)
Supplemental Figure Legends

Supplemental Figure I: Correlation analysis between aortic diameter and α-2 spectrin cleavage. Spectrin cleavage correlated significantly with increasing aortic diameter. MFS (●), BAV (▲) and control patients (○). Dashed lines indicate the 95% confidence interval.

Supplemental References


