

The role of extracellular and intracellular proteolytic systems in aneurysms of the ascending aorta

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Summary. Aneurysms of the ascending aorta are an outstanding challenge to clinicians as they may persist asymptomatic until they present with dissection or rupture. Intensive research is performed to reveal the molecular mechanisms causing aneurysm formation. Calpains are ubiquitous non-lysosomal cysteine proteases which are classically activated by calcium signaling. The two major forms of the calpain-family are calpain-I and calpain-II. Calpastatin specifically inhibits the proteolytic activity of calpain-I and -II. Recently it has been demonstrated in aneurysm tissues from ascending aortas obtained from Marfan syndrome patients that calpain-II expression is increased and calpastatin expression is decreased. Thus, we were interested in the probable role of calpains in aneurysms of ascending aorta in non-Marfan patients. Therefore, ascending aortic samples of dilated and non-dilated aortas were analyzed according to their calpain-I, -II and calpastatin content as well as the expression levels of MMPs and elastin as well as the infiltration of inflammatory cells.

We have found significant differences in calpain-I and calpastatin protein expression and serum levels in patients with aneurysm of the ascending aorta. Furthermore, MMP-1 and MMP-3 expression levels correlate with calpain-I protein levels. Due to our findings we conclude that calpain-1 seems to be related

to fibrotic alteration in aortic aneurysm tissue in our experimental group. The change in calpain-1 modulates the structure of aortic tissue causing alteration in elastin structure, thus enabling macrophage infiltration and elevation of MMP levels. Circulating levels of calpain-1 may be used as a prognostic marker in the future if further correlation analyses are done.

Key words: Calpain, Calpastatin, MMP, Aortic aneurysm, Elastin

Introduction

Aneurysms of the ascending aorta (AAA) are an outstanding challenge to primary care physicians, internists, and cardiac surgeons as they may persist asymptomatic until they present with either dissection or rupture (Lavall et al., 2012). The risk of developing an AAA is augmented by hypertension, cigarette smoking and advanced age (Vahanian et al., 2007). Most commonly AAA is caused by atherosclerosis, genetic predisposition and connective tissue disorders like Marfan syndrome (Filipovic et al., 2005; Judge and Dietz, 2005; Lemaire et al., 2011). Intensive research is performed to reveal the molecular mechanisms causing aneurysm formation. Marfan syndrome (MFS) is caused by a mutation in the extracellular matrix protein (ECM) fibrillin-1 (Barrett and Topol, 2013). These patients are susceptible to cardiovascular lesions, in particular aortic aneurysms, as a result of pathological remodeling of the medial and adventitial ECM (Barrett and Topol, 2013). Matrix metalloproteinases (MMPs) play a key role in the

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degradation of collagen and other ECM macromolecules. MMPs are expressed at low levels in normal adult tissue, whereas an up-regulation of MMPs can be observed in normal and pathological remodeling processes (Loftus and Thompson, 2002). Besides their known role in the processes of wound healing (Wysocki et al., 1993), MMPs are more often associated with their potential role in disease states where ECM breakdown is a dominant feature. There are increasing data supporting an essential role of MMPs in vascular disease, particularly aneurysm formation, intimal hyperplasia and atherosclerosis (Chistiakov et al., 2013). Even though there is currently no proven pharmacological therapy to treat AAAs, selective inhibition of MMPs has been found to impair aneurysm formation in animal models (Manning et al., 2003). It has been suggested that MMP action may facilitate the recruitment of circulating inflammatory cells and the subsequent leukocyte infiltration through endothelial cells (Galis and Khatri, 2002). Furthermore, MMP production can also be stimulated via activated macrophages in vascular cells (Galis et al., 1994). A study on inflamed vessel disease has provided further data which indicate that MMP-9 seems to be one of the critical molecules responsible for the elastin breakdown (Lau et al., 2008). Elastin is known to be essential for arterial wall mechanical resistance, tensile strength and elasticity. Changes in the elastin content have been shown to play a key role in the development of abdominal aortic aneurysm (Gandhi et al., 1994; Carmo et al., 2002).

Calpains are ubiquitous non-lysosomal cysteine proteases which are classically activated by calcium signaling (Goll et al., 2003). A number of studies also provided evidence that epidermal growth factor (EGF) (Glading et al., 2004) and vascular endothelial growth factor (VEGF) (Hoang et al., 2010) are potent calpain activators. Calpains cleave polypeptides at a restricted number of sites assembling large, often catalytically active fragments. This property indicates that the calpains have more a regulatory or signaling function in cells rather than a digestive function such as lysosomal proteases (Goll et al., 2003). Calpains are associated to a variety of physiological processes such as cell migration (Huttenlocher et al., 1997), cytoskeletal remodeling (Dourdin et al., 2001), cell growth (Raimbourg et al., 2013) and apoptotic cell death (Kidd et al., 2000). The two major forms of the calpain-family are calpain-I and calpain-II. Besides their different tertiary structure, they are mainly distinguished by their Ca^{2+} requirement to be activated, reflected in their early nomenclature μ -calpain for calpain-I and m-calpain for calpain-II (Goll et al., 2003). Besides Ca^{2+} , the activation of calpain-I and -II can be regulated by their endogenous inhibitor calpastatin and their intracellular localization (Gil-Parrado et al., 2003; Goll et al., 2003). Calpastatin is a protein that specifically inhibits the proteolytic activity of calpain-I and -II. Up to now, there are no further proteases known to be blocked by calpastatin (Goll et al., 2003). Animal models have shown that calpains are

involved in acute inflammatory processes. Aortic medial hypertrophy and perivascular inflammation in mice has been shown to be mediated by calpain activation (Letavernier et al., 2008). Furthermore, a rat model of organ injury and dysfunction associated with hemorrhagic shock has provided evidence that the inhibition of calpain leads to a reduced activation of NF- κ B and hence prevention of the expression of NF- κ B-dependent genes (McDonald et al., 2001). Besides animal studies, one study on human aortic samples by Pilop and colleagues demonstrated increasing calpain-II expression and decreasing calpastatin expression in aneurysm tissues from ascending aortas obtained from Marfan syndrome patients (Pilop et al., 2009). Therefore, we were interested in the probable role of the calpain family in aneurysms of ascending aorta in non-Marfan patients. For this purpose ascending aortic samples of dilated and non-dilated aortas were analyzed according to their calpain-I, -II and calpastatin content as well as the expression levels of MMPs.

Materials and methods

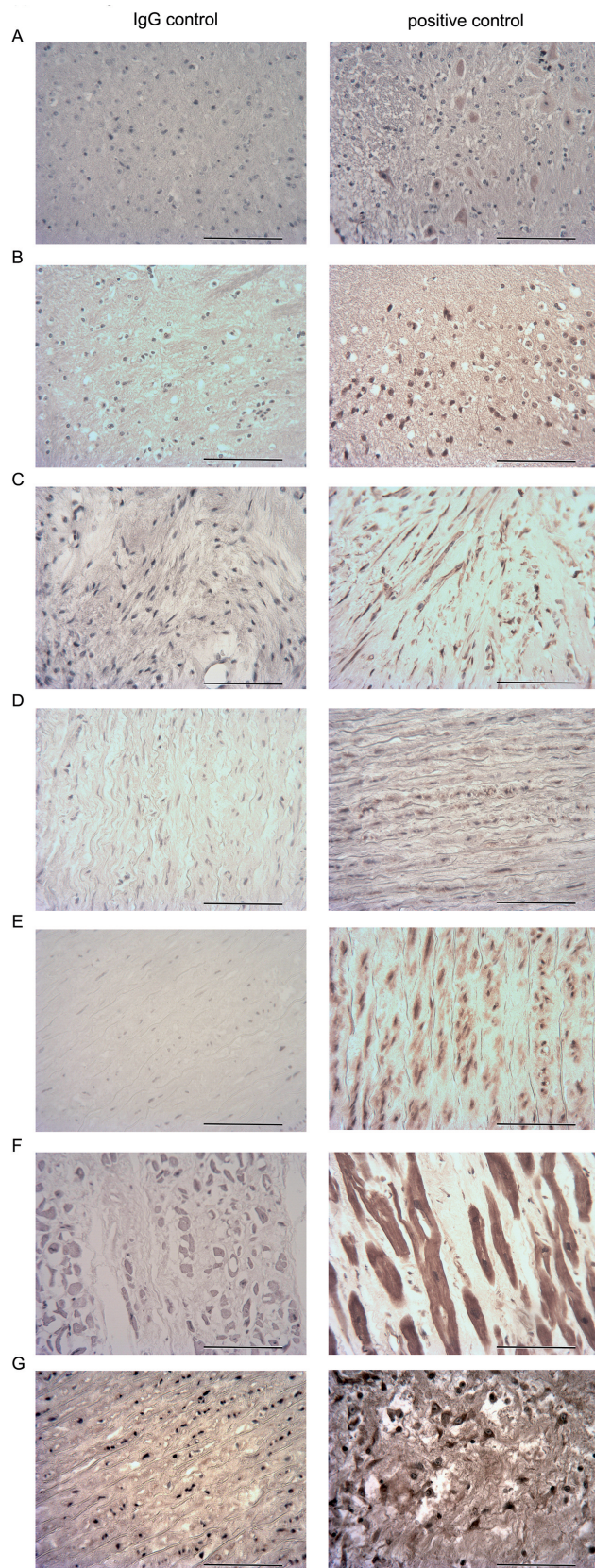
Patient characteristics and sample collection

The study was approved by the ethics committee of the University of Frankfurt, Germany (Nr.: 166/12). All samples were obtained according to the declaration of Helsinki. The ethic committee admitted that no written informed consent was required as all samples collected were declared as discarded material. Patients suffering from ascending aortic aneurysms were included in the experimental group (further referred to as AAA group). For the control group, patients without dilated aortas undergoing coronary bypass grafting (CABG) were included (further referred to as control group). Patients suffering from Marfan syndrome were not taken into consideration and excluded from the study. Aortic tissue samples were collected from the excised aneurysm in the AAA group, and from the CABG central anastomosis excision in the control group, directly stored in RNAlater for RT-PCR experiments and in 4% formaline for immunohistochemical analysis. For the control group, tissue samples from aorta without structural pathology were taken. Patients' blood was obtained immediately from discarded blood samples taken from clinical routine analyses, and stored in EDTA tubes. The blood was centrifuged at 3.000rpm for 10min and stored at -80°C until usage.

Histology and immunohistochemistry

Serial sections (6 per patient) of aortic tissue were stained to analyze calpain-I (Rabbit polyclonal to Calpain 1 - Carboxyterminal end domain IV, Abcam, Cambridge, UK), calpain-II (Rabbit polyclonal to Calpain 2 - Aminoterminal end domain III, Abcam, Cambridge, UK), calpastatin (Rabbit monoclonal [SP82] to Calpastatin, Abcam, Cambridge, UK), macrophage

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infiltration (anti-CD163, Abcam, Cambridge, UK; anti-CD-68, Santa Cruz Biotechnology, USA), MMP-1 (AbD Serotec, Oxfordshire, UK), MMP-3 (AbD Serotec, Oxfordshire, UK), MMP-9 (AbD Serotec, Oxfordshire, UK), elastin content (Abcam, Cambridge, UK) and spectrin (Santa Cruz Biotechnology, USA). For connective tissue evaluation, trichrome staining was performed according to the manufactures' instructions (Masson-Goldener-Trichrom-Staining Kit, Roth, Germany). For each antibody 6 slides per patient were stained. Five images per slide were taken for quantification. The sampling of the aortas was done in a randomized manner. Calpain-I, calpain-II, calpastatin, spectrin, MMPs and elastin expression are expressed as percentage of positive stained tissue area/field. The number of cell profiles per field were counted and expressed as number of cells/field. Representative images of the control staining for immunohistochemistry, including IgG and positive control, are summarized in Fig. 1.

Apoptosis assay

The evaluation of apoptotic cells in the tissue samples was done using an In Situ Apoptosis Detection Kit (TACS[®] 2 TdT-DAB In Situ Apoptosis Detection Kit, Trevigen, Gaithersburg, USA). The assay was performed according to the manufactures' protocol.

Total RNA isolation, cDNA synthesis and RT-PCR

Total RNA was isolated from aortic tissue samples with ZR RNA MiniPrep[™] Kit (Zymo Research, Irvine, USA) according to the manufacture's protocol. Total RNA concentration was measured with nanodrop (Nano Vue, General Electric, Connecticut, USA). An appropriate quantity of cDNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Applied Biosystems, Darmstadt, Germany) according to the manufacture's protocol. RT-PCR was performed using specific oligonucleotide primers for calpain-I, calpain-II and calpastatin (all SABioscience, Hilden, Germany) using MX3005P PCR cycler (Stratagene, Santa Clara, USA). GAPDH served as housekeeping gene in comparisons of gene expression data of calpain-I, calpain-II and calpastatin (SABioscience, Hilden, Germany).

Fig. 1. Representative images of immunohistochemical control staining. Left column images show IgG controls (negative controls), appropriate to the corresponding primary antibodies. The right column images show the positive control staining. **A.** Calpain-I control groups, stained on brain tissue. **B.** Calpain-II control groups, stained on brain tissue. **C.** Calpastatin control groups, stained on mammary carcinoma tissue. **D.** MMP-1 control groups, stained on aorta tissue. **E.** MMP-3 control groups, stained on aorta tissue. **F.** MMP-9 control groups, stained on myocardial tissue. Scale bar: 1 mm.

Enzyme-linked immunoadsorbent assay (ELISA)

Calpain-I, calpain-II and calpastatin levels in the serum of the AAA and control group were determined using human Calpain-I, Calpain-II and Calpastatin Enzyme Immunoassay Kit from Cusabio Biotech (Wuhan, China) in accordance with the manufacturer's protocol.

Statistical analysis

Data represent means $\pm$ SEM. Statistical analysis was performed with Prism 6 software (Graph Pad) using unpaired Students' T-test. Differences with $p < 0.05$ were considered statistically significant.

Results

Our patients did not differ in age (control group: 61.6 ± 9.2 years; AAA group: 63.9 ± 7.2 years) and sex (control group: 9 (90%) male; AAA group: 9 (90%) male).

The analysis of the aortic tissue samples by RT-PCR did not show any differences in expression levels of calpain-I, calpain-II nor calpastatin comparing controls with AAA (Fig. 2). Nevertheless, the control group presented significantly higher calpain-II levels compared to calpain-I and calpastatin (Calpain-II $0.101 \pm 0.015\%$ vs. calpain-I $0.014 \pm 0.002\%$ ($p < 0.0001$) and calpastatin $0.056 \pm 0.004\%$ ($p < 0.0151$)). Aortic calpain-I contents were even less significant than calpastatin contents (Calpain-I $0.014 \pm 0.002\%$ vs. calpastatin $0.056 \pm 0.004\%$ ($p < 0.0001$)). A similar observation was made in the AAA group. Calpain-I was significantly less expressed compared to calpain-II and calpastatin (Calpain-I $0.007 \pm 0.001\%$ vs. calpain-II $0.084 \pm 0.014\%$ ($p = 0.0001$) and calpastatin $0.063 \pm 0.008\%$ ($p < 0.0001$)). A difference in calpain-II and calpastatin was only observed by trend.

The same patient samples were used for immunohistochemical staining. The evaluation of calpain-I staining in aortic tissue showed a significant increase of calpain-I in the AAA group compared to controls (AAA: $22.69 \pm 1.04\%$ vs. control: $15.48 \pm 1.54\%$ ($p = 0.0001$)) (Fig. 3A). Analysis of calpain-II did not lead to a significant different expression level in the aortic tissue samples comparing both groups (control: $21.30 \pm 1.46\%$ vs. AAA: $23.78 \pm 1.58\%$ ($p = 0.2811$)) (Fig. 3B). Even though no changes were observed, a high level of calpain-II was found in both groups, comparable with the amount of calpain-I found in the AAA group. The aortic tissue of the control group contained significantly more calpain-II than calpain-I (calpain-I: $15.48 \pm 1.54\%$ vs. calpain-II: $21.30 \pm 1.46\%$ ($p < 0.0074$)). A significant up-regulation of calpastatin was observed in the AAA group compared to the control group (control: $0.132 \pm 0.051\%$ vs. AAA: $1.327 \pm 0.198\%$ ($p < 0.0001$)) (Fig. 3E). However, the level of calpastatin expressed in aortic tissue was relatively low compared to calpain-I and -II levels. The control group presented significantly less calpastatin compared to calpain-I and -II (Calpain-I: $15.48 \pm 1.54\%$ ($p < 0.0001$); Calpain-II $21.30 \pm 1.46\%$ ($p < 0.0074$) vs Calpastatin: $0.132 \pm 0.051\%$). The same observation was made in the AAA group (Calpain-I: $22.69 \pm 1.04\%$ ($p < 0.0001$); Calpain-II $23.78 \pm 1.58\%$ ($p < 0.0001$) vs Calpastatin: $1.327 \pm 0.198\%$). The representative images of the immunohistochemical staining (Fig. 3C-D,G-H) illustrate the calculated differences. In accordance with calpain-I expression, its specific-target protein spectrin is also significantly higher expressed in the AAA group compared to control (AAA: $11.39 \pm 0.47\%$ vs. control: $9.25 \pm 0.60\%$ ($p = 0.0069$)) (Fig. 3F).

The protein content in the serum samples was measured by ELISA. Significantly less calpain-I was detected in the AAA group compared to control samples ($p = 0.0019$) (Fig. 4A). The opposite results were detected

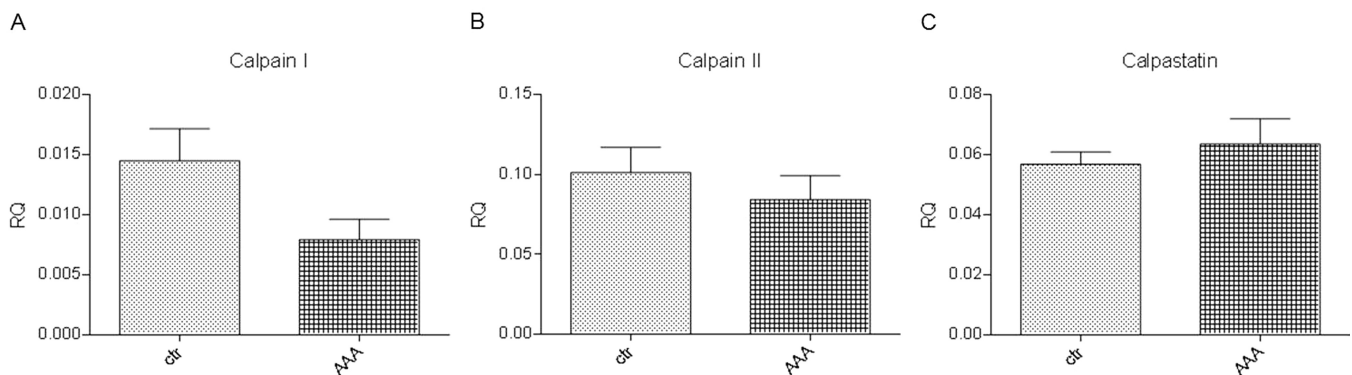


Fig. 2. No differences in the mRNA expression levels of Calpain I, and II as well as Calpastatin were observed in aortic tissue. **A.** Expression level of calpain-I in aortic tissue samples comparing control vs. AAA group. **B.** Expression level of calpain-II in aortic tissue samples comparing control vs. AAA group. **C.** Expression level of calpastatin in aortic tissue samples comparing control vs. AAA group. (Data represent mean $\pm$ SEM, * $p < 0.05$ is considered statistically significant)

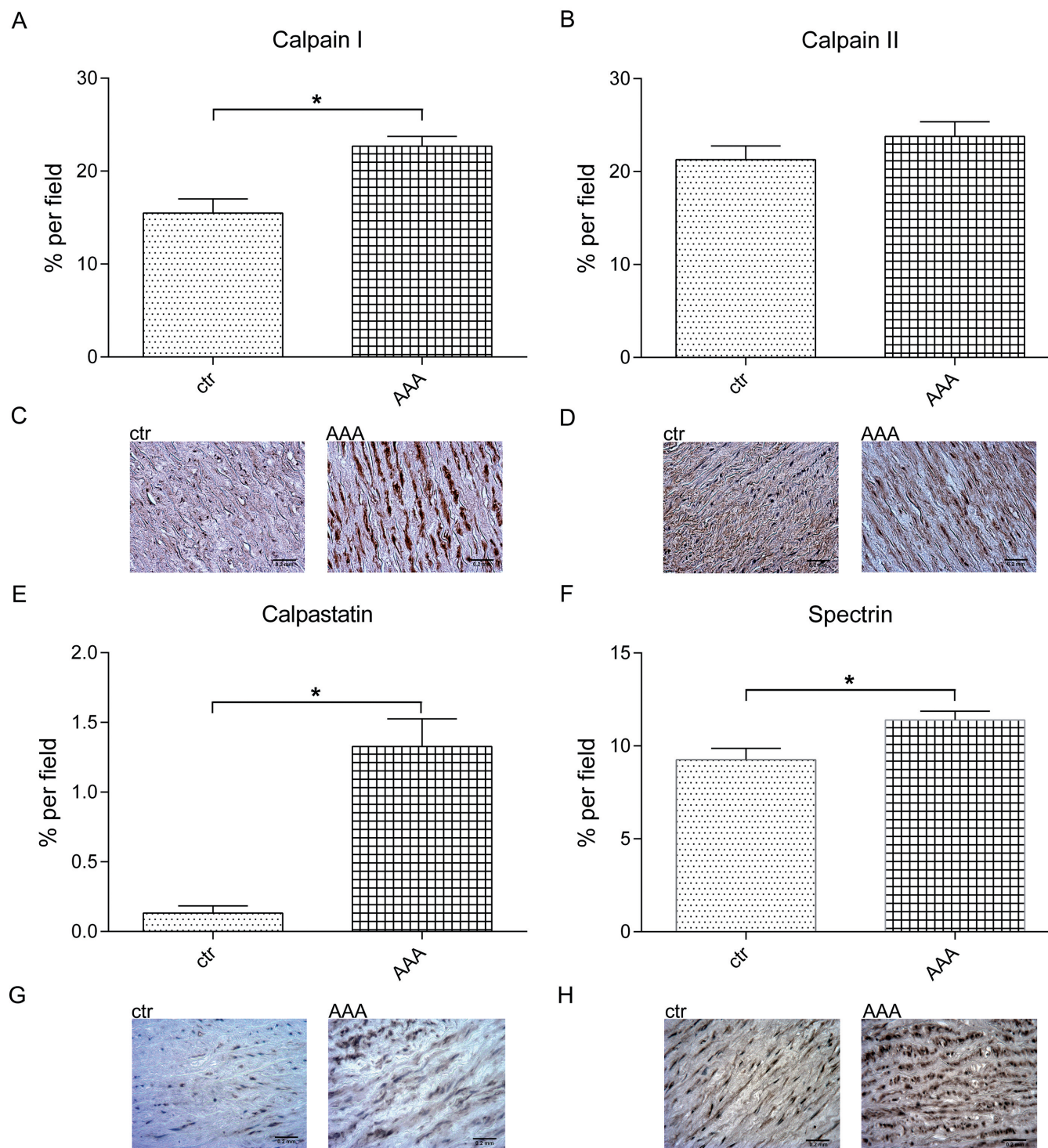


Fig. 3. Immunohistochemical staining revealed different expression levels of calpain-1 and calpastatin in dilated aortic tissue. **A.** Calpain-I is more highly expressed in aortic tissue samples in the AAA group compared to control group. **B.** Expression level of calpain-II in aortic tissue samples comparing control vs. AAA group were not different. **E.** Calpastatin is more highly expressed in aortic tissue samples in the AAA group compared to control group. **F.** Spectrin is significantly more highly expressed in the AAA group compared to control group. **C.** Representative images from **A.** **D.** Representative images from **B.** **G.** Representative images from **E.** **H.** Representative images from **F.** Data represent mean $\pm$ SEM, * $p < 0.05$ is considered statistically significant. Scale bar: 1 mm.

in the calpastatin levels ($p=0.0312$) (Fig. 4B). Furthermore, calpain-2 was measured in the AAA and control samples; however, we could neither detect calpain-2 in the AAA group nor in the control group.

Tissue structure analysis by immunohistochemical staining for elastin indicated no difference in elastin content between the groups (control: $18.20 \pm 1.29\%$ vs. AAA: $18.55 \pm 1.07\%$ ($p=0.8352$)) (Fig. 5A). However, the dilated aortic tissue from the AAA group showed severe structural impairments (Fig. 5B). In the AAA group, elastin seems to be more accumulated and is not anymore consistently distributed in the tissue in fine-spun fibers as seen in the control tissue. The number of inflammatory immune cell profiles did not differ in amount comparing controls and AAA (control: 9.02 ± 1.49 vs. AAA: 7.58 ± 1.03 ($p=0.4154$)) (Fig. 6). Connective tissue staining also revealed structural impairments (Fig. 5C-D). Nevertheless, the macrophages are more deeply infiltrated into the tissue in the AAA samples compared to the control group (Fig. 6C). The staining for apoptotic cells in our tissue samples only showed scarce positive reactions for infiltrating PMN leukocytes as shown in Fig. 7.

The analysis of MMPs in the aortic tissue via immunohistochemical staining revealed differences in MMP-1 and MMP-3 but not MMP-9 expression (Fig. 8). MMP-1 is significantly higher expressed in the tissue of dilated aortas (control: $1.74 \pm 0.32\%$ vs. AAA: $3.22 \pm 0.29\%$ ($p=0.0011$)). The same observation was made regarding MMP-3 (control: $0.98 \pm 0.23\%$ vs. AAA: $5.68 \pm 0.50\%$ ($p<0.0001$)). No alterations in MMP-9 content could be seen comparing control and AAA group

(control: $11.60 \pm 1.19\%$ vs. AAA: $11.99 \pm 0.59\%$ ($p=0.7528$)). Comparing the expression levels of MMP-1, -3 and -9 within the control and the AAA group changes worth mentioning have been observed. The control group showed significantly higher MMP-9 levels compared to MMP-1 and -3 (MMP-9: $11.60 \pm 1.19\%$ vs. MMP-1: $1.74 \pm 0.32\%$ ($p<0.0001$) and MMP-3: $0.98 \pm 0.23\%$ ($p<0.0001$)). Focusing the AAA group, we see the lowest expression levels in MMP-1, increased levels of MMP-3 and the highest expression of MMP-9, all of them significant to each other (MMP-1: $3.22 \pm 0.29\%$ vs. MMP-3: $5.68 \pm 0.50\%$ ($p<0.0001$) vs. MMP-9: $11.99 \pm 0.59\%$ ($p<0.0001$)).

Discussion

The research on the pathogenesis of ascending aortic aneurysm has predominantly been focused on aneurysms caused by MFS and other connective tissue disorders. However, molecular mechanisms causing aortic aneurysms of patients without genetic disorders are far from being understood. As aneurysms of the ascending aorta are still an unresolved issue to clinicians the necessity of diagnostic and prognostic markers is absolutely essential.

In the present study we analyzed the tissue or ascending aortas from patients suffering from aneurysms of the ascending aorta. We used RT-PCR analysis and immunohistochemical staining to evaluate the expression levels of calpain-I, -II and their inhibitor calpastatin. Additionally the tissue was investigated regarding spectrin, apoptosis, MMP and elastin

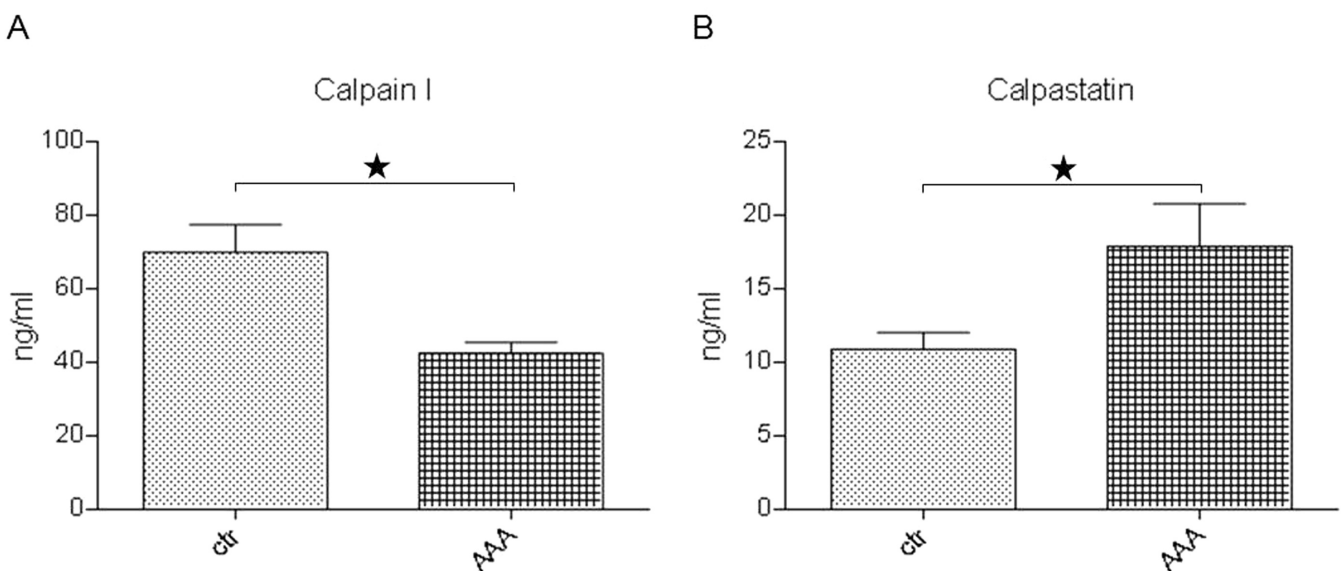


Fig. 4. ELISA measurements show opposed expression levels of calpain-1 and calpastatin. **A.** Calpain-1 is less detectable in the AAA group compared to controls. **B.** Calpastatin is more highly expressed in the AAA group compared to the control group. Data represent mean $\pm$ SEM, * $p<0.05$ is considered statistically significant.

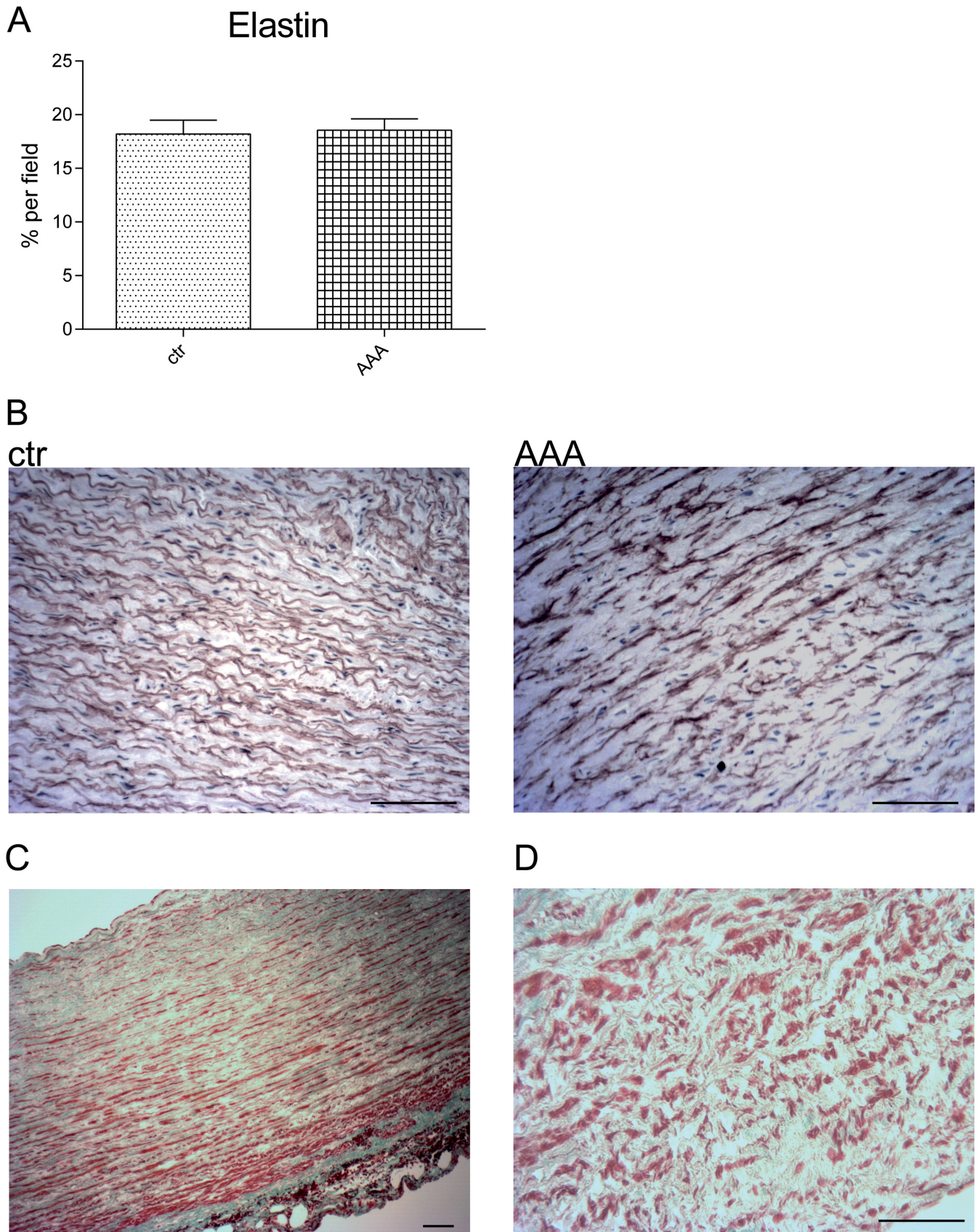
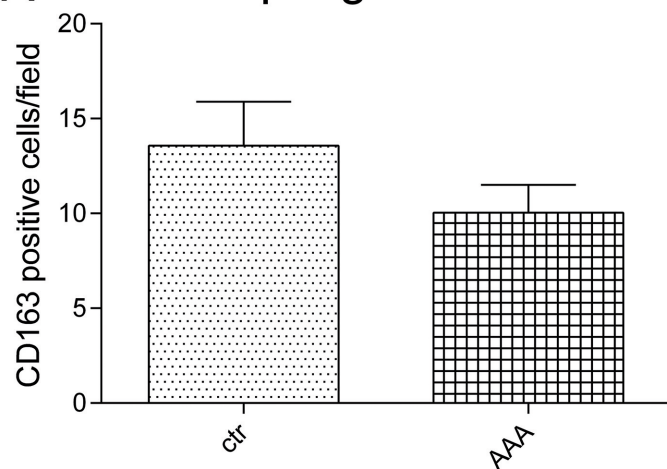
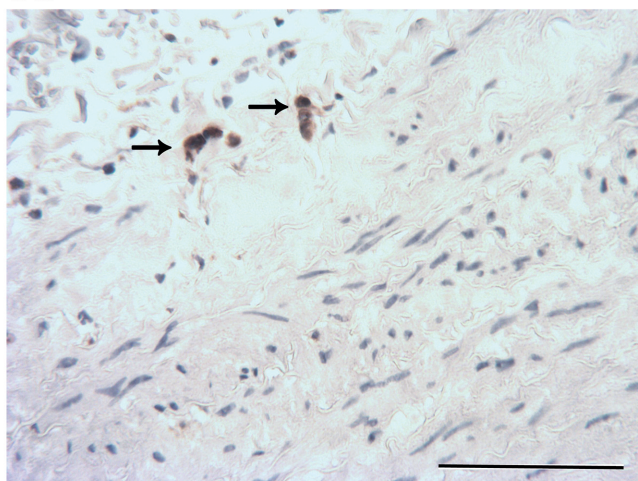


Fig. 5. Immunohistochemical staining of elastin and connective tissue. **A.** The level of elastin content in the dilated aortas does not differ between the groups. **B.** Representative images from **A** showing the altered elastin structure in the AAA group compared to control. **C.** Representative images from Masson-Goldner-Trichrom-Staining. **D.** Representative images from Masson-Goldner-Trichrom-Staining of AAA aortic tissue. Data represent mean $\pm$ SEM, * $p < 0.05$ is considered statistically significant. Scale bars: 1 mm.

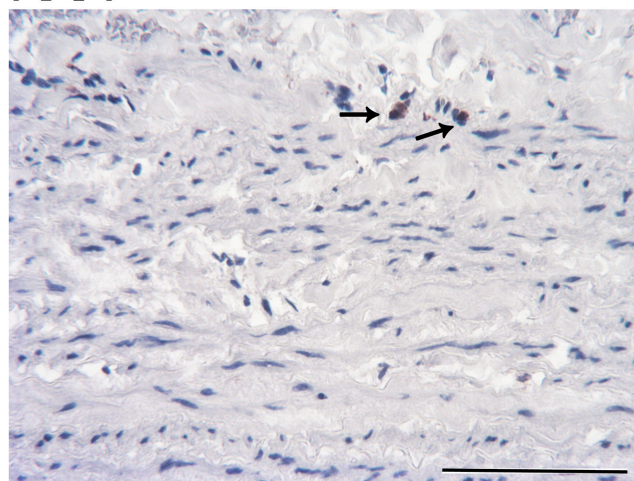
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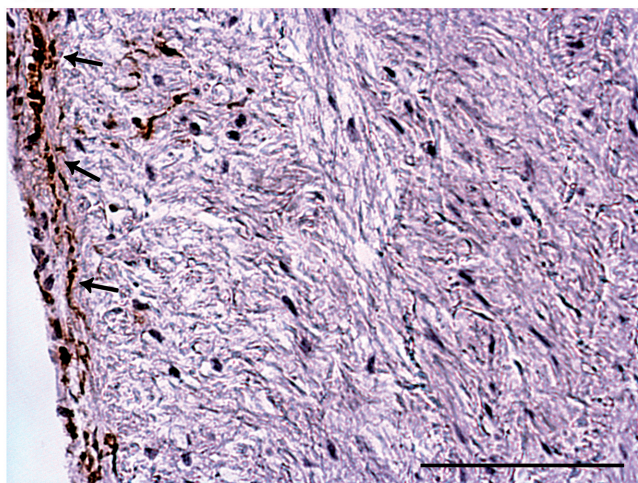
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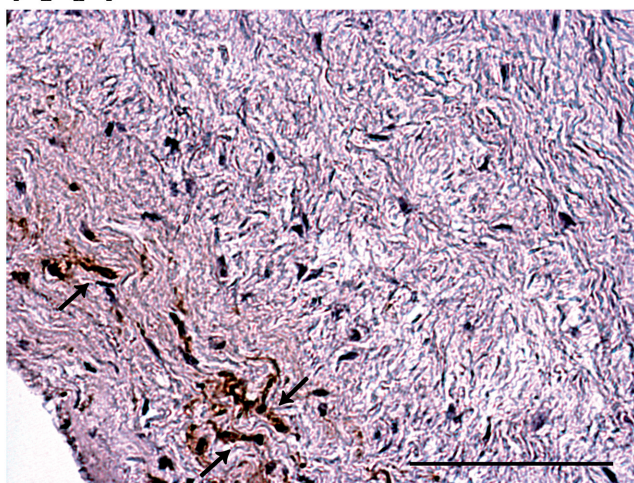


Fig. 6. Immunohistochemical staining of CD163 cells in non-dilated vs. dilated aortic tissue. **A.** The number of infiltrated macrophages is almost equal in both groups. **B.** Representative images from CD68 staining. **C.** The difference in macrophage localization is shown by CD163 staining. Data represent mean $\pm$ SEM, * $p < 0.05$ is considered statistically significant. Scale bars: 1 mm.

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expression as well as macrophage infiltration. Most interesting is the finding that calpain-I but not calpain-II seem to play a role in AAA compared to previous data on AAA in Marfan patients (Pilop et al., 2009). Pilop et al. assessed calpain activity by measuring the cleavage fragments of α -2 spectrin and found a significant upregulated cleavage in aneurysmal aortic media compared to control (Pilop et al., 2009). Evaluation of immunohistochemistry has shown that calpain-I and calpastatin are significantly higher expressed in dilated aortic tissue compared to non-dilated aortic tissue. Our

analysis on the calpain target protein spectrin also shows a significantly higher expression level in AAA tissue than in the control group, supporting the calpain analysis. The level of calpain-II did not differ between the AAA and control group. This finding indicates that in patients without Marfan, calpain is differently expressed and seems to play a different role in AAA. Even though calpain-I is elevated in AAA, calpastatin is also significantly raised in AAA tissue. This discrepancy can be explained by the constitutive extreme low tissue expression levels of calpastatin (control below 0.15%

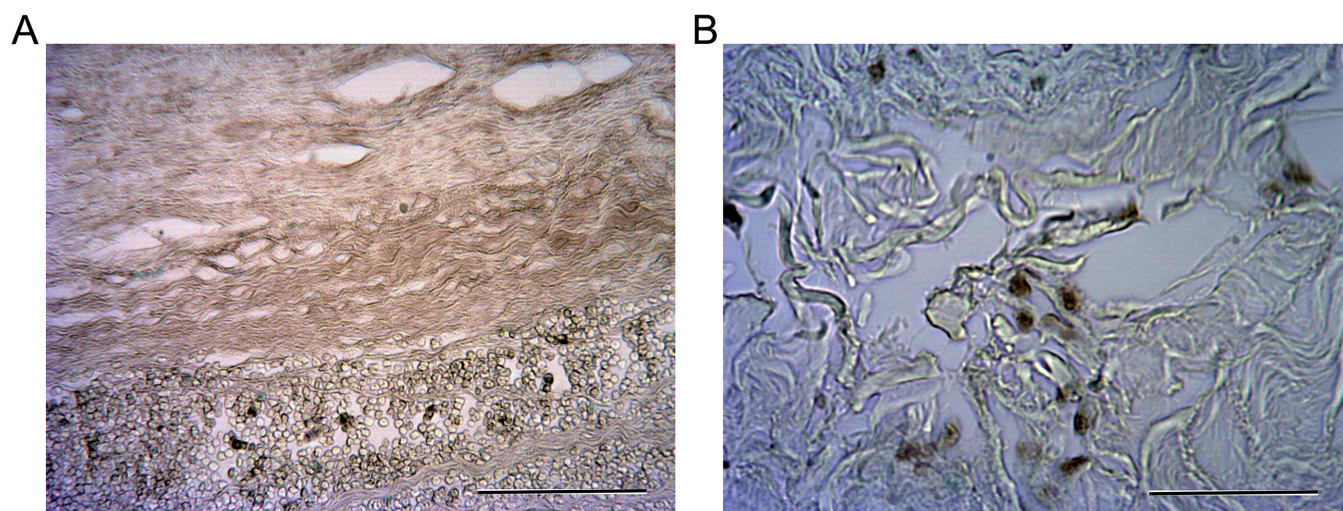


Fig. 7. Representative images of apoptosis staining. **A.** Aortic tissue sample with scarce positive reactions. **B.** Positive control staining of aortic tissue using TACS nucleases. Scale bar: 1 mm.

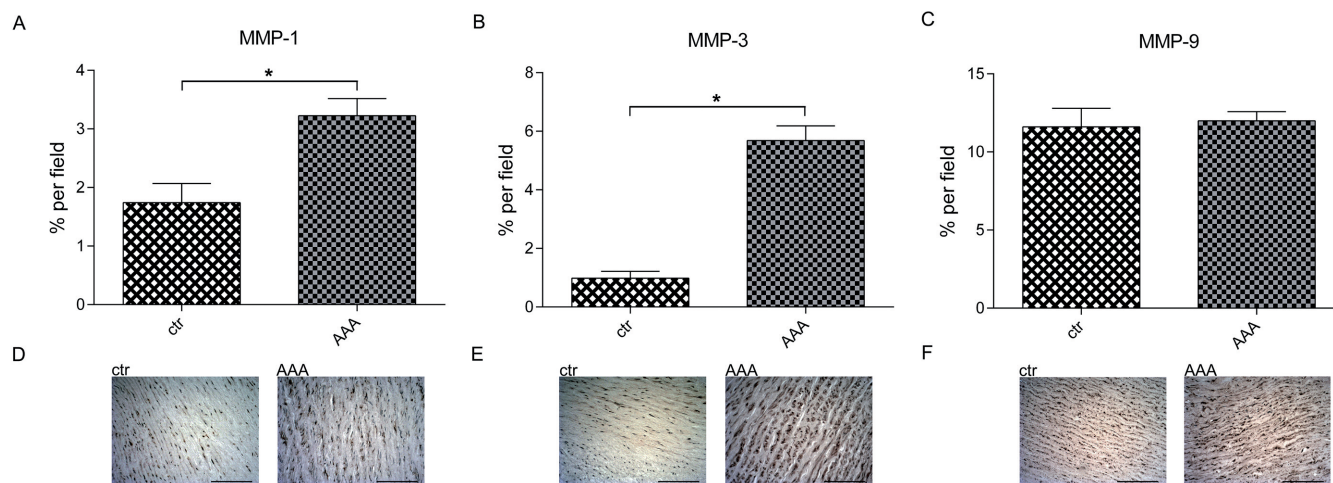


Fig. 8. Evaluation of immunohistochemical staining of MMPs in aortic tissue comparing controls and AAA. **A.** MMP-1 is more highly expressed in aortic tissue samples in the AAA group compared to control group. **B.** MMP-3 is more highly expressed in aortic tissue samples in the AAA group compared to control group. **C.** Expression level of MMP-9 in aortic tissue samples comparing control vs. AAA group were not different. **D.** Representative images from **A.** **E.** Representative images from **B.** **F.** Representative images from **C.** Data represent mean $\pm$ SEM, * $p < 0.05$ is considered statistically significant. Scale bars: 1 mm.

and AAA below 1.40%) compared to the calpain-I and -II expression levels (both above 15-20%). Additionally, previous studies have provided evidence that calpains and calpastatin are not co-localized within the cell until calcium-related cell stimulation leads to calpain-calpastatin interaction (De Tullio et al., 1999). Not much is known about the circulating levels of calpain-1 and -2 and their inhibitor calpastatin in the pathology of ascending aortic aneurysms. It has been shown that calpain-1 and -2 have a relatively long half-life of 5 or more days, thus providing the potential to be used as prognostic marker (Zhang et al., 1996). We see that the circulating levels of calpain-1 and calpastatin are opposite detected in the AAA and control group. Calpain-1 is less found in the serum of the patients suffering from AAA. This does not correlate with the protein level of calpain-1 in the tissue samples. It seems that the role of calpain-1 on the development and/or progression on aneurysm of the ascending aorta is not a systemic effect but a more local effect. As previously mentioned calpains and calpastatin do not interact with each other as long as no calcium-related cell stimulation occurs (De Tullio et al., 1999). Due to the negative correlation in calpain-1 and calpastatin levels in the circulation, it seems that the inhibitory effect of calpastatin can work in the circulation in a more independent way.

Elastin belongs to the dominant extracellular matrix (ECM) proteins in the aortic wall. Vascular smooth muscle cells (VSMCs) are responsible for elastin synthesis in the media after mechanical stimuli such as stretch and pressure. Furthermore, elastin can control cellular components and interact with VSMCs via elastin-binding proteins and thus tune the cytoskeletal actin alignment and restrict cell migration and proliferation (Mochizuki et al., 2002; Karnik et al., 2003). Our data on macrophage infiltration can confirm the disturbance in elastin structure as the macrophages in the AAA tissue samples were much more deeply infiltrated compared to the non-dilated aortic tissues, and thus the protective property of elastin regarding cell migration already started to fail. This could also explain that no difference in the number of migrated macrophages is observed but a clear difference in location. Furthermore, elastin has been postulated to be chemotactic and could therefore play a role in the inflammatory response (Houghton et al., 2006), further explaining the difference in macrophage localization in the AAA and control group. The histological staining for elastin demonstrates normal elastin content and organization within the non-dilated aortic segment and confirmed previous data on the disrupted and degraded elastin structures in dilated aortas (Nataatmadja et al., 2003; Padang et al., 2012). It is well known that the destruction of connective tissue plays an essential role in aortic aneurysm formation and development (Davies, 1998). Besides the alterations in elastin in our experimental groups, we can also confirm severely

manipulated connective tissue formation and alignment in both groups. This fact is probably also responsible for the lack of positive reaction for our apoptosis assays. The infiltrated inflammatory cells in the tissue can trigger and increase the production of MMPs. It is well known that MMPs are important for homeostasis and turnover of the ECM in health and disease. Initially it was thought that MMPs were mainly involved in matrix turnover and degradation, however there are recent data providing evidence that MMPs are actively involved in the inflammatory process (Birrell et al., 2006). Also data on the abdominal aortic aneurysm has provided evidence that inflammatory processes in the aortic wall are tightly linked to ruptured abdominal aortic aneurysms (Treska et al., 2002). A prospective, non-randomised study, comparing asymptomatic, symptomatic and ruptured abdominal aortic aneurysm, measured MMP expression and assigned them a potential role in inflammatory responses and fibrinolysis (Eberlova et al., 2013). However, even though abdominal and ascending aortic aneurysms share some similar pathological phenotypes, they also display several central differences (Guo et al., 2006), thus differentiated conclusions are essential. In vitro it has been shown that the combination of monocytes and VSM or endothelial cells can cause an up-regulation of MMP-1 (Zhu et al., 2000) and MMP-9 (Amorino and Hoover, 1998), respectively. Hence, it was concluded that they may play a crucial role in monocyte adherence on the endothelium. These findings are in line with our MMP-1 expression levels. The AAA group has shown significant higher MMP-1 levels than the controls, possibly favoring the deeper macrophage infiltration into the aortic aneurysm tissue. MMP-3 has been assigned to be involved in the development of dilative pathology of ascending thoracic aorta and a protective property has been postulated in combination with angiotensin-converting enzyme (Lesauskaite et al., 2011). Even though studies have identified MMP-3 within aneurysms, the role of MMP-3 is not clarified in this pathology. Our data provide additional information about the expression levels of MMP-3 in ascending aortic aneurysms. To us, it seems that MMP-3 is involved in aneurysm formation or progression, although this needs further clarification. Surprisingly, we did not detect a difference in MMP-9 expression levels between the AAA and control group. Previous studies have shown higher MMP-9 levels in aneurysms compared to non-diseased patients (Davis et al., 1998). The discrepancy could be due to the patients collective and their anamnesis.

Due to our findings we conclude that calpain-1 seems to provoke the fibrotic alteration in aortic aneurysm tissue in our experimental group. The change in calpain-1 modulates the structure of aortic tissue causing alteration in elastin structure, thus provoking macrophage infiltration and elevation of MMP levels. In the case of a more detailed look at the correlation between disease stage and circulating levels of calpain-1

it may be used as prognostic marker in the future.

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