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ABSTRACT

Aims

Thoracic Ascending Aortic Aneurysms (TAA) are characterized by elastic fibre breakdown and cystic medial degeneration within the aortic media, associated with progressive SMC rarefaction. The TGF-β/Smad2 signaling pathway is involved in this process. Since pericellular fibrinolytic system activation is able to degrade adhesive proteins, activate MMPs, induce SMC disappearance and increase the bio-availability of TGF-β, we explored the plasminergic system in TAA.

Methods and Results

Ascending aortas (21 controls and 19 TAAs (of 3 different aetiologies)) were analyzed. Immunohistochemistry showed accumulation of t-PA, u-PA & plasmin in TAAs, associated with remaining SMCs. Over-expression of t-PA, and u-PA was confirmed by RT-PCR, immunoblotting and zymography on TAA extracts and culture medium conditioned by TAA. Plasminogen was present on the SMC surface and inside cytoplasmic vesicles, but plasminogen mRNA was undetectable in TAA medial layer. Plasmin-antiplasmin complexes were detected in TAA-conditioned medium and activation of the fibrinolytic system was associated with an increased fibronectin turnover. Fibronectin-related material was detected immunohistochamically in dense clumps around SMCs and co-localized with Latent TGF-β Binding Protein-1.

Conclusions

These observations indicate that the fibrinolytic pathway could play a critical role in TAA progression, via direct or indirect impact on ECM and consecutive modulation of TGF-β bio-availability.

***Key words*:** plasmin; plasminogen activators; LTBP-1; TGF-β1; smooth muscle cells; fibronectin; electron microscopy

INTRODUCTION

Proteolytic degradation of the extracellular matrix, including adhesive glycoproteins and microfibrils[1, 2], of the medial layer, leading to elastin fibre network breakdown, is necessary for aneurysm formation in large arteries and is therefore a common pathophysiological pathway regardless of aneurysmal aetiology and localization[3]. In thoracic ascending aneurysm (TAA), this proteolytic injury is promoted by smooth muscle cell (SMC) rarefaction [4] and the subsequent development of areas of cystic medial degeneration [5, 6, 7]. Studies of mediators of extracellular matrix breakdown in human TAA[8, 9] of various aetiologies[10-12] have mainly focused on matrix metalloproteinases[13], but the serine proteases, including fibrinolytic proteases, have received less attention. Plasmin generation could participate in MMP activation and smooth muscle cell (SMC) detachment via degradation of adhesive proteins including fibronectin[14]; and is also claimed to be a major player regulating TGF-β1 bio-availability (see[15] for review).

Recent data obtained in Marfan syndrome have focused interest on TGF-β1 bio-availability/Smad2 signalling in vascular SMCs, as a molecular link between the initial *FBN1* gene mutation and secondary dilatation of the aorta[16]. We recently showed that Latent TGF-β Binding Protein-1 (LTBP-1) and Smad2 are overexpressed at both transcript and protein levels in human TAA. In contrast, mRNA coding for TGF-β1 is not increased although there is an increased in the storage of its latent form [17]. These observations were made in human TAA of diverse etiologies: (i) Marfan syndrome related to *FBN1* gene mutations, but also mutations *of TGFBR2*, (ii) associated with bicuspid aortic valve (BAV), and (iii) degenerative forms. Contrasting with

atherothrombotic aneurysms of the abdominal aorta[18], TAA are devoid of any intraluminal thrombus[19] and present no major leukocyte infiltrate[20].

In order to further characterize the potential involvement of plasmin activation in the development of TAA, we explored on samples of the aortic wall the tissue expression and release of t-PA, u-PA and plasmin, as well as fibronectin degradation fragments as end-products. We showed that t-PA and u-PA are overexpressed in TAA tissue samples leading to plasminogen activation. Furthermore fibronectin is overexpressed but also overdegraded, and co-localizes with Latent TGF-β Binding Protein-1.

MATERIALS AND METHODS

Aneurysmal aortic wall taken during surgery from 19 patients with aortic aneurysm of the ascending aorta of various aetiologies (Marfan, TAA associated with biscuspid aortic valve (BAV) and degenerative form) were collected over a 2 year period (Table 1)[19] in Xavier Bichat hospital (Paris, France) and in the Heart Institute (INCOR) of Sao Paulo, Brazil. Since the outer curvature of the ascending aorta (usual site of maximal dilatation) and the inner curvature (usually less or not dilated) could differ by several morphological aspects and biological mediators, only the outer curvature was used for histological and biochemical studies in the present study. Acute dissections of the ascending aorta were excluded from the present study because thrombus formation in the false channel involves plasminogen and plasminogen activator retention and activation,.This study was approved by the Ethical Committee (Ambroise Paré Hospital (Boulogne, France) 04/14/2005) and by the Heart Institute of Sao Paulo, Brazil. All patients signed informed consent. Samples of normal walls of the ascending aorta and aortic arch were obtained in 21 subjects at the time of organ collection for heart/lung transplantation after authorization from the French Biomedicine Agency. Unfortunately, anonymity was required during this process and so no clinical data is available for the controls. Measurements of plasma concentrations of plasmin-antiplasmin complexes were performed on citrated plasma. Plasma was obtained from normal volunteers (Centre d'Investigation Preventive et Clinique", IPC, Paris, France), [21] and, prior to surgery, from patients with a thoracic aortic aneurysm. These 2 populations were matched for age and sex (Table 1).

Histology

Samples of ascending aortic wall, taken from the outer curvature (n=19 for TAA, n=21 for controls) were fixed in 3.7% paraformaldehyde for 24 hours and then processed for routine paraffin embedding. Aortic convexity wall samples were oriented to obtain cross-sections, perpendicular to the axis of blood flow *in vivo*. Five micrometer thick serial sections were stained with haematoxylin/eosin, nuclear red/Alcian blue (proteoglycans), Sirius red (collagen fibres)/haematoxylin or orcein (elastic fibres). Serial sections of these tissues were also used for immunohistochemical staining. Standard histology was performed to evaluate features characteristic of TAA disease: disruption of the elastic and collagen fibre network, smooth muscle cell rarefaction and presence of cystic medial degeneration areas.

Immunohistochemistry

Serial sections (n=10 per group) were deparaffinized, rehydrated and antigen retrieval was performed in citrate buffer, at pH 7 for plasminogen, tissue- and urokinase-type plasminogen activators (t-PA, u-PA). Endogenous peroxidase was blocked with 3% H_2O_2 in aqueous solution for 5 minutes, and non-specific binding was blocked with normal horse serum for 30 minutes (except for plasminogen detection). We used polyclonal antibodies for α-actin (Dako, 10µg/ml), fibronectin (Sigma, 5µg/ml), human smooth muscle myosin heavy chains, SM2 (Abcam, 10µg/ml), and t-PA (Abcam, 5µg/ml) and monoclonal antibodies for plasmin/plasminogen (Technoclone, 40µg/ml) and u-PA (Calbiochem, 10µg/ml). All sections were incubated overnight with the

primary antibody in TBS/TC solution (Tris-buffered saline – 0.2% Tween – 0.6% casein, pH6), followed by the secondary antibody (Kit LSAB-DAKO) for 30 minutes. The binding reaction was detected using DAB. Slides were then counterstained with Mayer's hematoxylin. Negative control immunohistochemical procedures consisted of substitution of the primary antibody by equivalent amounts of naive mouse and/or rabbit IgG. This procedure gave consistently negative results. Sections of pathological aortic tissue were compared to normal aortas.

Since Latent TGF-β Binding Protein-1 (LTBP-1) antibodies could not be used on fixed tissue, cryostat sections (5 µm) were used for studying fluorescent localization of fibronectin, LTBP-1 and TGF-β1 . Briefly, cryostat sections were post-fixed in acetone, washed, blocked and incubated with the specific anti-fibronectin polyclonal antibody (Sigma, 5µg/ml) for one hour at room temperature and revealed by anti-rabbit Alexa-488 (green, 4µg/ml). A similar procedure was performed for LTBP-1 (R&D system,5 µg/ml) and TGF-β1 (Santa Cruz, 5 µg/ml) by using specific monoclonal antibodies for each and an anti-mouse Alexa-555 (red, 4µg/ml) for revelation. The spontaneous autofluorescence of elastin was revealed by ultraviolet transillumination.

Immunoeclectron microscopy for fibrinolytic serine-proteases

For immunoelectron microscopy (n=5 per goup), small pieces of tissue were fixed in 3% glutaraldehyde in 0.15M phosphate buffer at pH 7.2 for 1 hour, followed by postfixation in 1% osmium tetroxide in 0.9% sodium chloride for 1 hour. Fixed material was stained *en bloc* in 0.5% aqueous uranyl acetate overnight. After this procedure, the samples were dehydrated in a graded acetone series, and embedded in Araldite resin.

Ultrathin sections, at 70nm thick, were obtained using an LKB ultramicrotome, equipped with a diamond knife and placed on formvar-coated, 200-mesh, nickel grids. Sections were treated with 0.5M Sodium Periodate in aqueous solution for 15 minutes to remove the resin and to reduce any free aldehyde groups present.

The sections were incubated overnight with the primary antibody at a dilution of 40µg/ml for plasmin/plasminogen in TBS/TC solution (Tris-buffered saline – 0.2% Tween – 0.6% casein, pH6). After rinsing in TBS/TC solution (8 times; 1min), the sections were incubated in a solution containing the gold-conjugate (bridging antibody RαG/M. DAKO]) for 2hr. After a new rinsing with TBS/TC solution (4 times; 1min) and distilled water (8 times; 5min), sections were double-stained with uranyl acetate and lead citrate. The grids were studied and photomicrographed in a Philips TECHNAI 12 transmission electron microscope, operating at 80kV.

Biochemistry

Preparation of tissue culture media

Since the adventitia of samples from surgery is always contaminated by coagulated blood, adventitial removal was gently performed, and the aortic media was washed 3 times before use. Aortic media samples from 19 TAA and 21 control aortas were washed 3 times in PBS solution, cut into small pieces, weighed and incubated in RPMI buffer (6mL buffer per g of sample), for 18 h at 37°C as previously described[19]. The sample of culture medium, containing the proteins released by the aortic tissue were then collected, centrifuged and frozen.

Tissue extraction

Protein extraction was performed directly from frozen aortic medial tissue at -80 °C. The aortic medial samples were first cryogenically pulverized in liquid nitrogen, using a freezer mill (model 6750 SPEX SamplePrep). About 100 to 300 mg of crushed powder per sample were subsequently used. The powder was homogenised using a potter in 5 times weight-to-volume of extraction buffer (5M urea, 2M thiourea, 2% CHAPS, 2% SB3-10, 20mM Tris pH 7 and 1% of protease inhibitor cocktail) at 4°C with sporadic vortexing. Homogenates were centrifuged at 6000 g for 10 min. Supernatants were transferred to new tubes and centrifuge at 14000 g for 2 min. All centrifugation steps were carried out at 4°C. The final supernatant was divided into 50µL aliquots and stored at -20°C until analysis. Total proteins were quantified by the method of Bradford.

Immunoblotting

Equal amounts of protein (25 µg) were loaded onto 4-12% bis-tris acrylamide gels (Criterion Gels, BioRad) under reducing conditions, as well as 2.5 ng of recombinant human tPA (r-TPA from actilyse, Boehringer Ingelheim and from HYPEN BioMed) and 2.5 ng of the complex between tPA and the plasminogen activator inhibitor type 1 (PAI-1) as positive control. The complex was formed by mixing equal amounts of both compounds at neutral pH and incubating at room temperature for 30 min. Gels were subjected to electrophoresis. Protein transfer to nitrocellulose membrane was performed at 300 mA for 1h. Membranes were blocked for 1h in TBS-T solution containing 5% (w/v) non-fat dry milk. Several anti t-PA antibodies were tested on tissue extracts and all give several bands. Among these, sheep-derived polyclonal antibody against human tPA (Abcam) was chosen as the most specific. Incubation in 2 µg/mL of primary antibody in 1% of blocking buffer was performed overnight at 4°C in a sealed plastic bag under

agitation. Secondary anti-sheep IgG antibodies conjugated with horseradish peroxidase (Jackson Immuno Research Laboratories) at 1µg/mL were incubated for 1h at room temperature. Antigen visualisation was achieved by a 20 min X-ray film exposure using luminescent ECL (GE Healthcare) substrate. Negative quality control of primary antibody specificity was done by loading in duplicate one sample from the TAA group in which the primary antibody incubation was omitted. Finally, to control for equal loading of protein on SDS-PAGE gels the membrane was reblotted against β-actin (Sigma). For each sample, t-PA was normalized to the quantity of β-actin and values were reported as a percentage. Quantification of bands was performed with Image J software (http://rsbweb.nih.gov/ij/). The non-specific band was excluded.

Immunoblotting of uPA and fibronectin was carried out similarly to that of tPA, but gel loading was done at constant volume (3 µL for fibronectin and 20 µL for uPA) of tissue-culture medium for each sample. Primary antibodies were monoclonal against: human uPA-B chain (American Diagnostica, 1 µg/mL)a and against fibronectin (Sigma).

Casein zymography

Samples of tissue culture medium, containing the proteins released by the aortic tissue during incubation, were analyzed by electrophoresis on 10% SDS-polyacrylamide gels containing 1mg/ml of α-casein for detection of u-PA (Sigma)[22]. Molecular mass determinations were made with reference to pre-stained protein standards (BioRad) co-electrophoresed in the gels. Serine–proteases were inhibited by aprotinin and u-PA selectively by 2 µM amiloride.

ELISA

Plasmin-antiplasmin (PAP) complexes were detected in samples of tissue-conditionned culture medium and plasma using a commercially available kit (American Diagnostica) according to the manufacturer's instructions.

Real-Time PCR

Powder of crushed aortic tissue was incubated in lysis buffer (OMEGA - vWR) and the solution then filtered. Total RNA from aneurysmal and control tissue was extracted, using the EZNA kit (OMEGA - vWR), according to the manufacturer's instructions. 0.2µg of RNA was reverse-transcribed using Taq Polymerase (Invitrogen). Real-time PCR was performed in the LightCycler system with SYBR Green detection (Roche Applied Science) using 5ng of cDNA. mRNA levels were normalized to GAPDH mRNA[17]. The primers used are presented in Table 2.

Statistical analysis

Results are expressed as box plots, in which the median is shown. Upper and lower limits of boxes represent interquartiles (25^{th} and 75^{th}), whereas upper and lower bars show percentiles (10^{th} and 90^{th}). Statistical analysis was performed with Statview® software using the nonparametric Mann-Whitney test. Differences between measured variables were considered significant for $p \leq 0.05$.

RESULTS

Patient characteristics

In this study, we included 19 patients with TAA [7 patients with Marfan syndrome (7 males), 6 patients with TAA associated with a bicuspid aortic valve (BAV, 5 males, 1 female) and 6 older patients with the degenerative form (5 males, 1 female)]. These 3 aetiologies differ by the age of surgical aortic root replacement (Table 1). One Marfan and one BAV patient had aortic valve insufficiency and one BAV patient had calcified stenosis; no aortic valve disease was associated with the degenerative form of TAA. At surgery the mean aortic root diameter was slightly smaller in Marfan patients than in patients with TAA of the other aetiologies (Table 1). No cases of syphilitic aneurysm or dissection were included in this series.

Histological characterization

Aortic sections from controls (n=21) showed a normal arrangement of the media in which the layers of SMCs were separated by prominent elastic lamellae. These lamellae were interconnected by a network of small elastic and collagen fibres and proteoglycans[4].

Aortic sections from all aneurysmal aortas (n=19) showed disorganization and important breakdown of the elastic and collagen network (Fig 1 A, B and C, Fig 2 A,B and C), associated with cell rarefaction corresponding to more or less diffuse areas (cystic medial degeneration) of vacuolated basophilic material (Fig. 1 C). The accumulation of this amorphous, Alcian blue-positive material often involved several elastic lamellar units[12]. Some morphological differences were observed between the different etiologies: Marfan patients showed more pronounced and diffuse cystic medial degeneration despite

their young age (Table 1), corresponding to more diffuse elastic fibre breakdown, whereas cystic media degeneration and corresponding elastic fibre breakdowns was usually less diffuse and more localized in TAA associated with BAV (Fig 2A & B).

Age related degenerative forms were characterized by diffuse degradation of the medial extracellular matrix usually associated with intimal proliferation (data not shown) as previously reported[23]. Occasionally fatty streaks were present, but did not differ morphologically from those in control aorta, and did not predominated in the outer curvature.

Viable medial cells stained positively for SM α-actin (not shown) and SM myosin (Fig. 1D) in both controls and TAA, indicating that they were smooth muscle cells (SMCs). In addition, some isolated retracted SMCs were present within the cystic medial degeneration areas in aneurysmal aortic wall (Fig. 1C & D). No inflammatory cells were present (data not shown).

Electron microscopy revealed of SMCs of a contractile phenotype in the normal aortic wall (Fig 1E, F) whereas SMCs from the aneurysmal aorta exhibited a more synthetic phenotype with a well developed rough endoplasmic reticulum and Golgi apparatus (Fig 1G, H).

Plasminogen activators

t-PA was mainly associated with the surviving SMCs present in aneurysmal aortic wall whereas it was only present in small amounts in association with normal SMCs in normal aortic wall (Fig. 3A & B). Immunoblot revealed a clear increase in free tPA from aneurysmal aortic wall compared to normal aorta (Fig. 3C). A band corresponding to free

tPA (64 KDa), which is the only form that has fibrinolytic activity, indicated an almost three-fold increase in the protein concentration (Fig. 3D). A faint band corresponding to the antiprotease-tPA complex was also observed (100 kDa, Fig. 3C). The negative control revealed the presence of a non-specific band around 50kDa that, consequently, was not used for the tPA analysis. These data, obtained at the protein level, were confirmed at the transcript level by quantitative RT-PCR showing a significant ($p<0.001$) increase in t-PA mRNA expression in aneurysmal as compared to normal aortic wall (Fig. 3E).

u-PA was also associated with the surviving SMCs in aneurysmal aortic wall (Fig. 4 A, B & C). u-PA activity was visualized by casein zymography[24] in tissue-conditioned medium. A 33.5 kDa protein was observed in conditioned medium (Fig. 4D) which predominated in aneurysmal tissue as compared to controls ($p<0.01$, Fig. 4E). This LMW serine protease activity was inhibited by amiloride (2 µM), an inhibitor of u-PA[25](data not shown). A similar band could be generated by incubating urine, a source of two-chain u-PA, with active MMP-7[26], or plasmin (data not shown) and was identified as LMW urokinase by immunoblot (Fig. 4E). Similarly to t-PA, u-PA mRNA was significantly increased in TAA tissue compared to controls ($p= 0.005$, Fig. 4F).

Plasminogen uptake and plasmin generation

In normal aortic tissue, immunoreactivity for plasmin/plasminogen was detected on the luminal side and presented a negative gradient across the media to the more external layers (Fig. 5A). In the medial layer of TAA, plasmin/plasminogen immunostaining was associated with the remaining SMCs, but also with extracellular material (Fig. 5B &

inset). In contrast, plasminogen/plasmin were not retained in areas of cystic medial degeneration. Extracellular matrix components were positive for plasmin/plasminogen when analyzed by electron microscopy, in both normal and aneurismal aorta although the staining was more intense in the latter. Plasminogen/plasmin labeling was associated with the cell surface and was also present inside the cytoplasmic vesicles near to the cell membrane in SMCs from aneurysmal aorta (Fig. 4C).

Immunoblots using an anti-plasminogen antibody directed towards the kringle 1-3 region allowing detection of both plasminogen and plasmin, confirmed the immunohistological data. In normal aorta, only very faint bands were observed at the expected position for plasminogen and plasmin (Fig. 4D), whereas in aneurysmal aorta, both plasminogen and plasmin could be detected, and the intensity of the bands was considerably higher (O.D. 84 +/- 10 in TAA versus 21 +/- 3.5 in controls, $p<0.01$). Plasminogen mRNA was not detected in normal or aneurysmal aortic wall in usual conditions (PCR > 50 cycles).

Plasmin-Antiplasmin (PAP) complex concentration was higher in samples of tissue-conditioned medium obtained from aneurysmal aorta than in those from normal aorta ($p<0.05$, Fig. 5D). Interestingly, the concentration of PAP complexes was also significantly higher in plasma from patients with TAA (n=37, 1459 +/- 39 ng/ml) than in plasma from age- and gender-paired normal volunteers (n=30, 956+/- 30 ng/ml) ($p<0.05$).

Fibronectin as a pericellular target for plasmin

Immunostaining of cellular fibronectin was homogeneous in normal aortic tissue throughout the extracellular space between the elastic lamellae (Fig. 6A & B). In contrast,

fibronectin distribution was heterogeneous in aneurysmal aortic wall. Areas of acellular cystic medial degeneration were devoid of fibronectin (Fig. 6C), whereas some clumps of compact fibronectin immunostaining were observed around the remaining retracted SMCs (Fig. 6C & D). This abnormal distribution was associated with a significant global increase in fibronectin mRNA levels in TAA tissue compared to controls ($p<0.001$, Fig. 6E).

Fibronectin degradation products were detected by immunoblot in cuture medium conditioned by both normal and aneurysmal aortas. Quantification of fibronectin bands showed that degradation products were significantly increased in TAA-conditioned medium compared to controls ($p<0.01$) (Fig. 6F).

It has already been shown that Latent TGF-β Binding Protein-1 (LTBP-1) is associated with fibronectin in cell cultures[27]. Since plasmin is capable of cleaving fibronectin, and as a consequence, of mobilizing latent TGF-β1/LTBP-1 complexes[28, 29], we explored the localization of LTBP-1, TGF-β1 and fibronectin in TAA by immunoflurorescence (Figs. 2 and Fig. 7). In controls, fibronectin, LTBP-1 and TGF-β1 were regularly distributed and co-localized around SMCs (Fig. 7A). In contrast, the distribution of fibronectin, LTBP-1 and TGF-β1 was more heterogeneous in TAA but these 3 proteins remained co-localized, exhibiting the distribution described above for fibronectin (Fig. 7B).

Comparison of TAA of Marfan origin to that associated with BAV (Fig 2) showed that despite the differences observed in the morphology of cystic media degeneration, there was no difference in the co-localization of compacted fibronectin and LTBP-1 in the edge of the cystic degeneration areas (Fig 2 E/F, H/I, K/L).

DISCUSSION

Our morphological data confirmed, in TAA having reached the stage requiring surgery, that cystic medial degeneration co-localizes with extracellular matrix breakdown, including elastic fibre disruption, and vacuolated areas of mucoid material retention[12, 20] and smooth muscle cell rarefaction [10, 30-32].

In the normal aortic wall, smooth muscle cells have a contractile phenotype recognized by electron microscopy[33]. In the aneurysmal wall, SMCs are quite different, with a significant enrichment in rough endoplasmic reticulum, Golgi apparatus and vesicles, suggesting a switch to a more secretory and phagocytic phenotype[34]. The presence of cytoplasmic vesicles in close proximity to the cell membrane, suggests endocytic activity, which is in agreement with the well established capacity of SMCs to acquire accessory endocytic/phagocytic activities in pathological conditions[35, 36].

We explored the fibrinolytic pathway in the aneurysmal aortic wall because plasmin, produced by t-PA or u-PA, is able to activate MMPs and TGF-β1, and to cause, either directly or indirectly pericellular fibronectin degradation and SMC rarefaction[15,14]. Both t-PA and u-PA were associated with the remaining SMCs in aneurysmal aortic wall as previously described in aortic dissection[37]. This suggests that plasminogen activation could occur in the pericellular space[14]. In addition, increased expression of both t-PA and u-PA at the transcription as well as at the protein level were observed, and LMW-uPA was found to be active in the TAA samples. LMW-uPA can be generated from sc-uPA by plasmin and also by MMP-7[26], that we have recently reported to be localized in areas of cystic medial degeneration[7]. Therefore, more conditions favoring pericellular activation of plasminogen appear to be present in aneurysmal aorta than in normal aorta.

Nevertheless, the stimuli responsible for plasminogen activator overexpression by smooth muscle cells remain to be elucidated.

In normal aortic wall, plasmin/plasminogen immunoreactivity presented a clear, consistent, negative gradient across the media from the luminal side outwards and was predominantly associated with SMCs. This is compatible with a convection process from the plasma to the outer aortic wall[38] which is probably increased in TAA due to perturbation of endothelial permeability[19, 39] and elastic lamellar degradation[40]. Plasminogen is a plasma protein, synthesized by the liver and circulating in the micromolar range; and we could not detect plasminogen mRNA in the aortic media. In pathological areas, the plasminogen/plasmin immunoreactivity was intense, always associated with the remaining SMCs, whereas the cystic medial degeneraion areas were strictly negative. In addition, biochemical analysis indicated that TAA samples contained increased amounts of plasminogen/plasmin compared to control aorta. Plasminogen and t-PA could bind to external cell surface via annexin II[41] leading to pericellular plasmin activation and fibronectin degradation[14]. Annexin II overexpression has been already reported in human[42] and experimental[43] abdominal aneurysm. Furthermore, the increased plasmin/antiplasmin complexes provide evidence of plasminogen activation by t-PA or u-PA synthesized by smooth muscle cells within the pathological aortic samples.

The localization of plasminogen/plasmin in close association with SMC membranes and their presence within cytoplasmic vesicles was demonstrated by electron microscopy. Indeed, the pericellular localization of plasmin/plasminogen could be related to its binding to free lysine residues on membrane proteins[14]. Cell membrane-bound plasminogen can be cleared from the extracellular space by endocytosis[44]. The presence

of plasminogen/plasmin within vesicles indicates that, in TAA, SMCs can endocytose plasmin-antiprotease complexes[45].

Analysis of fibronectin is in keeping with the possible involvement of plasmin activation. Fibronectin is the main adhesive protein involved in SMC spreading and adhesion[46]. Its expression is under the control of the TGF-β1 and Smad2 signaling pathway[47, 48] which is recognized to be involved in TAA development [49]. The *in vivo* relevance of this point was further suggested by the increased fibronectin mRNA in TAA compared to controls. These data confirm earlier studies showing an increased expression of fibronectin in the outer curvature of TAA associated with BAV as compared to the aneurysmal inner curvature[50, 51]. The compacted fibronectin observed in the pericellular space of the remaining, retracted SMCs provides evidence for important modifications of the SMC micro-environment in TAA. The increased quantity of degraded fibronectin and solubilized products observed in TAA conditioned-medium compared to control, also provides evidence of proteolytic degradation. Plasmin is a proteolytic enzyme [14], capable of degrading and solubilizing fibronectin in the pericellular space. The proteolytic degradation of fibronectin could be one of the links between pericellular matrix protein degradation and SMC apoptosis[46]. In parallel, the microfibrillar structure of fibrillin, responsible for elastic fibre assembly[52], is strictly dependent on the integrity of fibronectin[53, 54]. Therefore, pericellular plasmin activation could also indirectly impact elastic fibre structure in TAA.

The degradation of fibronectin in TAA could have another important consequence by increasing LTBP-1/TGF-β1 release. Since LTBP-1 sequesters TGF-β1 in the extracellular matrix and is bound to pericellular fibronectin with a high affinity[27] via an

integrin-dependent process[55,56], we explored the localization of fibronectin, LTBP-1 and TGF-β1 in human aortic wall. We demonstrated here, for the first time, a co-localization of LTBP-1 with fibronectin in the pericellular space of SMCs in the normal human aortic wall. In diseased aortic wall, the association persists between compacted fibronectin, LTBP-1, and TGFβ-1, suggesting that plasmin-dependent fibronectin degradation could release LTBP-1/latent TGF-β1 complexes[28, 29]. These findings are in agreement with our previous study[17], showing an increased storage of latent TGF-β1 by the aneurysmal wall as compared to normal aorta, without a parallel increase in TGF-β1 mRNA expression.

In conclusion, the present observational study suggests, for the first time, that plasmin and plasminogen activators accumulate in the vicinity of the remaining SMCs in the medial layer of TAA. In parallel, fibronectin is both overexpressed, abnormally accumulating around suffering SMCs in close association with LTBP-1, and overdegraded, indicating an increased turnover.

Our results indicate that the fibrinolytic pathway is activated in TAA, and may participate in proteolysis of the extracellular matrix, resulting in disorganization of the aortic wall and the subsequent increase in LTBP-1/latent TGF-β1 release.

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FIGURE LEGENDS

Figure 1. Morphology of the ECM and SMCs: Sections of medial layer of human aortic aneurysmal wall stained with orcein (A), picrosirius red (B), alcian blue (C) or immunolabeled for human smooth muscle myosin heavy chains SM2 (D). E to H; electron micrographs of longitudinal ultrathin sections showing smooth muscle cells from normal aorta (E and G) and from aneurysmal aorta (F and H).

A- Disrupted elastic lamellae are visible in aneurysmal wall; the negative stain indicating the absence of elastic lamellae and fibres corresponds to the area of cystic medial degeneration (star). **B**- Collagen fibres, stained in red, are also absent or disrupted within the areas of cystic medial degeneration (star). **C**– A large area of typical cystic medial degeneration (blue), observed in human aortic aneurysmal media. **D** – Immunostaining for human smooth muscle myosin (SMCs) heavy chains, SM2, indicating the presence of smooth muscle cells in the aortic media. Retracted cells predominate in the vicinity of areas of mucoïd degeneration. Orientation: intima top and adventitia bottom in A & B and intima right and adventitia left in C &D

E – Typical SMC of a contractile phenotype, showing myofilaments (actin and myosin) present in the sarcoplasm (Sc) and the presence of a surrounding basement membrane (arrows). The extracellular matrix (ECM) shows an arrangement of collagen fibrils; nucleus (N). **F** – Higher magnification of (E) showing the SMC sarcoplasm (Sc) in details, with the presence of myofilaments (Sc) typical of the contractile phenotype. The arrows show the basement membrane. **G** – The cytoplasm of a SMC in the TAA group exhibits a typical synthetic phenotype with the presence of well developed rough endoplasmic reticulum (RER) and few myofilaments (MF) restricted to the cell

periphery. Extracellular matrix (ECM); nucleus (N). **H** - Higher magnification showing details of the cytoplasm. Note the presence of ribosomes (arrows) adhering to the outside of the membrane of the endoplasmic reticulum (RER). Myofilaments (MF); nucleus (N).

Figure 2: Immunohistological characterization of TAA from Marfan and BAV patients: A – Normal aorta showing preserved elastic lamellae (blue spontaneous fluorescence). **B –** Marfan patients showing diffuse degradation of elastic lamellae. **C** – BAV patients showing focaldegradation of elastic fibres . **D, E & F –** Fibronectin immunostaining showing compacted fibronectin around zones of cystic medial degeneration, in both TAA aetiologies (Marfan and BAV, E, F) as compared to normal aorta (D).**– G, H & I-** similar clumpsof LTBP in Marfan and BAV (E, F) as compared to normal aortas (G) **J, K & L –** Merge of fibronectin and LTBP-1 showing their colocalization in Marfan and BAV aortas.

Figure 3. **Tissue-Plasminogen activator (t-PA): A -** Normal aorta showing a regular distribution of small amounts of t-PA, associated with SMCs throughout the media. **B** – In the TAA group, t-PA immunostaining was more intense and also associated with the remaining SMCs (arrows) whereas the mucoid areas (star) were always negative.

C – t-PA western blot from protein extracts of aorta medial layer. Line: NS: negative control, controls & TAA samples, rt-PA and PAI-1/tPA complex (2.5 ng). * Band of PAI-1/tPA complex 100 kDa; ** Band of tPA 64 kDa; NS- non-specific band, bottom β-actin loading control band.

D- Densitometric quantification showing the overexpression of t-PA in TAA.

E – Real Time PCR of t-PA mRNA, showing a significant increase in t-PA mRNA expression in TAA taken together compared to control (Control: 0.34±0.75; TAA: 0.93±0.88; $p<0.0001$).

Figure 4. Urokinase (u-PA): A - normal aorta: the distribution of u-PA immunostaining was less homogeneous than that of t-PA. **B & C** - Aneurysmal aortic wall: u-PA staining is intense and clearly associated with SMCs (arrows in **Fig. 3C**). Orientation: intima right and adventitia left in all cases.

D – Zymography detected a 33.5 KDa band which was more intense in TAA-conditioned medium than control. This band was amiloride-sensitive and could be generated by co-incubation of urine (containing urokinase) and active MMP-7. This band was also observed by western blot in MMP-7 treated urine.

E- Densitometric quantification of the caseinolytic LMW band showing the over activity of u-PA in TAA.

F – Real Time PCR of u-PA mRNA showing a significant increase in u-PA mRNA expression in TAA groups taken together compared to controls (Control: 0.25±0.32; TAA: 0.91±1.1; $p<0.005$).

Figure 5. Cellular localization of plasminogen/plasmin: Histological sections of normal (**A**) and aneurysmal (**B**) aorta immunostained for plasminogen/plasmin. In both samples, positive immunostaining for plasminogen is present in the intima and is associated with SMCs in the media. A negative gradient is present from the lumen to the more external parts of the aortic wall. No immunostaining is present in the mucoid areas (stars). **Inset**: Plasminogen is localized both inside and outside the SMCs surrounding the

mucoid area (arrow). Orientation: intima top and adventitia bottom in both cases. **C –** Ultrathin section of human aorta of the TAA group treated with an antibody against plasminogen/plasmin labeled with small gold particles and viewed under the transmission electron microscope. Note the positivity for this protease inside the endocytic vesicles (Cv; double arrows), at the cell surface (arrows) and labeling the extracellular molecules (small arrows), ECM: extracellular matrix. Bar represents 0.5µm.

D- Plasminogen/Plasmin immunoblotting on tissue extracts: Extracts from control and TAA aortic samples were analyzed using an antibody that recognizes both plasminogen and plasmin, in non-reducing conditions. The intensity of the bands was significantly greater in TAA samples, as indicated by densitometric quantification. Plasmin/anti-plasmin was measured in tissue-culture media by ELISA.

Figure 6. Fibronectin overexpression, clumping and degradation: Immunostaining of fibronectin (hematoxylin counterstaining, blue). Normal aorta: a regular distribution of fibronectin between the elastic lamellae is observed (**A**), in the extracellular space surrrounding the SMCs (**B**). In aneurysmal aorta, immunostaining of fibronectin was heterogeneous: areas of cystic medial degeneration were devoid of fibronectin (star) (**C**), whereas clumps of fibronectin appeared around retracted SMCs in the vicinity of mucoid areas (**D**).

E- Real Time PCR of fibronectin mRNA showing a significant increase in fibronectin mRNA expression in TAA groups taken together compared to controls (Control: 0.16±0.21; TAA: 0.9±0.8; $p<0.0001$).

F - Fibronectin western blot of samples of aortic tissue-conditioned medium. Line: 1: negative control, 2 to 6: control group, 7 to 11: TAA group, 12: molecular weight marker (kDa), 13: Aortic SMC supernatant incubated without plasmin, 14: Aortic SMC supernatant incubated with plasmin.

Figure 7: Colocalisation of LTBP-1, TGF-β1 and fibronectin: Elastin in blue (ultraviolet), fibronectin in green and Latent TGF-β Binding Protein-1 and TGF-β1 in red; overlay of the two fluorophores in yellow.

A: Normal aorta shows intact elastin, and the regular distribution of pericellular fibronectin, LTBP-1 and TGF-β1;

B: Aneurysmal aorta shows degraded elastin and clumps of fibronectin colocalizing with LTBP-1 and TGF-β1 accumulation (X20).

Figure 1

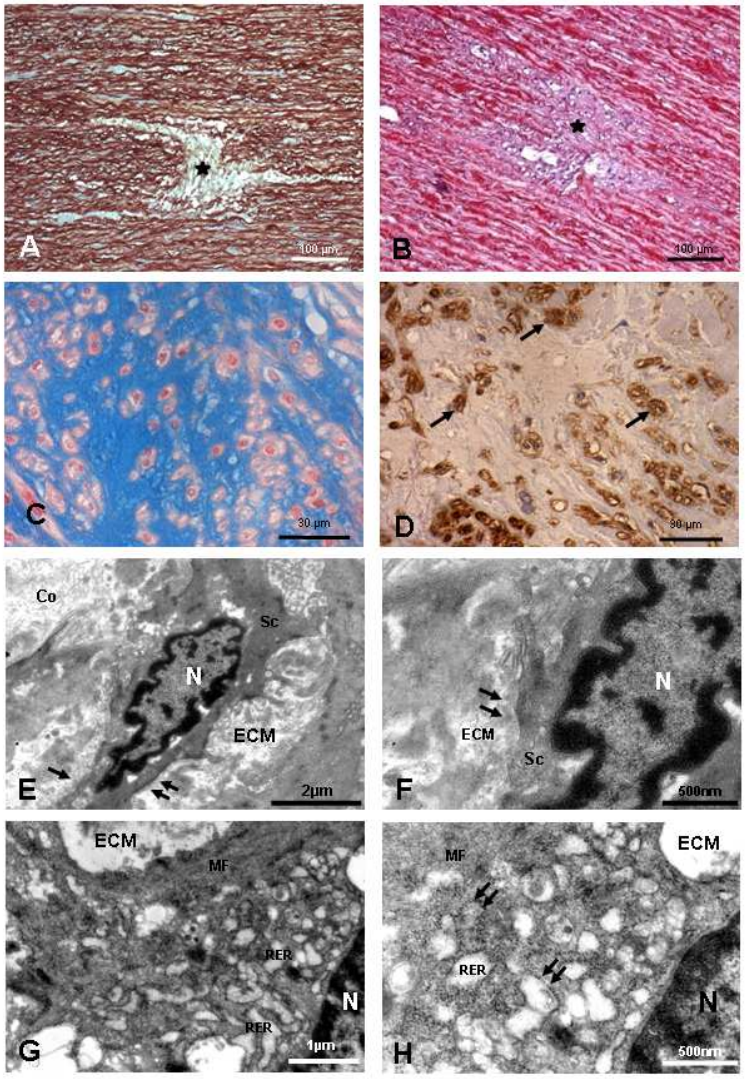

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Figure 2

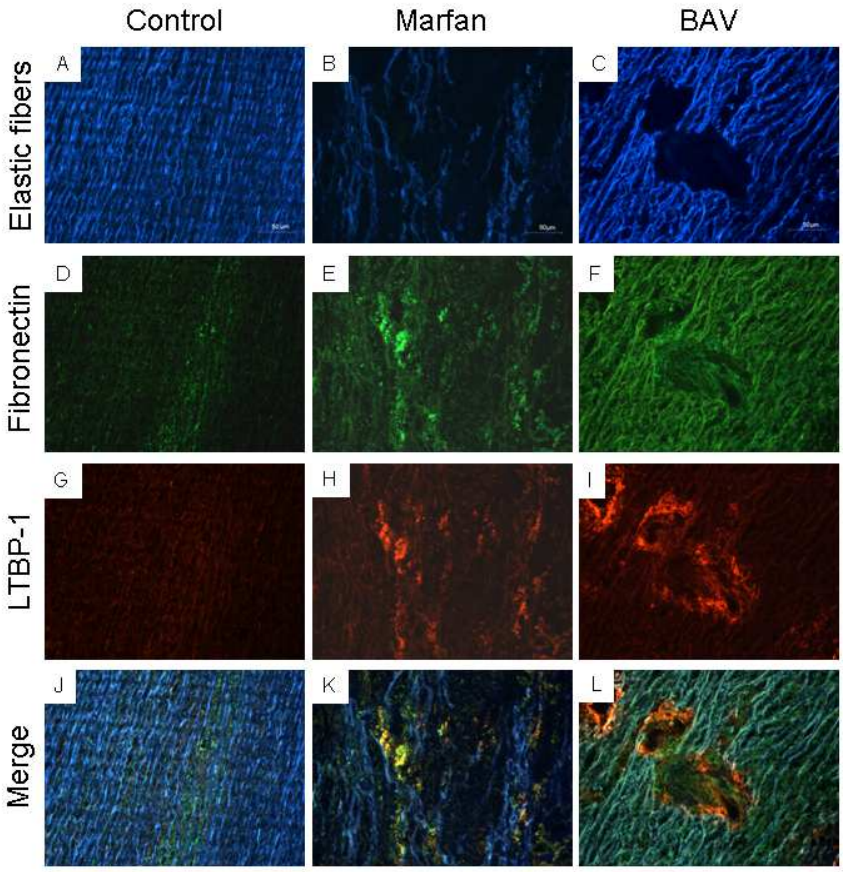

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Figure 3

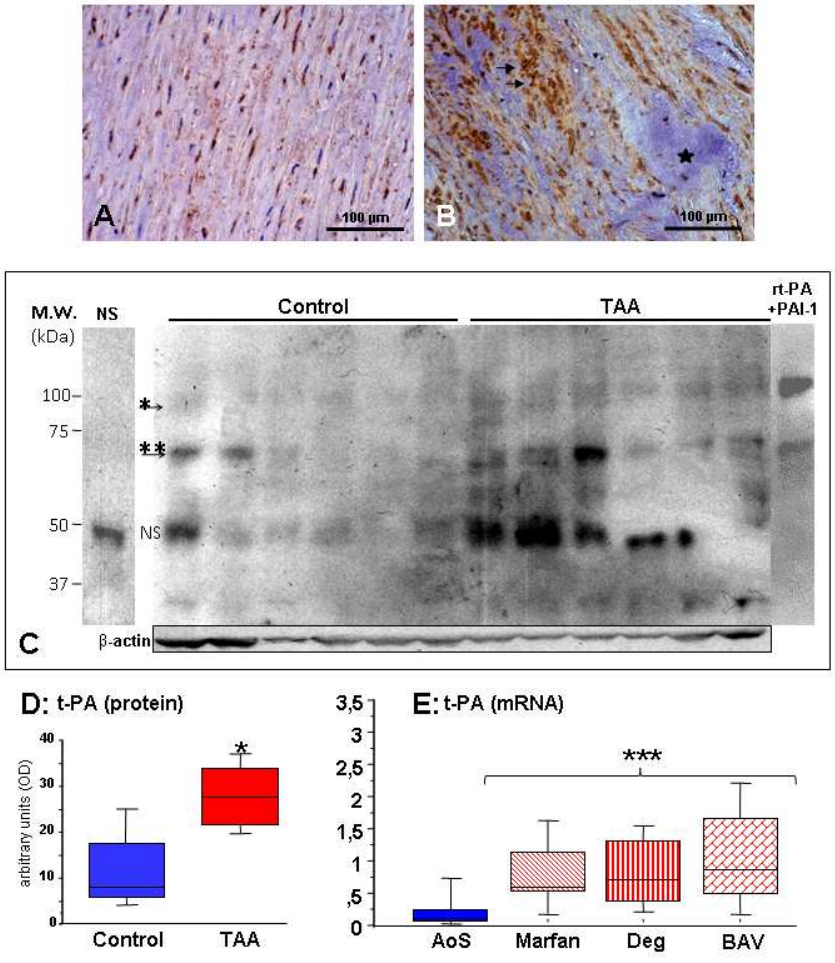

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Figure 4

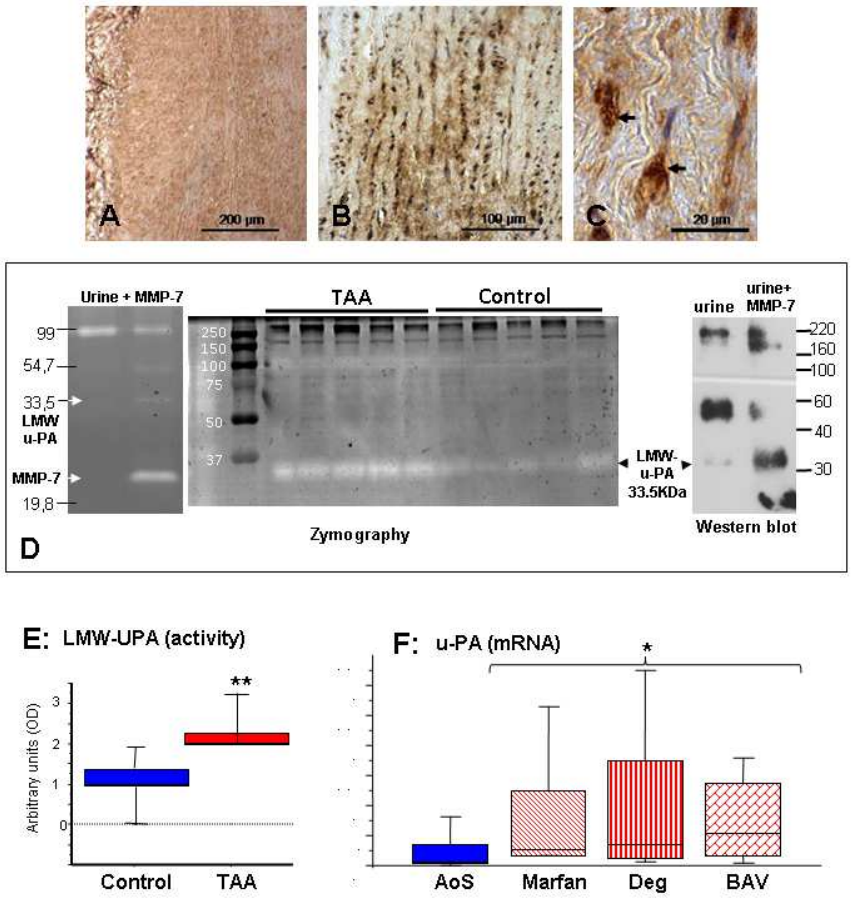

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Figure 5

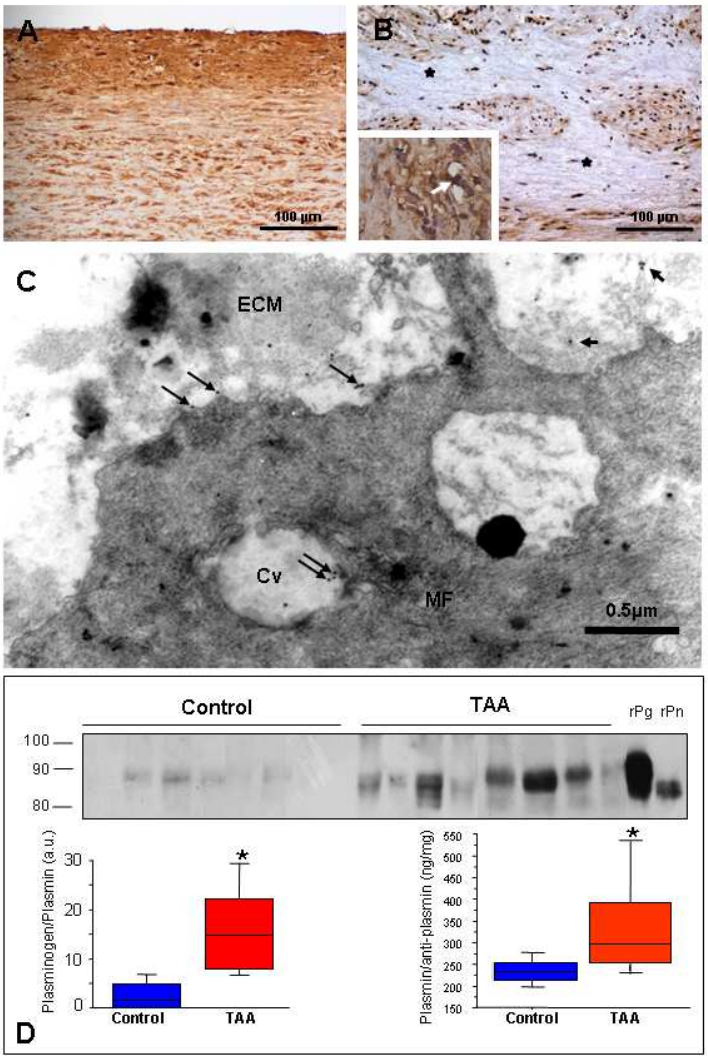

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Figure 6

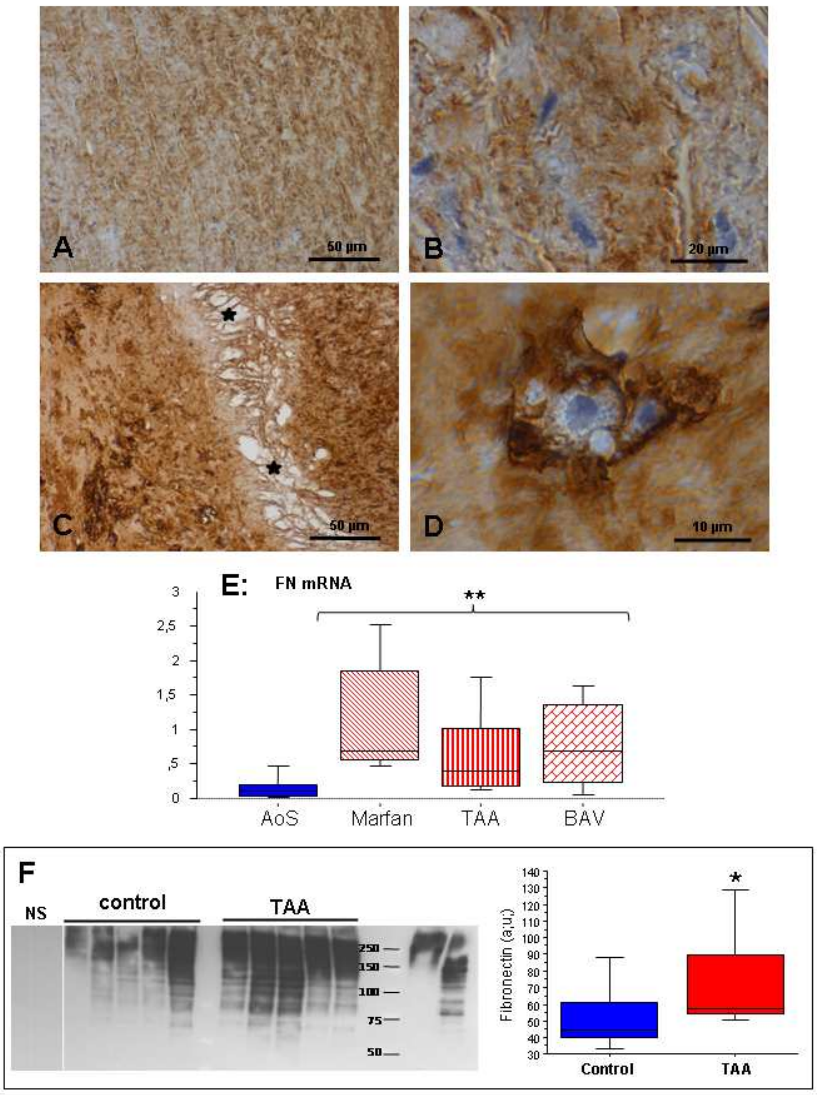

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Figure 7

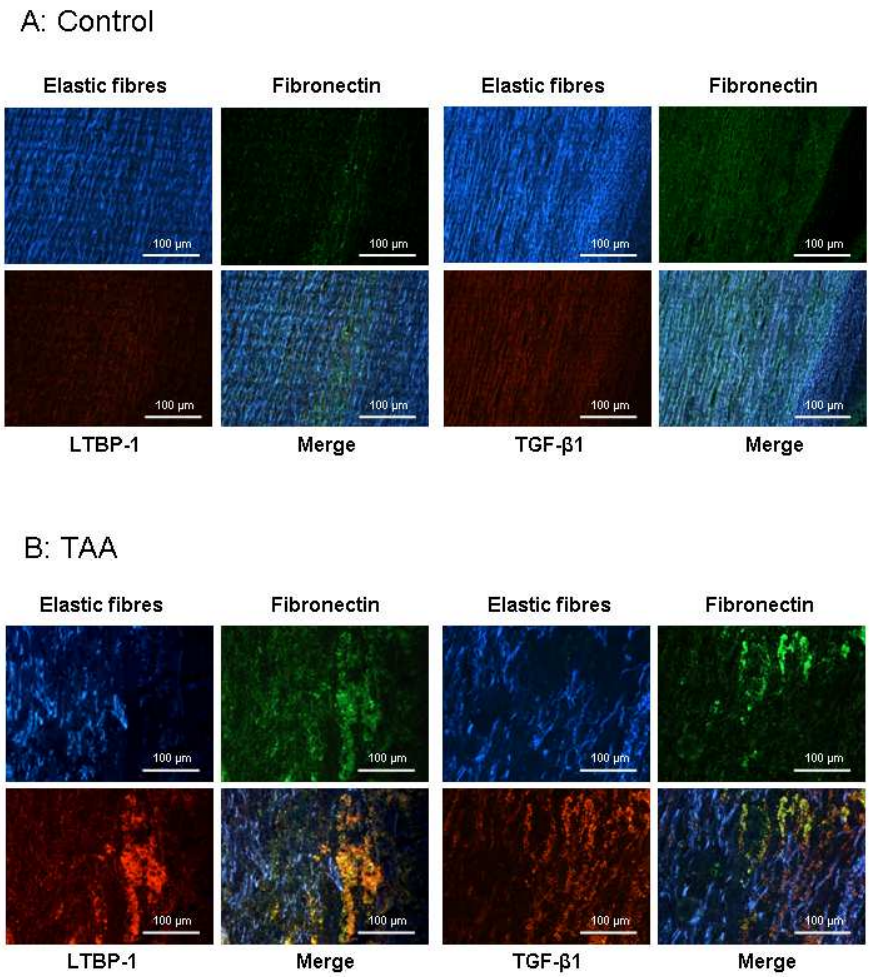

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Table 1: Patient characteristics

	controls	TAA	Monogenic	BAV	Degenerative
Total number	21	19	7	6	6
Males	18	17	7	5	5
Mean age ± SD	47.3 ± 4.6	52.8 ± 7.4	36 ± 8.4	52 ± 4.8	71 ± 6.6
Aortic Diameter (mm)	33.7±4.4	54.8±7.8	51.2±3.6	54±6.8	57.8±8.6

Table 2: list of q-PCR primers used for mRNA quantification in tissue

Plasminogen	forward	5'-GCCCCATAGACACAGCATTT-3'
	reverse	5'-CCTACAACCCTTCCAGGACA-3'
t-PA	forward	5'-CCAGATCGAGACTCAAAGCC-3'
	reverse	5'-GACCCATTCCCAAAGTAGCA-3'
u-PA	forward	5'-ACTACTACGGCTCTGAAGTCACCA-3'
	reverse	5'-GAAGTGTGAGACTCTCGTGTAGAC-3'
Fibronectin	forward	5'-AGATAAATCAACAGTGGGAGC-3'
	reverse	5'-CCAGATCATGGAGTCTTTAGG-3'
GAPDH	forward	5'-GGGCAYCCTGGGCTAMACTGA-3'
	reverse	5'-TGCTCTTGCTGGGGCTGGT-3'