



**HAL**  
open science

## Altered TGFbeta signaling and cardiovascular manifestations in patients with autosomal recessive cutis laxa type I caused by fibulin-4 deficiency

Bart Loeys, Marjolijn Renard, Tammy Holm, Regan Veith, Bert L. Callewaert, Lesley C. Adès, Osman Baspinar, Angela Pickart, Majed Dasouki, Juliane Hoyer, et al.

### ► To cite this version:

Bart Loeys, Marjolijn Renard, Tammy Holm, Regan Veith, Bert L. Callewaert, et al.. Altered TGF-beta signaling and cardiovascular manifestations in patients with autosomal recessive cutis laxa type I caused by fibulin-4 deficiency. *European Journal of Human Genetics*, 2010, 10.1038/ejhg.2010.45 . hal-00526205

**HAL Id: hal-00526205**

**<https://hal.science/hal-00526205>**

Submitted on 14 Oct 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Altered TGFbeta signaling and cardiovascular manifestations in patients with**  
2 **autosomal recessive cutis laxa type I caused by fibulin-4 deficiency**

3 Marjolijn Renard<sup>1</sup>, Tammy Holm<sup>2</sup>, Regan Veith<sup>3</sup>, Bert L. Callewaert<sup>1</sup>, Lesley C. Adès<sup>4</sup>,  
4 Osman Baspinar<sup>5</sup>, Angela Pickart<sup>3</sup>, Majed Dasouki<sup>6</sup>, Juliane Hoyer<sup>7</sup>, Anita Rauch<sup>8</sup>, Pamela  
5 Trapane<sup>9</sup>, Michael G. Earing<sup>9</sup>, Paul J. Coucke<sup>1</sup>, Lynn Y. Sakai<sup>10</sup>, Harry C. Dietz<sup>2,11</sup>, Anne  
6 M. De Paepe<sup>1</sup>, Bart L. Loeys<sup>1</sup>

7 <sup>1</sup> Center for Medical Genetics, Ghent University, Ghent, Belgium, <sup>2</sup> McKusick-Nathans  
8 Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore,  
9 MD, USA, <sup>3</sup> Department of Medical Genetics, Children's Hospital of Wisconsin,  
10 Milwaukee, WI, USA, <sup>4</sup> Department of Clinical Genetics, The Children's Hospital at  
11 Westmead, and Discipline of Paediatrics and Child Health, Sydney, Australia, <sup>5</sup> Department  
12 of Paediatrics, Gaziantep University, Gaziantep, Turkey, <sup>6</sup> Departments of Paediatrics and  
13 Internal Medicine, University of Kansas Medical Center, Kansas City, KS, USA, <sup>7</sup> Institute  
14 of Human Genetics, Friedrich-Alexander University of Erlangen Nuremberg, Erlangen,  
15 Germany, <sup>8</sup> Institute of Human Genetics, University of Zurich, Zurich-Schwerzenbach,  
16 Switzerland, <sup>9</sup> Genetics Center, Department of Paediatrics, Medical College of Wisconsin,  
17 Milwaukee, WI, USA, <sup>10</sup> Department of Biochemistry and Molecular Biology and Shriners  
18 Hospital for Children, Oregon Health & Science University, Portland, OR, USA, <sup>11</sup> Howard  
19 Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD,  
20 USA

21 \* To whom correspondence should be addressed at: Bart L. Loeys, Center for Medical  
22 Genetics, De Pintelaan 185, B-9000 Ghent, Belgium, phone 0032-9-332.36.03, fax 0032-9-  
23 332.49.70, e-mail [Bart.Loeys@ugent.be](mailto:Bart.Loeys@ugent.be)

24

25 Running title: TGF $\beta$  signaling in patients with *FBLN4* deficiency

26

27 **ABSTRACT**

28

29 Fibulin-4 is a member of the fibulin family, a group of extracellular matrix proteins  
30 prominently expressed in medial layers of large veins and arteries. Involvement of the  
31 *FBLN4* gene in cardiovascular pathology was shown in a murine model and in three  
32 patients affected with cutis laxa in association with systemic involvement. In order to  
33 elucidate the contribution of *FBLN4* in human disease, we investigated two cohorts of  
34 patients. Direct sequencing of 17 patients with cutis laxa revealed no *FBLN4* mutations. In  
35 a second group of 22 patients presenting with arterial tortuosity, stenosis and aneurysms,  
36 *FBLN4* mutations were identified in three patients, two homozygous missense mutations  
37 (*p.Glu126Lys* and *p.Ala397Thr*) and compound heterozygosity for missense mutation  
38 *p.Glu126Val* and frameshift mutation *c.577delC*. Immunoblotting analysis showed a  
39 decreased amount of fibulin-4 protein in the fibroblast culture media of two patients, a  
40 finding sustained by diminished fibulin-4 in the extracellular matrix of the aortic wall on  
41 immunohistochemistry. pSmad2 and CTGF immunostaining of aortic and lung tissue  
42 revealed an increase in TGF $\beta$  signaling. This was confirmed by pSmad2 immunoblotting  
43 of fibroblast cultures. In conclusion, patients with recessive *FBLN4* mutations are  
44 predominantly characterized by aortic aneurysms, arterial tortuosity and stenosis. This  
45 confirms the important role of fibulin-4 in vascular elastic fiber assembly. Furthermore, we

46 provide the first evidence for the involvement of altered TGFbeta signaling in the  
47 pathogenesis of *FBLN4* mutations in humans.

48

#### 49 **KEYWORDS**

50 TGFbeta, fibulin-4, cutis laxa, aortic aneurysm, arterial tortuosity

51

#### 52 **INTRODUCTION**

53

54       Fibulins are a seven-member family of extracellular matrix proteins that play a role  
55 in both elastic fiber assembly and function <sup>1-4</sup>. Fibulin-4 is prominently expressed in the  
56 medial layers of large veins and arteries and in small capillaries <sup>5</sup>. The importance of  
57 fibulin-4 to elastogenesis was shown in studies of *fibulin-4*<sup>-/-</sup> mice which have severe lung  
58 and vascular defects including emphysema, aortic/arterial aneurysms or stenosis and  
59 arterial tortuosity <sup>6-7</sup>. In the aorta of the *fibulin-4*<sup>-/-</sup> mouse, an increase in TGFbeta signaling  
60 was shown <sup>6</sup>, a phenomenon that has previously been described in other human aortic  
61 aneurysm syndromes, including Marfan syndrome (MFS, MIM 154700), Loeys-Dietz  
62 syndrome (LDS, MIM 610168 and 609192) and Arterial Tortuosity Syndrome (ATS, MIM  
63 208050) <sup>8-10</sup>. Recently, it was shown that fibulin-4 deficiency not only leads to altered  
64 elastic fiber assembly due to its role in elastogenesis, but also results in a defective smooth  
65 muscle cell terminal differentiation with downregulation of smooth muscle contractile  
66 proteins <sup>11</sup>. Both processes can potentially contribute, separately or in concert, to aneurysm  
67 formation.

68           So far, *FBLN4* (also called *EFEMP2*) mutations have been described in three  
69 patients with autosomal recessive cutis laxa type I (MIM 219100)<sup>12-14</sup>. These patients  
70 presented prenatally with fragmented elastic tissues, cutis laxa and variable pulmonary and  
71 cardiovascular involvement (Table 1). Cardiorespiratory complications often lead to death  
72 during childhood. Mutations in *FBLN5*, the gene encoding the fibulin-5 protein, also cause  
73 autosomal recessive and autosomal dominant cutis laxa type I<sup>15-18</sup> and are typically  
74 associated with pulmonary emphysema. On the other hand, the X-linked form (MIM  
75 304150), caused by mutations in the gene encoding a copper transporting ATPase (*ATP7A*),  
76 is now classified within the group of copper deficiency syndromes. Type II autosomal  
77 recessive cutis laxa (MIM 219200) presents with joint laxity and developmental delay.  
78 Recently, it was shown that mutations in the *ATP6V0A2* gene, encoding the  $\alpha 2$  unit of the  
79 V-type H<sup>+</sup> ATPase, resulted in this cutis laxa subtype by causing impaired glycosylation<sup>19</sup>.  
80 An additional form of autosomal recessive cutis laxa resembling type II is De Barsy  
81 syndrome (MIM 219150). Patients have a progeroid-like appearance, typical facial features,  
82 growth delay and cutis laxa<sup>20-21</sup>. Recently, disease causing mutations were identified in  
83 *PYCRI*, the gene encoding pyrroline-5-carboxylate reductase 1<sup>22-23</sup>. Patients presenting  
84 with autosomal dominant cutis laxa (MIM 123700), caused by alterations in the gene  
85 encoding the elastin protein, *ELN*<sup>24-27</sup>, have a normal life span and internal organ  
86 abnormalities are less prominent.

87           In this study we screened *FBLN4*, which encodes the fibulin-4 protein, in two  
88 phenotypically distinct cohorts, the first with cutis laxa, and the second with arterial  
89 tortuosity, stenosis and aneurysms. A potential link with TGFbeta-signaling and the effect  
90 of the different mutations on fibulin-4 protein expression were investigated.

91

## 92 **MATERIALS AND METHODS**

### 93 **Patient data**

94           Two patient cohorts were investigated in this study. The first cohort consisted of 17  
95 patients with predominant cutis laxa without major cardiovascular findings (supplementary  
96 table S1). The 22 patients in the second cohort had mild skin involvement but significant  
97 cardiovascular features, such as arterial tortuosity, stenosis and aneurysms (supplementary  
98 table S1). We describe here the clinical details of all three patients in whom we found  
99 *FBLN4* mutations, and summarize the clinical data of two previously reported patients from  
100 whom we studied fibroblasts or tissues.

101           A currently 20-year-old female (patient 1) presented at the age of 2 months with  
102 airway compression, ascending aortic aneurysm, proximal pulmonary arterial stenosis,  
103 distal pulmonary arterial dilatation and innominate artery dilatation. She underwent  
104 cardiovascular surgery at 2.5, 7 and 8 months of age. By age 39 months, she had abdominal  
105 arterial tortuosity and dilatation. Clinical findings included a high arched palate,  
106 micrognathia, mild joint hypermobility, velvety skin, no cutis laxa and normal scarring. A  
107 cerebral angiogram at the age of 5 years showed arterial tortuosity of the internal carotid,  
108 anterior/middle cerebral, and vertebral arteries. Histology of aortic biopsies showed  
109 disrupted elastic fibers and increased deposition of glycosaminoglycans. Detailed clinical  
110 description has been published previously<sup>28</sup>.

111           Patient 2 is a 3-year-old boy with a flat face, prominent forehead, hypertelorism,  
112 highly arched palate, pectus excavatum and joint hypermobility. He had a wide  
113 mediastinum on routine chest radiography. Echocardiography showed a dilated ascending

114 aorta and aortography showed pseudocoarctation and aortic tortuosity. The clinical features  
115 of this patient have been reported previously <sup>29</sup>. To date, the patient is 7 years old and the  
116 dilatation of his ascending aorta increased to 54 mm. The patient is asymptomatic.

117 Patient 3 was born at 37 weeks of gestation. Clinical features included a prominent  
118 forehead, mild hypertelorism, down-slanting palpebral fissures, depressed nasal bridge,  
119 low-set and distorted external ears and long fingers. An echocardiogram showed severe  
120 dilatation of the ascending aorta and severe tortuosity of the entire aorta with hypoplasia of  
121 the transverse aortic arch, proximal descending, thoracic, and abdominal aorta. MRI  
122 showed severe tortuosity of the carotid and cerebral arteries. At 5 weeks of age, she  
123 suffered a large hemispheric stroke. She died from cardiorespiratory failure at  
124 approximately 18 months. Autopsy was declined. Neither parent of patient 3 had  
125 phenotypic characteristics of ARCL type I.

126 Patient 4 was described recently <sup>12</sup>. This female patient presented with mild cutis  
127 laxa, arachnodactyly and systemic involvement, including pulmonary hypertension, mild  
128 tricuspid valve insufficiency, abdominal aortic tortuosity, and dilatation of the ascending  
129 aorta and the main branches of the pulmonary arteries, with dissection. The parents denied  
130 consanguinity. She died at 27 days of age from respiratory distress and inoperable systemic  
131 vascular abnormalities.

132 Patient 5 is a child from consanguineous parents. She died shortly after birth from  
133 profound bradycardia. Clinical features included facial dysmorphism, cutis laxa, joint  
134 contractures and arachnodactyly. She had a thickened myocardium with a minimal  
135 pericardial effusion, emphysema and fragile vessels. A detailed clinical description has  
136 been reported recently <sup>14</sup>.

137

### 138 **Molecular analysis**

139 For patients 1-3, genomic DNA was extracted from EDTA blood samples using the  
140 Puregene method (Qiagen, Venlo, The Netherlands). All coding exons and flanking introns  
141 of *FBLN4* were amplified by PCR on genomic DNA level (primer sequences are available  
142 upon request). Products were sequenced using the BigDye terminator cycle sequencing  
143 method on the ABI3730 XL automatic sequencer (Applied Biosystems, Halle, Belgium).

144

### 145 **Cell culture**

146 Skin fibroblasts were available from patients 1 and 5, and age- and sex-matched  
147 controls. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM,  
148 Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS,  
149 Invitrogen) in the presence of antibiotics at 37°C in 5% CO<sub>2</sub>.

150

### 151 **Protein analysis**

152 Confluent fibroblasts of patient 1 and patient 5 and age- and sex-matched controls  
153 were grown in DMEM medium in the absence of FCS for 24 hours. Media were harvested  
154 and concentrated (Centriprep-30, Millipore, Brussels, Belgium) in the presence of protease  
155 inhibitors (Complete Mini tablet, Roche Applied Science, Vilvoorde, Belgium). Protein  
156 samples were loaded on gel together with 5x non-reducing lane marker sample buffer  
157 (Thermo Fisher Scientific, Aalst, Belgium). SDS-PAGE electrophoresis was performed  
158 using NuPage 4-12% Bis-Tris precast gels (Invitrogen) followed by electrotransfer of the  
159 proteins onto a Hybond-ECL nitrocellulose membrane (GE healthcare) using the iBlot



160 system (Invitrogen). Blocking of the membrane was done in 2% ECL-advantage buffer (GE  
161 healthcare). Immunoblotting was carried out using a mouse monoclonal anti-human fibulin-  
162 4 antibody (clone 226,347,597) (1:500) in combination with a mouse monoclonal anti-  
163 human fibronectin-1 antibody (clone 84) (1:500), followed by incubation with the  
164 secondary antibody ECLplex goat-anti-mouse IgG-Cy5 (1/2000) (GE healthcare).  
165 Monoclonal antibodies were generated in mice immunized with full-length recombinant  
166 human fibulin-4<sup>30</sup>. Epitopes for all three monoclonal antibodies are located in the N-  
167 terminal half of fibulin-4. Membranes were scanned using the Typhoon 9400 (GE  
168 healthcare). Immunoblotting of pSmad2 was performed as previously described<sup>31</sup>.  
169 Quantification of the immunoblots was performed using ImageJ software.

170

### 171 **Histological analysis**

172 Paraffin-embedded lung and aortic tissue were available from patient 4. For  
173 immunohistochemical staining of fibulin-4 (clone 347), connective tissue growth factor  
174 (CTGF) and pSmad2 (the phosphorylated form of Smad2) in lung and aortic tissue, we  
175 selected representative specimens from three healthy control individuals. From these  
176 formalin-fixed, paraffin-embedded specimens, 5µm thick sections were cut, deparaffinized  
177 and rehydrated. Antigens were unmasked using 10mM sodium citrate buffer and auto-  
178 peroxidase activity was inhibited. Sections were blocked with normal goat serum (pSmad2  
179 and CTGF) or normal horse serum (fibulin-4) (Vectastain, Burlingame, CA, USA).  
180 Antibodies directed against CTGF (Abcam, Cambridge, UK), pSmad2 (Ser465/467) (Cell  
181 Signaling Technology, Boston, MA, USA), and fibulin-4 (see protein analysis section)  
182 were used. Subsequently, sections were incubated with a secondary antibody, goat-anti-

183 rabbit IgG (pSmad2 and CTGF) or horse-anti-mouse (fibulin-4) (Vectastain), ABC (Avidin:  
184 Biotinylated enzyme Complex) reagent (Vectastain) and DAB (3,3'-Diaminobenzidine)  
185 peroxidase (Vectastain). Sections were dehydrated in xylene and mounted. Light  
186 microscopy was performed on a Zeiss Axio Imager A1 microscope.

187

## 188 **RESULTS**

### 189 ***FBLN4* mutation screening**

190 We sequenced *FBLN4* in two cohorts of patients. The 17 patients of the first cohort  
191 presented predominantly with cutis laxa and had screened negative previously for *ELN* and  
192 *FBLN5* mutations. The second cohort included 22 patients who were diagnosed with  
193 arterial tortuosity, stenosis and aneurysms. This cohort screened negative previously for  
194 *SLC2A10*, *FBN1*, *TGFBR1* and *TGFBR2* mutations. Molecular study of the first cohort did  
195 not identify any *FBLN4* mutations. In the second cohort *FBLN4* mutations were identified  
196 in three patients. Patients 1 and 2 have homozygous missense mutations, *c.376G>A*  
197 (*p.Glu126Lys*) and *c.1189G>A* (*p.Ala397Thr*) (Figure 2A and B). The first mutation  
198 replaces the same glutamic acid residue from the EGF consensus sequence as the  
199 previously reported *p.Glu57Lys*<sup>13</sup> but in a different cbEGF domain, whereas the second  
200 mutation affects a highly conserved amino acid in the fibulin-type module. The parents of  
201 patient 2 were heterozygous for the *p.Ala397Thr* mutation. The third patient (Figure 1)  
202 harbors a missense mutation, *c.377A>T* (*p.Glu126Val*), in combination with a frameshift  
203 mutation, *c.577delC* leading to a premature termination codon (*p.Gln193Ser fs X12*)  
204 (Figure 2C). Parental studies confirmed that these mutations occurred in *trans*. The  
205 missense mutation in patient 3 affects the same glutamic acid residue (at position 126) as

206 identified in patient 1, but leads to a different amino acid substitution. Parental samples  
207 were not available for molecular analysis. None of the mutations were identified in 200  
208 control chromosomes. An overview of the molecular and clinical data of these three  
209 patients and the *FBLN4*-mutation positive patients described previously in the literature  
210 (patients 4-6) is given in Table 1.

211

### 212 **Fibulin-4 expression in human skin fibroblasts and aortic tissue**

213 To investigate the effect of the *FBLN4* mutations on the expression of fibulin-4  
214 protein, an immunoblotting experiment was performed on the culture medium of dermal  
215 fibroblasts of patient 1 (homozygous *c.376G>A* (*p.Glu126Lys*) missense mutation), patient  
216 5 (homozygous *c.800G>A* (*p.Cys267Tyr*) missense mutation), and two age- and sex-  
217 matched controls. Immunoblotting showed a distinct protein band of approximately 49 kDa  
218 in the culture medium of the control samples. In patient 5 a complete absence and in patient  
219 1 a slightly diminished amount of fibulin-4 in the culture medium was observed (Figure  
220 3B). In addition, we performed immunohistochemical staining of fibulin-4 on aortic tissue  
221 of a control individual and patient 4, which confirmed the near-absence of extracellular  
222 fibulin-4 in the aortic wall of patient 4 in comparison to its control. Similar intracellular  
223 fibulin-4 staining was observed in both individuals (Figure 3A). These results suggest that  
224 the mutant proteins are either not secreted, or are secreted into the extracellular space to a  
225 lesser extent. The impaired secretion of mutant fibulin-4 leads to a decrease of available  
226 fibulin-4 protein in the extracellular matrix for elastogenesis, and disturbed elastic fiber  
227 formation.

228

229 **Increased TGFbeta signaling in patients with *FBLN4* mutations**

230 To determine whether mutations in the human *FBLN4* gene have an effect on the  
231 TGFbeta signaling pathway, immunohistochemical staining for phosphorylated Smad2  
232 (pSmad2, an effector of TGFbeta signaling) and CTGF (a TGFbeta-driven gene product) in  
233 the aorta and lungs of a normal individual and patient 4 was performed. We demonstrated a  
234 more intense and increased nuclear pSmad2 staining in the aorta and lung of the patient that  
235 was not present in the control tissues (Figure 4). Following TGFbeta stimulation,  
236 immunoblotting indicated a significantly increased pSmad2 signal in fibroblast cultures of  
237 patient 1 and patient 5 in comparison with their respective controls (Figure 5). These  
238 findings suggest that mutations in *FBLN4* have an effect on the TGFbeta signaling  
239 pathway. This hypothesis is supported by increased intensity of staining for CTGF in the  
240 aortic and lung tissue of patient 4.

241

242 **DISCUSSION**

243

244 We identified *FBLN4* mutations in three unrelated patients clinically characterized  
245 by major cardiovascular defects, including aortic aneurysms, arterial tortuosity and  
246 stenosis, and minor skin involvement. This phenotype is different from that seen in patients  
247 with mutations in the genes encoding the elastin protein, *ELN*, and the fibulin-5 protein,  
248 *FBLN5*, in which skin involvement is the most prominent clinical finding. Although the  
249 patients described in this paper share clinical characteristics with previously reported  
250 *FBLN4* mutation positive patients<sup>12-14</sup>, there is considerable clinical overlap with Loeys-  
251 Dietz syndrome (LDS) and Arterial Tortuosity syndrome (ATS). LDS is an autosomal

252 dominant disorder characterized by a triad of arterial tortuosity and aneurysms,  
253 hypertelorism and bifid uvula or cleft palate. The disorder is caused by mutations in the  
254 genes encoding transforming growth factor beta receptor 1 and 2 (*TGFBRI* and *TGFBR2*,  
255 respectively) <sup>9,31</sup>. The autosomal recessive disorder, ATS, is characterized by tortuosity,  
256 elongation, stenosis and aneurysm formation of the major arteries <sup>32</sup>. Coucke *et al.* <sup>8</sup>  
257 identified *SLC2A10*, the gene encoding a facilitative glucose transporter GLUT10, as the  
258 causative gene. The patients investigated in this study were referred because of clinical  
259 features reminiscent of one or more of these disorders. This report doubles the number of  
260 known patients with *FBLN4* mutations <sup>12-14</sup>. Our findings underscore the prominent  
261 vascular involvement and the rather mild cutaneous findings associated with *FBLN4*  
262 mutations. As such, screening of *FBLN4* should be considered particularly in patients with  
263 findings suggestive of LDS or ATS, in whom *TGFBRI*, *TGFBR2* or *SLC2A10* mutation  
264 screening is negative. We report for the first time long-term survival in patient 1 and patient  
265 2, 20 and 7 years old, respectively. This is an interesting observation in view of the early  
266 lethality previously reported in *FBLN4*-mutation positive patients and *Fbln4*-deficient mice  
267 <sup>7</sup>.

268 We report four novel *FBLN4* mutations. Several lines of evidence suggest the  
269 disease causing nature of these mutations. First, the three missense mutations cause either a  
270 change of charge (*p.Glu126Lys* and *p.Glu126Val*), a change of hydrophilic side chain to  
271 hydrophobic side chain (*p.Glu126Val*), or the addition of a hydroxyl group (*p.Ala397Thr*).  
272 Secondly, these missense mutations modify highly conserved residues within the fibulin-4  
273 sequence. The glutamic acid residue at position 126 is located in the DINE consensus  
274 sequence of the second cbEFG-like module, which is essential for Ca<sup>2+</sup> binding. Ca<sup>2+</sup>

275 binding of EGF modules is crucial for module-module interactions, and hence, for the  
276 structural and binding properties of the intact protein <sup>33</sup>. Third, *in silico* prediction  
277 programs, Polyphen and SIFT, classify the missense mutations as probably (*p.Glu126Lys*  
278 and *p.Glu126Val*) or possibly (*p.Ala397Thr*) damaging and not tolerated, respectively.  
279 Finally, the alanine residue at position 397 is highly conserved among species and between  
280 the homologous fibulin-3, -4 and -5 proteins (Figure 2B).

281 Immunoblotting analysis showed the absence (patient 5) or a slightly decreased  
282 amount (patient 1) of the mutant fibulin-4 protein in the culture media of the patients in  
283 comparison with the controls. This result mirrors the difference in disease severity  
284 manifested clinically between these two patients. A possible explanation for the difference  
285 in secretion between these two patients is that the homozygous alteration of a cysteine  
286 residue in patient 5 (*p.Cys267Tyr*) has a more dramatic effect on protein folding and  
287 trafficking by the altered disulfide bridge formation compared to the glutamic acid to lysine  
288 alteration in patient 1. However, two previously reported *FBLN4* mutations, *p.Glu57Lys* <sup>13</sup>  
289 and *p.Arg279Cys* in combination with *c.1070\_1073dupCCGC* <sup>12</sup> show complete absence of  
290 the mutant protein in the extracellular matrix, the latter was confirmed in this report by  
291 immunohistochemical staining of fibulin-4 on aortic tissue. This indicates that the  
292 diminished amount of mutant fibulin-4 present in the extracellular matrix of patient 1 is the  
293 first example of a patient with *FBLN4* mutation with the production and secretion of the  
294 mutant protein. This might be in accordance with the longer survival of the patient. Given  
295 these observations we suggest that mutations in *FBLN4* impair the stability and/or secretion  
296 of fibulin-4, similarly to prior findings for fibulin-5 mutations <sup>34</sup>. The resulting decrease of  
297 protein in the extracellular matrix leads to altered interactions with fibulin-4 binding

298 partners and subsequently, to impaired elastogenesis. Fibulin-4 is known to associate with  
299 tropoelastin, possibly connecting elastin to the microfibrils to form the elastic fibers <sup>30</sup>.  
300 Secondly, fibulin-4 also strongly binds to the N-terminal region of fibrillin-1 in the  
301 presence of Ca<sup>2+</sup> <sup>35-36</sup>. As suggested by Ono and colleagues <sup>36</sup>, fibulin-4 competes with  
302 latent TGFbeta binding proteins (LTBPs) for binding to fibrillin-1. As such, this fibulin  
303 protein may play a role in the modulation of LTBP sequestration by the extracellular  
304 matrix, an event important in the regulation of the bioavailability of TGFbeta for activation  
305 and signaling. As a result of impaired mutant fibulin-4 secretion and/or stability and the  
306 resulting impaired elastogenesis, we believe that LTBP is no longer able to bind the  
307 fragmented elastic fibers. The TGFbeta-LAP (latency associated peptide) complex is  
308 released and TGFbeta is subsequently more prone to activation. An analogous mechanism  
309 has previously been suggested to occur in MFS and LDS <sup>10,31,37</sup>. Additionally, the resulting  
310 decrease of mutant fibulin-4 protein in the extracellular matrix may lead to defective  
311 smooth muscle terminal differentiation as recently suggested by Huang and colleagues.  
312 They hypothesized that fibulin-4 deficiency not only results in aneurysm formation through  
313 defective elastic fiber formation, but also through extracellular regulation of smooth muscle  
314 cell differentiation genes <sup>11</sup>.

315 To investigate whether increased TGFbeta signaling is involved in the pathogenesis  
316 of autosomal recessive cutis laxa type I in humans, as predicted based on the observations  
317 of impaired TGFbeta signaling in *Fbln4*<sup>-/-</sup> mice by Hanada and colleagues <sup>6</sup>, we assessed  
318 the level of pSmad2 and CTGF expression in aorta and lung and subsequently performed  
319 pSmad2 immunoblotting on fibroblast cultures before and after TGFbeta stimulation.  
320 TGFbeta-stimulated fibroblast cultures of patients with *FBLN4* mutations in this study

321 showed an increased and more intense level of phosphorylated Smad2 compared to  
322 controls. This finding is in line with an “in vivo” increase in TGFbeta signaling  
323 demonstrated in the aorta and lung of the fibulin-4 deficient patient 4. The increased  
324 TGFbeta signaling in aortic tissue of the *FBLN4*-mutation positive patients confirms the  
325 key role of this signaling pathway in the pathogenesis of aortic and arterial aneurysms and  
326 tortuosity, as demonstrated previously in other human diseases such as MFS, LDS and ATS  
327 <sup>8-10,32</sup>. As already shown in Marfan mouse models, this finding offers the potential for  
328 treatment by blocking TGFbeta signaling through the use of angiotensin receptor type 1  
329 blockers such as losartan <sup>38</sup>.

330

### 331 **ACKNOWLEDGEMENTS**

332 We are indebted to P. Willems for providing skin fibroblasts of *FBLN4*-mutation positive  
333 patients, to D. Zwick and A. Kats for providing sections of aorta and lung tissue of patient  
334 5, and L. Myers for the optimized pSmad2 immunohistochemical protocol. We are very  
335 grateful to N. Charbonneau for generating the antibodies to fibulin-4, T. Sasaki for  
336 recombinant human fibulin-4, and the National Marfan Foundation for providing funding to  
337 generate the fibulin-4 monoclonal antibodies. We thank K. Wettinck for excellent technical  
338 assistance with the molecular analyses. B. Loeys and M. Renard are, respectively, senior  
339 clinical investigator and junior scientific investigator supported by the Fund for Scientific  
340 Research, Flanders (Belgium). This work was supported by the Fund for Scientific  
341 Research, Flanders (Belgium) [G.0094.06]; Fighting Aneurysmal Disease [EC-FP7]; and  
342 Methusalem [08/01M01108 to A.D.P.].

343



344 **CONFLICT OF INTEREST**

345 The authors declare no conflict of interest.

346

347 Supplementary information is available at European Journal of Human Genetics' website.

348

349 **REFERENCES**

- 350 1. Argraves WS, Greene LM, Cooley MA, Gallagher WM: Fibulins: physiological and disease  
351 perspectives. *EMBO Rep* 2003; **4**: 1127-1131.  
352
- 353 2. Nakamura T, Lozano PR, Ikeda Y *et al*: Fibulin-5/DANCE is essential for elastogenesis in vivo.  
354 *Nature* 2002; **415**: 171-175.  
355
- 356 3. Sasaki T, Gohring W, Miosge N, Abrams WR, Rosenbloom J, Timpl R: Tropoelastin binding  
357 to fibulins, nidogen-2 and other extracellular matrix proteins. *FEBS Lett* 1999; **460**: 280-  
358 284.  
359
- 360 4. Yanagisawa H, Davis EC, Starcher BC *et al*: Fibulin-5 is an elastin-binding protein essential  
361 for elastic fibre development in vivo. *Nature* 2002; **415**: 168-171.  
362
- 363 5. Giltay R, Timpl R, Kostka G: Sequence, recombinant expression and tissue localization of  
364 two novel extracellular matrix proteins, fibulin-3 and fibulin-4. *Matrix Biol* 1999; **18**: 469-  
365 480.  
366
- 367 6. Hanada K, Vermeij M, Garinis GA *et al*: Perturbations of vascular homeostasis and aortic  
368 valve abnormalities in fibulin-4 deficient mice. *Circ Res* 2007; **100**: 738-746.  
369
- 370 7. McLaughlin PJ, Chen Q, Horiguchi M *et al*: Targeted disruption of fibulin-4 abolishes  
371 elastogenesis and causes perinatal lethality in mice. *Mol Cell Biol* 2006; **26**: 1700-1709.  
372
- 373 8. Coucke PJ, Willaert A, Wessels MW *et al*: Mutations in the facilitative glucose transporter  
374 GLUT10 alter angiogenesis and cause arterial tortuosity syndrome. *Nat Genet* 2006; **38**:  
375 452-457.  
376
- 377 9. Loeys BL, Schwarze U, Holm T *et al*: Aneurysm syndromes caused by mutations in the TGF-  
378 beta receptor. *N Engl J Med* 2006; **355**: 788-798.  
379
- 380 10. Neptune ER, Frischmeyer PA, Arking DE *et al*: Dysregulation of TGF-beta activation  
381 contributes to pathogenesis in Marfan syndrome. *Nat Genet* 2003; **33**: 407-411.  
382

- 383 11. Huang J, Davis EC, Chapman SL *et al*: Fibulin-4 Deficiency Results in Ascending Aortic  
384 Aneurysms. A Potential Link Between Abnormal Smooth Muscle Cell Phenotype and  
385 Aneurysm Progression. *Circ Res* 2009.  
386
- 387 12. Dasouki M, Markova D, Garola R *et al*: Compound heterozygous mutations in fibulin-4  
388 causing neonatal lethal pulmonary artery occlusion, aortic aneurysm, arachnodactyly, and  
389 mild cutis laxa. *Am J Med Genet A* 2007; **143**: 2635-2641.  
390
- 391 13. Huchtagowder V, Sausgruber N, Kim KH, Angle B, Marmorstein LY, Urban Z: Fibulin-4: a  
392 novel gene for an autosomal recessive cutis laxa syndrome. *Am J Hum Genet* 2006; **78**:  
393 1075-1080.  
394
- 395 14. Hoyer J, Kraus C, Hammersen G, Geppert JP, Rauch A: Lethal cutis laxa with contractural  
396 arachnodactyly, overgrowth and soft tissue bleeding due to a novel homozygous fibulin-4  
397 gene mutation. *Clin Genet* 2009.  
398
- 399 15. Claus S, Fischer J, Megarbane H *et al*: A p.C217R mutation in fibulin-5 from cutis laxa  
400 patients is associated with incomplete extracellular matrix formation in a skin equivalent  
401 model. *J Invest Dermatol* 2008; **128**: 1442-1450.  
402
- 403 16. Elahi E, Kalhor R, Banihosseini SS *et al*: Homozygous missense mutation in fibulin-5 in an  
404 Iranian autosomal recessive cutis laxa pedigree and associated haplotype. *J Invest*  
405 *Dermatol* 2006; **126**: 1506-1509.  
406
- 407 17. Loeys B, Van Maldergem L, Mortier G *et al*: Homozygosity for a missense mutation in  
408 fibulin-5 (FBLN5) results in a severe form of cutis laxa. *Hum Mol Genet* 2002; **11**: 2113-  
409 2118.  
410
- 411 18. Markova D, Zou Y, Ringpfeil F *et al*: Genetic heterogeneity of cutis laxa: a heterozygous  
412 tandem duplication within the fibulin-5 (FBLN5) gene. *Am J Hum Genet* 2003; **72**: 998-1004.  
413
- 414 19. Kornak U, Reynders E, Dimopoulou A *et al*: Impaired glycosylation and cutis laxa caused by  
415 mutations in the vesicular H<sup>+</sup>-ATPase subunit ATP6V0A2. *Nat Genet* 2008; **40**: 32-34.  
416
- 417 20. Kivuva EC, Parker MJ, Cohen MC, Wagner BE, Sobey G: De Barsy syndrome: a review of the  
418 phenotype. *Clin Dysmorphol* 2008; **17**: 99-107.  
419
- 420 21. Morava E, Guillard M, Lefeber DJ, Wevers RA: Autosomal recessive cutis laxa syndrome  
421 revisited. *Eur J Hum Genet* 2009.  
422
- 423 22. Guernsey DL, Jiang H, Evans SC *et al*: Mutation in pyrroline-5-carboxylate reductase 1 gene  
424 in families with cutis laxa type 2. *Am J Hum Genet* 2009; **85**: 120-129.  
425
- 426 23. Reversade B, Escande-Beillard N, Dimopoulou A *et al*: Mutations in PYCR1 cause cutis laxa  
427 with progeroid features. *Nat Genet* 2009; **41**: 1016-1021.  
428

- 429 24. Rodriguez-Reventa L, Iranzo P, Badenas C, Puig S, Carrio A, Mila M: A novel elastin gene  
430 mutation resulting in an autosomal dominant form of cutis laxa. *Arch Dermatol* 2004; **140**:  
431 1135-1139.  
432
- 433 25. Szabo Z, Crepeau MW, Mitchell AL *et al*: Aortic aneurysmal disease and cutis laxa caused  
434 by defects in the elastin gene. *J Med Genet* 2006; **43**: 255-258.  
435
- 436 26. Tassabehji M, Metcalfe K, Hurst J *et al*: An elastin gene mutation producing abnormal  
437 tropoelastin and abnormal elastic fibres in a patient with autosomal dominant cutis laxa.  
438 *Hum Mol Genet* 1998; **7**: 1021-1028.  
439
- 440 27. Zhang MC, He L, Giro M, Yong SL, Tiller GE, Davidson JM: Cutis laxa arising from frameshift  
441 mutations in exon 30 of the elastin gene (ELN). *J Biol Chem* 1999; **274**: 981-986.  
442
- 443 28. Ades LC, Knight WB, Byard RW *et al*: Clinicopathologic findings in congenital aneurysms of  
444 the great vessels. *Am J Med Genet* 1996; **66**: 289-299.  
445
- 446 29. Baspinar O, Kilinc M, Balat A, Celkan MA, Coskun Y: Long tortuous aorta in a child with  
447 Larsen syndrome. *Can J Cardiol* 2005; **21**: 299-301.  
448
- 449 30. Kobayashi N, Kostka G, Garbe JH *et al*: A comparative analysis of the fibulin protein family.  
450 Biochemical characterization, binding interactions, and tissue localization. *J Biol Chem*  
451 2007; **282**: 11805-11816.  
452
- 453 31. Loeys BL, Chen J, Neptune ER *et al*: A syndrome of altered cardiovascular, craniofacial,  
454 neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat*  
455 *Genet* 2005; **37**: 275-281.  
456
- 457 32. Callewaert BL, Willaert A, Kerstjens-Frederikse WS *et al*: Arterial tortuosity syndrome:  
458 clinical and molecular findings in 12 newly identified families. *Hum Mutat* 2008; **29**: 150-  
459 158.  
460
- 461 33. Rao Z, Handford P, Mayhew M, Knott V, Brownlee GG, Stuart D: The structure of a Ca(2+)-  
462 binding epidermal growth factor-like domain: its role in protein-protein interactions. *Cell*  
463 1995; **82**: 131-141.  
464
- 465 34. Hu Q, Reymond JL, Pinel N, Zobot MT, Urban Z: Inflammatory destruction of elastic fibers  
466 in acquired cutis laxa is associated with missense alleles in the elastin and fibulin-5 genes. *J*  
467 *Invest Dermatol* 2006; **126**: 283-290.  
468
- 469 35. Freeman LJ, Lomas A, Hodson N *et al*: Fibulin-5 interacts with fibrillin-1 molecules and  
470 microfibrils. *Biochem J* 2005; **388**: 1-5.  
471
- 472 36. Ono RN, Sengle G, Charbonneau NL *et al*: LTBPS and fibulins compete for fibrillin-1 and  
473 exhibit exquisite specificities in binding sites. *J Biol Chem* 2009.  
474

475 37. Ng CM, Cheng A, Myers LA *et al*: TGF-beta-dependent pathogenesis of mitral valve  
476 prolapse in a mouse model of Marfan syndrome. *J Clin Invest* 2004; **114**: 1586-1592.  
477  
478 38. Habashi JP, Judge DP, Holm TM *et al*: Losartan, an AT1 antagonist, prevents aortic  
479 aneurysm in a mouse model of Marfan syndrome. *Science* 2006; **312**: 117-121.  
480  
481  
482  
  
483  
  
484  
  
485  
  
486  
  
487  
  
488  
  
489  
  
490  
  
491  
  
492  
  
493  
  
494  
  
495  
  
496  
  
497  
  
498  
  
499  
  
500

501 **TITLES AND LEGENDS TO FIGURES**

502

503 **Figure 1. A.** Clinical photograph of patient 3 showing a prominent forehead with depressed  
504 nasal bridge, mild hypertelorism and downslanting palpebral fissures. The patient has a  
505 trachea canulae. **B.** MRI shows severe tortuosity of the carotid arteries (anterolateral  
506 oblique view). **C.** A posterior view of a 3D reconstruction of an MR angio showing  
507 successively, the dilated aorta ascendens (indicated with an arrow), hypoplasia of the  
508 transverse aorta (indicated with an arrowhead) and tortuous descending aorta.

509

510 **Figure 2.** DNA sequence of *FBLN4* in **A.** patient 1, showing the missense *c.376G>A*  
511 mutation, leading to the *Glu126Lys* substitution in the protein. The glutamic acid residue at  
512 position 126 is evolutionarily conserved and located in the DINE consensus sequence of the  
513 second cbEGF-like domain, in **B.** patient 2, showing the *c.1189G>A* mutation, leading to  
514 the *Ala397Thr* substitution. The alanine residue is located in the C-terminal fibulin-like  
515 module and is conserved between fibulin homologues and highly conserved among species,  
516 and in **C.** patient 3, showing the compound heterozygous *c.377A>T* missense (leading to  
517 the *Glu126Val* substitution) and *c.577delC* mutations. The missense mutation affects the  
518 same conserved glutamic acid residue as identified in patient 1 (see panel A for alignment  
519 of the conserved sequence).

520

521 **Figure 3. A.** Immunohistochemical staining of aortic tissue of patient 4 and its age- and  
522 sex-matched control. Intracellular fibulin-4 staining is seen in both individuals (examples of  
523 intracellular staining are indicated with a black arrow). No extracellular fibulin-4 is seen in

524 the aorta of the patient as compared to its control (extracellular staining is indicated with  
525 white arrows). Scale bars = 100µm. **B.** Immunoblot analysis of the cultured media using  
526 fibroblasts of age- and sex-matched controls (C1 and C5) and two patients (P1 and P5).  
527 Top panel: anti-fibulin-4 monoclonal antibody (clone 347) demonstrating impaired fibulin-  
528 4 secretion in patient 1 and slightly impaired fibulin-4 secretion in patient 5 in contrast to  
529 their matched control. Bottom panel: anti-fibronectin-1 monoclonal antibody indicating the  
530 amounts of samples loaded on the gel. The fibulin-4 secretion to the extracellular space as  
531 seen on the gel images was quantified using ImageJ software.

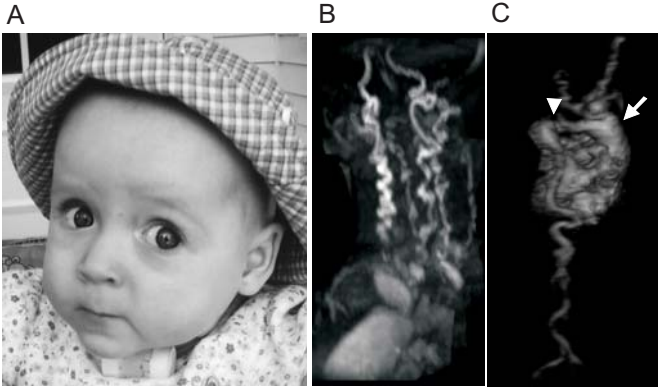
532

533 **Figure 4.** Immunohistochemical staining of aorta (A-D and I-L) and lung (E-H and M-P)  
534 tissue in *FBLN4*-mutation positive patient 4 (B,D,F,H,J,L,N,P) and in control  
535 (A,C,E,G,I,K,M,O). Increased nuclear accumulation of phosphorylated Smad2 is present in  
536 both the aorta (B,D) (some of the nuclei are indicated with an arrow) and the lung (F,H) of  
537 the patient, as compared with the control aorta (A,C) and lung (E,G). Similarly, increased  
538 levels of CTGF expression are noted in the aorta (J,L) and lung (N,P) of the patient,  
539 compared to control aorta (I,K) and lung (M,O). These results are indicative of increased  
540 TGFbeta signaling in the *FBLN4*-mutation positive patient. Scale bars = 100 µm.

541

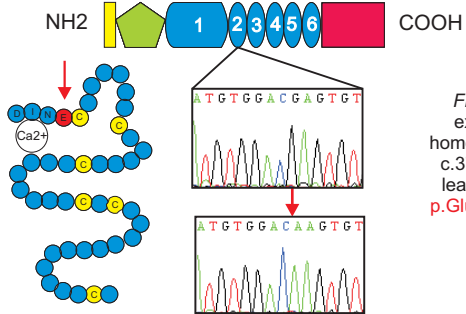
542 **Figure 5.** Immunoblot analysis of the fibroblast cell extracts of age- and sex-matched  
543 controls (C1 and C5) and patients 1 (P1) and 5 (P5). The graph and the pSmad2  
544 immunoblotting panel show an increase in pSmad2 level in patient 1 and patient 5 when  
545 cells were stimulated with TGFbeta protein in contrast to their controls. When cells are not  
546 stimulated basal expression of pSmad2 is seen. Bottom panel: anti-vinculin antibody,

547 indicating the amounts of samples loaded on the gel. This immunoblotting analysis is  
548 representative of three independent experiments (see supplemental figure S1).





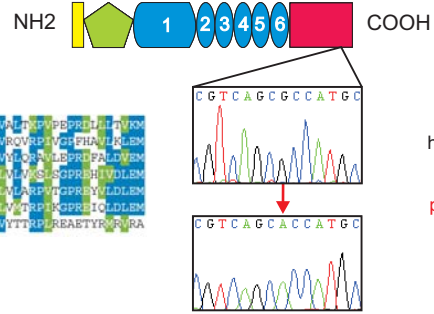
A



*FBLN4*  
exon 5  
homozygous  
c.376G>A  
leading to  
p.Glu126Lys

Homo	EPD	--	DQDSCVDVDE	CAQALHDCRPSQDCHNLPGSYQCTCPDGYRKIGPECVDIDECRYR	171
Pan	EPD	--	NQESCVDVDE	CAQALHDCRPSQDCHNLPGSYQCTCPDGYRKIGPECVDIDECRYR	170
Bos	EPD	--	EQE-----	-----RCVDIDECRYR	130
Mus	EPD	-	EQESCVDVDE	CTQALHDCRPSQDCHNLPGSYQCTCPDGYRKIGPECVDIDECRYR	171
Xenopus	QLDPHGQGT	CVDFE	EC	ENERHDCLPSEQVNCMLGSHCKCPDGYRKIGNECVDIDECRYR	178

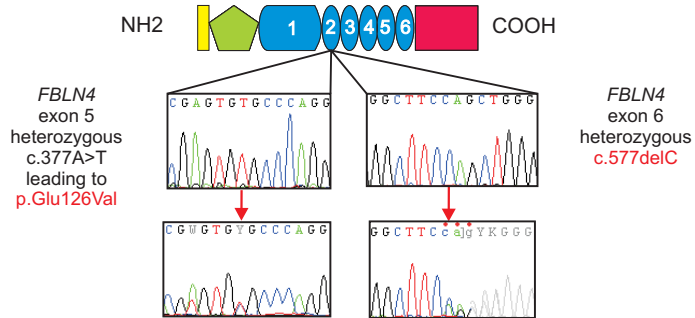
B



*FBLN4*  
exon 11  
homozygous  
c.1189G>A  
leading to  
p.Ala397Thr

Homo	TIT	SERSVPADV	FQIQATS	VYPGAYNAFQIRAGNSQGGDFYIRQINN	SA	MLVLARPVTGP	408	
Pan	TIT	SERSVPADV	FQIQATS	VYPGAYNAFQIRAGNSQGGDFYIRQINN	SA	MLVLARPVTGP	407	
Bos	SIT	SERSVPADV	FQIQATS	VYPGAYNAFQIRAGNSQGGDFYIRQINN	SA	MLVLARPVTGP	369	
Mus	SIT	SERSVPADV	FQIQATS	VYPGAYNAFQIRSGNTQGGDFYIRQINN	SA	MLVLARPVTGP	408	
Xenopus	SIT	SDRSVPSE	IFQIQATS	VYPGAYNTFQIRSGNEEGEFFI	RQINNL	SA	MLVLARPVIGP	416

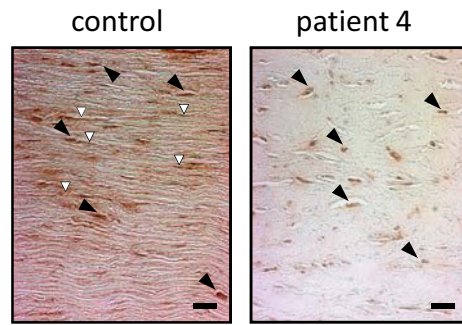
C



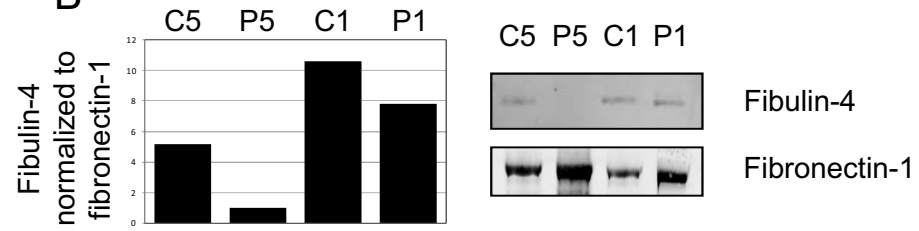
*FBLN4*  
exon 5  
heterozygous  
c.377A>T  
leading to  
p.Glu126Val

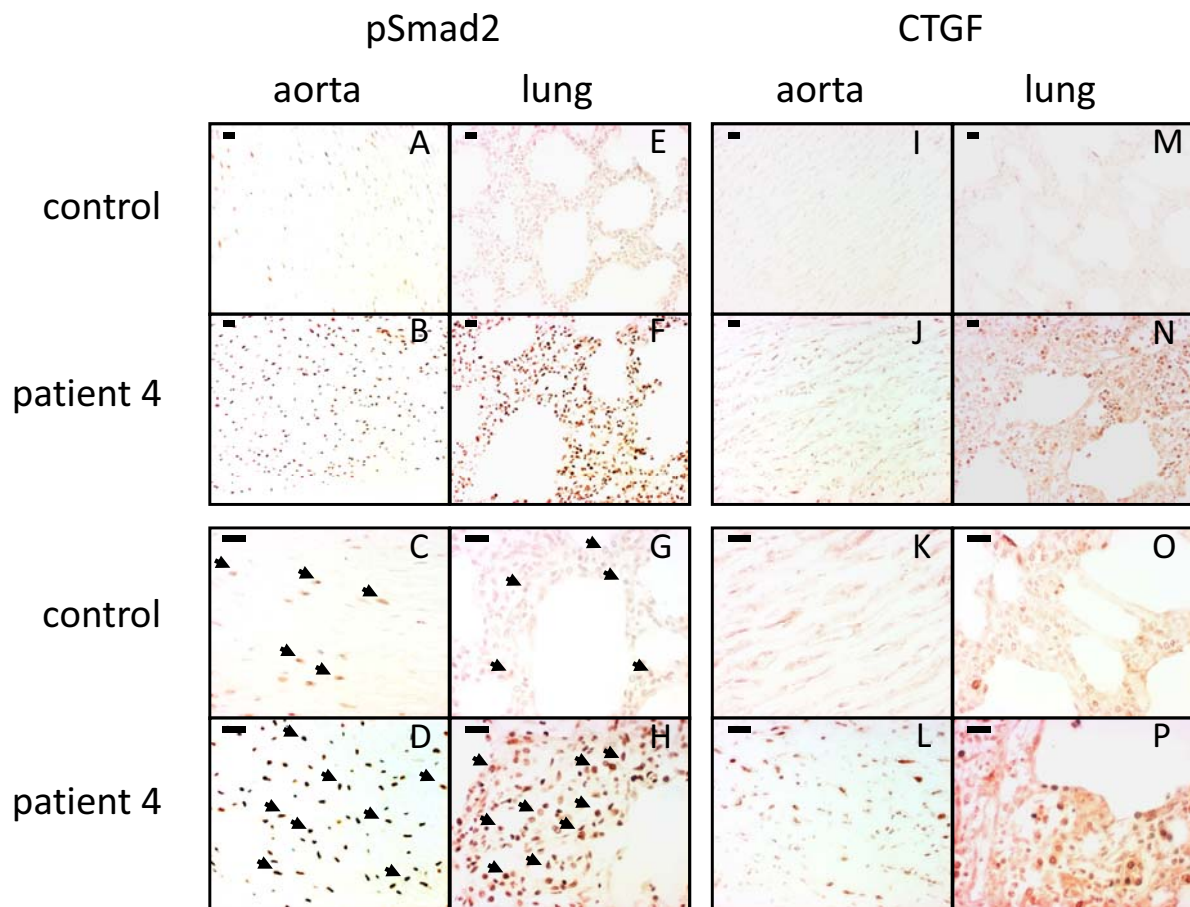
*FBLN4*  
exon 6  
heterozygous  
c.577delC

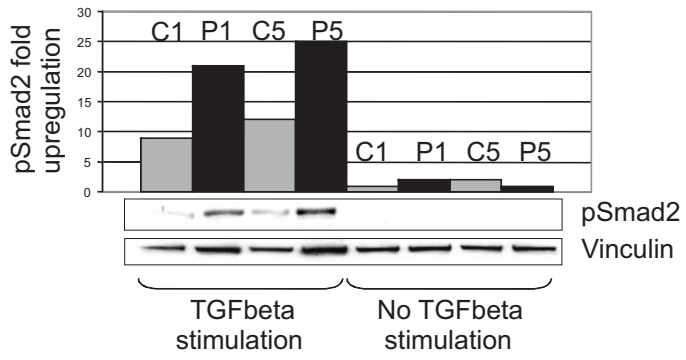
**A**



**B**







## TABLES

	patient 1	patient 2	patient 3	patient 4	patient 5	patient 6
	current report	current report	current report	Dasouki et al, 2006 (12)	Hoyer et al, 2009 (14)	Huchtagowder et al, 2006 (13)
mutation	<i>p.Glu126Lys</i> homozygous	<i>p.Ala397Thr</i> homozygous	<i>p.Glu126Val</i> <i>c.577delC</i> compound heterozygous	<i>p.Arg279Cys</i> <i>c.1070_1073dupCCGC</i> compound heterozygous	<i>p.Cys267Tyr</i> homozygous	<i>p.Glu57Lys</i> homozygous
sex	female	male	female	female	female	female
current age	20 years	7 years	died at 18 months	died at 27 days	died at birth	2 years
skin	velvety skin normal scars	-	-	mild cutis laxa	generalized cutis laxa hyperextensibility	cutis laxa
arteries	tortuosity aneurysm stenosis	tortuosity aneurysm	tortuosity aneurysm	tortuosity aneurysm dissection	-	tortuosity aneurysm
emphysema	-	-	-	-	++	++
joint laxity	-	+	-	-	-	++
other	arched palate retrognathia	hypertelorism flat facies	hypertelorism long fingers hemispheric stroke	arachnodactyly	hypertelorism retrognathia cardiorespiratory insufficiency	fractures hernia diaphragmatica

**Table 1.** Overview of the clinical and molecular data of the three patients reported in this article compared to previously described patients<sup>11-13</sup>.