

Serine protease inhibitor A3 in atherosclerosis and aneurysm disease

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Abstract. Remodeling of extracellular matrix (ECM) plays an important role in both atherosclerosis and aneurysm disease. Serine protease inhibitor A3 (serpinA3) is an inhibitor of several proteases such as elastase, cathepsin G and chymase derived from mast cells and neutrophils. In this study, we investigated the putative role of serpinA3 in atherosclerosis and aneurysm formation. SerpinA3 was expressed in endothelial cells and medial smooth muscle cells in human atherosclerotic lesions and a 14-fold increased expression of serpinA3n mRNA was found in lesions from Apoe-/- mice compared to lesion-free vessels. In contrast, decreased mRNA expression (-80%) of serpinA3 was found in biopsies of human abdominal aortic aneurysm (AAA) compared to non-dilated aortas. Overexpression of serpinA3n in transgenic mice did not influence the development of atherosclerosis or CaCl₂-induced aneurysm formation. In situ zymography analysis showed that the transgenic mice had lower cathepsin G and elastase activity, and more elastin in the aortas compared to wild-type mice, which could indicate a more stable aortic phenotype. Differential vascular expression of serpinA3 is clearly associated with human atherosclerosis and AAA but serpinA3 had

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no major effect on experimentally induced atherosclerosis or AAA development in mouse. However, serpinA3 may be involved in a phenotypic stabilization of the aorta.

Introduction

Degradation and remodeling of extracellular matrix (ECM) play an important role in both atherosclerosis and aneurysm disease, two chronic inflammatory diseases (1,2). Neutrophils and mast cells produce several serine proteases such as tryptase, chymase, cathepsins and elastase, which are enzymes that are able to degrade different components of the ECM, a process involved in both diseases (3-5). Inhibition of mast cell chymase reduces atherosclerotic plaque progression and improves plaque stability in Apoe^{-/-} mice (6) and aneurysmal dilatation is inhibited in mast cell deficient animals (4,7,8). Also, neutrophils are major vascular component in atherosclerotic lesions (9) and an important source of proteases in the vessel wall of abdominal aortic aneurysm (AAA) (10). Furthermore, neutrophil depletion in mice inhibits elastase induced experimental AAA formation, an effect independent of MMP-2, MMP-8 or MMP-9 (11). The discovery of mediators of neutrophil recruitment and neutrophil derived factors in vascular samples, supports the involvement of neutrophils in vascular inflammatory disease (12).

Serine protease inhibitor A3 (serpinA3) inhibits several proteases derived from neutrophils and mast cells, such as human leukocyte elastase, cathepsin G and chymase (13). SerpinA3, also referred to as α 1-antichymotrypsin, was first characterized as an acute phase plasma protease inhibitor. It is synthesized in a range of tissues including hepatocytes, bronchial epithelial cells and neuronal cells (14,15). In mice, the serpinA3 gene has undergone extensive duplication and diversification resulting in a family of 13 closely related inhibitors with differing tissue distribution and protease specificity

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(16,17). Nevertheless, gene expression and functional studies suggest that serpinA3n is the closest murine ortholog of human serpinA3 (13,17).

Because mast cell- and neutrophil-derived proteases have been implicated in the remodelling of ECM and in the progression of atherosclerosis and aortic aneurysm formation, we investigated the putative involvement of serpinA3 in these two diseases.

Materials and methods

All human and mouse protocols were approved by the local ethics review board and the study was conducted according to the Helsinki Declaration.

Human subjects. Patients scheduled for elective surgery for infrarenal AAA (n=9) at Karolinska University Hospital (Stockholm, Sweden) where preoperative computer tomography demonstrated an eccentric intraluminal thrombus were included in the study. The intima/media and adventitia layers of AAA were separated by adventicectomy. The Biobank of Karolinska Endarterectomies (BiKE) study included atherosclerotic tissue collected from asymptomatic patients and patients with minor stroke or transient ischemic attack (TIA), undergoing carotid endarterectomy at the Karolinska University Hospital. Patients were included after informed, written and signed consent.

Control ascending aorta samples for RNA studies were obtained from 8 organ donors without clinical or macroscopic signs of aortic atherosclerosis. Infrarenal control aortic samples for histology were collected from 14 medicolegal autopsies performed in the Department of Forensic Medicine, University of Helsinki. The sections were immediately fixed in 4% formaldehyde for light microscopy or snap-frozen in liquid nitrogen for RNA isolation. The use of organ donor and autopsy tissues was approved by The National Authority for Medicolegal Affairs of Finland.

CaCl₂ induced aneurysm model in mice. CaCl₂-induced aneurysm is an inflammatory driven model and is a frequently used method to experimentally induce abdominal aortic aneurysm in a controlled fashion (18). Mice were anesthetized with standard doze of sodium pentobarbital intraperitoneally (6 mg/100 g). The adequacy of anesthesia was monitored by the absence of the corneal reflex, and adequate levels of anesthesia and analgesia were ensured with supplemental intraperitoneal injection of pentobarbital sodium given as required. Body temperature was maintained using a blanket control unit. A small compress strip with 0.5 M CaCl₂ or NaCl as control was applied to the isolated abdominal aorta which was covered for 15 min. Then the compress was removed and the treated area was washed with PBS twice. Mice were treated with Temgesic (0.1 mg/kg) 30 min before wakening 2 times/day for 2 days. Operated animals were monitored daily for any sign of pain or disability. After 2 weeks the mice were anesthetized with 2% isoflurane over a nose mask, whole blood was drawn by cardiac puncture and the aorta was removed for further analysis. Mice were then euthanized with carbon dioxide. The investigation conforms to the directive 2010/63/EU of the European Parliament.

Atherosclerotic lesion size. To study the effect of serpinA3n in atherosclerotic disease development, Apoe-1- (backcrossed 10 times with C57bl/6) and serpinA3n transgenic mice (on C57bl/6 background) crossed with Apoe^{-/-} were fed a chow diet and sacrificed at 20 weeks of age. After vascular perfusion with sterile RNase-free PBS, thoracic aortas and hearts were dissected and preserved for lesion analysis. Five $10-\mu m$ sections were collected at $100 - \mu m$ intervals starting at a $100 - \mu m$ distance from the aortic valves. Formaldehyde-fixed sections were stained with oil red-O and lesion size was analyzed using Leica Q500MC image analysis software. For each mouse, a mean lesion area was calculated from 5 sections, reflecting the cross-section area covered by atherosclerosis. The descending thoracic aorta was fixed in 4% formaldehyde, opened longitudinally, pinned onto black wax plates and stained with Sudan IV (Merck KGaA, Darmstadt, Germany). The lesion areas and total aortic areas were calculated using Image J software (NIH, Bethesda, MD, USA).

Cell culture. EA.hy 926 human endothelial cells were maintained in high-glucose (4.5 g/l) Dulbecco's modified Eagle's medium, supplemented with 10% FCS, sodium bicarbonate solution, 1X HAT (mixture of sodium hypoxanthine, aminopterin and thymidine) (Invitrogen Life Technologies), and penicillin-streptomycin. Human aortic smooth muscle cells (SMCs) were maintained in growth medium containing supplements (CC-3182, Clonetics, Cambrex, Walkersville, MD). The cells were used at passages 3-5. All cells were incubated in 5% CO₂ at 37°C and stimulated with lipopolysaccharide (LPS, 10 μ g/ml; Sigma-Aldrich, Stockholm, Sweden), IFN- γ , IL-1 β or TNF- α (20 μ g/ml, PeproTech, Inc., London, UK).

Creation of mice transgenic for serpinA3n. The murine serpinA3n cDNA sequence was amplified from C57Bl/6 mice and cloned into the chicken β -actin promoter plasmid pCAGIPuro, in which serpinA3n expression is driven by a human cytomegalovirus immediate early enhancer (HCMVIEE) coupled to the chicken β -actin promoter. The sequence of *serpinA3n* was confirmed by sequencing. Using DNA microinjection into C57Bl/6 fertilized oocytes of the β -actin promoter-HCMVIEE-*serpinA3n* fragment, we obtained 2 transgene-positive *serpinA3n* mice. The founder mice were identified using PCR. Genotyping primers were: F, 5'-GAGGACCTGACCACACCCTA-3' (exon 3) and R, 5'-TTATCAGGAAAGGCCGATTG-3' (exon 5). The 2 founder mice were crossed with C57Bl/6 mice (purchased from Taconic, Denmark).

ELISA of serpinA3. Blood samples were collected in Vacutainer tubes containing EDTA and then centrifuged to separate plasma, which was then stored at -70°C until analyzed. EDTA plasma samples were available from 49 healthy control subjects (69 ± 3.7 years), 40 patients diagnosed with AAA (71 ± 7.4 years) and 28 patients with myocardial infarction (70 ± 6.2 years). Plasma serpinA3 concentration was measured using a commercially available ELISA kit (#E-80CYT; ICL, Inc., Portland, OR, USA). Briefly, EDTA plasma samples were loaded into the serpinA3 pre-coated ELISA wells and incubated for 1 h. After washing, HRP-conjugated serpinA3 detection antibody was added, followed by washing and addition of tetramethylbenzidine substrate. Samples were run in duplicate and the results were quantified against a standard curve created using the recombinant standard that was included in the kit.

Protein preparation and western blot analysis. Aortic tissue from mice was homogenized in ice-cold lysis buffer containing PBS and proteins in lysates or plasma were separated under reducing conditions by electrophoresis using 10% SDS-PAGE (polyacrylamide gel electrophoresis). The blot was incubated with primary antibodies against murine serpinA3n (Agrisera, Vännäs, Sweden), overnight and then for 1 h with horseradish peroxidase-labeled secondary antibody. Signals were detected with enhanced chemiluminescence western blot detection reagent (GE Healthcare, Uppsala, Sweden).

Gene expression analysis. Tissue samples were homogenized with FastPrep using Lysing Matrix D tubes (MP Biomedicals, Illkirch, France). Total-RNA from tissue or cells in culture was isolated with RNeasy (Qiagen, Germany) and cDNA (1.0 ng) was amplified by RT-PCR reactions as described before (19). The following probe was used for analysis of human serpinA3 expression: Hs00153674 and murine serpinA3n expression: Mm00776439, and results were normalized to values of human RPLP0: Hs99999902 or murine β 2 microglobulin: Mm00437762. All probes were obtained from Applied biosystems, Foster City, CA, USA.

The BiKE RNA samples were hybridized and scanned at the Karolinska Institute Affymetrix core facility. For samples from the BiKE biobank Affymetrix HG-U133 plus 2.0 arrays and protocols were used as described before (20).

Immunohistochemistry. Human and mouse abdominal aortas were cryosectioned and fixed in acetone. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide for 5 min followed by incubation in 5% blocking bovine serum albumin solution. Sections were then incubated with a polyclonal rabbit anti-human serpinA3 antibody (Abcam) or an isotype control (Abcam) at the same concentration at 4°C overnight. Consecutive sections were incubated with rabbit polyclonal anti-human Von Willebrand factor (Dako, Glostrup, Denmark), mouse monoclonal CD66b (Fitzgerald, North Acton, MA, USA), mouse monoclonal CD68 (Leica Microsystems, Newcastle, UK), mouse monoclonal CD163 (Acris, Herford, Germany), mouse monoclonal CD3 (Santa Cruz Biotechnology, Inc.), mouse monoclonal mast cell tryptase (Dako) and mouse monoclonal anti-human α -actin (clone 1A4; Sigma-Aldrich) antibodies at 4°C overnight followed by secondary biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (Dako) antibody. Avidin-biotin peroxidase complexes (Dako) were added, followed by visualization with 3,3'-diaminobenzidine tetrahydrochloride (Dako). All sections were counterstained with Mayer's hematoxylin (Histolab Products, Göteborg, Sweden).

Mast cell chymase activity. For detection of mast cell chymase activity in mouse serum a chymase assay kit was used according to the manufactures instruction (Sigma-Aldrich). Briefly, serum was added, followed by assay buffer and the substrate N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide. The absorbance was detected at 405 nm. Serum without the addition of substrate was used as internal blanking for each sample.



Figure 1. SerpinA3 expression in aortic aneurysm. Immunohistochemical analysis of serpinA3 (A) in atherosclerotic lesions, (B and C) in AAA, (D and F) in control abdominal aorta and (E) in control thoracic aorta. Original magnification x200 (A, B, D and E) or x400 (C and F). AAA, abdominal aortic aneurysm; I, intima; L, lumen.

In situ zymography. In situ zymography was used to analyse activity of elastase and cathepsin G in mice aortas. Fluorogenic DQ-substrate for elastin (EnzCheck[®] Elastase assay kit; Invitrogen Life Technologies) and cathepsin G (SensoLyte[®] 520 cathepsin G assay kit; AnaSpec, Inc., Fremont, CA, USA) was mixed with 1% agarose in assay buffer and DAPI for nuclear localisation and incubated overnight at 37°C. Fluorescence was detected using a fluorescence microscope.

Statistical analysis. The statistical analysis was performed with the StatView for Windows software (release 5.0.1; SAS Institute, Inc., Cary, NC, USA). P-values <0.05 were considered significant. Comparisons were done with a Mann-Whitney U test. The Bonferroni correction was used to adjust for multiple comparisons of correlations.

Results

SerpinA3 expression in atherosclerosis. Immunohistochemistry was used to localize serpinA3 expression in human atherosclerotic lesions. As shown in Fig. 1A, serpinA3 was expressed in endothelial cells and medial smooth muscle cells in the atherosclerotic lesions. When investigating the mRNA expression of serpinA3 in human carotid lesions from asymptomatic patients and patients with a minor stroke or TIA we detected a significant increase in the expression of serpinA3 in the symptomatic patients (Fig. 2A).

Using gene arrays, we identified serpinA3n as one of the most induced genes in lesions from *Apoe^{-/-}* mice as compared to non-atherosclerotic control vessels. When verifying this





Figure 2. (A) Expression of serpinA3 in human atherosclerotic lesions from asymptomatic patients and patients with minor stroke and TIA. (B) Expression of serpinA3n mRNA in murine vessels and atherosclerotic lesions. (C) Expression of serpinA3 in abdominal control aortas and AAA samples from intima/media and adventitia layer with Th- or NTh-covered wall. AAA, abdominal aortic aneurysm; adv, adventitia; med, intima/media; NTh, non-thrombus-covered wall; TIA, transient ischemic attack; Th, thrombus-covered wall. *P<0.05 as compared to non-atherosclerotic vessels; ***P<0.001 as compared to control aortas.

result we observed a 14-fold increase in the serpinA3n mRNA expression in aortic root lesions from 20-week-old Apoe^{-/-} mice as compared to aortic vessels without atherosclerosis from 20-week-old *Apoe^{-/-}* mice and from 20-week-old C57bl/6 mice (Fig. 2B).

To document the role of serpinA3n in atherosclerosis we developed transgenic mice overexpressing serpinA3n and crossed these mice with Apoe^{-/-} mice. In aortas from transgenic serpinA3n mice, the serpinA3n mRNA level was 10-fold higher compared to aortas of wild-type C57Bl/6 mice (data not shown). Also, serpinA3n protein was increased in plasma and abdominal aortas of transgenic mice compared to wild-type mice, which had barely detectable serpinA3 expression as examined by western blot analysis (data not shown). However, *Apoe^{-/-}* x *serpinA3n*^{+/-} mice did not have any impact on the lesion development compared to Apoe^{-/-} at 20 week of age (Fig. 3) or inflammatory cell content (data not shown).

Figure 3. Effect of serpinA3n overexpression on atherosclerotic lesion development and AAA formation. (A) Morphometric quantitation of lesion size (μm^2) in the aortic root. (B) Thoracic aortas stained with Sudan IV, % lesion area of total vessel area. No significance in lesion size between *serpinA3n* transgenic *Apoe^{-/-}* and *Apoe^{-/-}* for each gender. (C) Abdominal aorta diameter in wild-type and *serpinA3n* transgenic mice treated with CaCl₂. No significance between CaCl₂-treated C57bl/6 and transgenic mice. ***P<0.001. TG, serpinA3n transgenic mice.

SerpinA3 expression in human AAA. SerpinA3 was expressed in the subendothelial layer of control aortas (Fig. 1D and E). There was a marked decrease in the staining of serpinA3 in AAA samples (Fig. 1B) as compared with abdominal (Fig. 1D) and thoracic (Fig. 1E) control aorta. Furthermore, serpinA3 was also expressed in small vessels present in the adventitia of the abdominal control aorta (Fig. 1F) but less expressed in small vessels present in the adventitia of AAA (Fig. 1C). Isotypic IgG served as negative control and showed no staining (data not shown). Serial sections were stained for T-cells (CD3), macrophages (CD163 and CD68), neutrophils (CD66b) and mast cells (tryptase) but did not associate to serpinA3 staining (data not shown). Similar to the immunohistological analysis, serpinA3 mRNA expression in human AAA was significantly decreased by 70-80% in the AAA samples compared to healthy aorta (Fig. 2C).

Since serpinA3 is also produced by the liver as an acute phase protein and could interfere with the immunohistochemical analysis we subsequently analyzed human plasma





Figure 4. Elastin layer fragmentation is decreased in CaCl₂-treated *serpinA3n* transgenic mice. (A) Verhoeffs-Van Gieson elastic fiber staining and (B) quantification. (C) Elastin and mast cell tryptase content, as determined by western blot analysis using a pool of aortas from 3 animals. (D) Quantification of western blot analysis as expressed in CaCl₂-treated wt mice in % vs. CaCl₂-treated *serpinA3n* mice. (E) Elastase and (F) cathepsin G activity in abdominal aorta as determined by *in situ* analysis is representative from 3 different samples. TG, serpinA3n transgenic mice; wt, wild-type. *P<0.05.



Figure 5. Expression of serpinA3 mRNA in endothelial cells at 4, 24 and 48 h after stimulation with LPS, IFN-7, TNF-a and IL-1β in % of control.

concentrations of serpinA3 in healthy controls (n=49), AAA patients (n=40) and patients with myocardial infarction (n=28) by ELISA. We detected no difference between the 3 groups (data not shown).

Overexpression of serpinA3n inhibits $CaCl_2$ -induced elastinolytic activity. Mice transgenic for serpinA3n (on C57bl/6 background) were also used to study the role of serpinA3 in aneurysm disease. Since serpinA3 expression was reduced in AAA samples we speculated whether overexpression of serpinA3n could inhibit aneurysm development. Abdominal aorta was dilated in CaCl₂-treated littermate wild-type C57bl/6 mice as compared to NaCl treated controls. However, no inhibition of the aortic diameter was observed in serpinA3n transgenic animals (Fig. 3C). The aortas of wild-type C57bl/6 mice treated with CaCl₂ had lower amounts of elastin compared with the aortas of NaCl treated controls and *serpinA3* transgenic mice on C57bl/6 background (Fig. 4A and B). We found a higher mast cell chymase activity (P<0.05) in the serum from CaCl₂-treated wild-type mice (detected in 6/9 animals with a mean of 0.13 mU) as compared to CaCl₂-treated serpinA3n mice (not detected in any of 5 animals). Also, *in situ* zymography showed a reduced elastase (Fig. 4C) and cathepsin G (Fig. 4D) activity in CaCl₂-treated *serpinA3n* mice as compared to CaCl₂-treated wild-type mice.

Regulation of serpinA3 by proinflammatory cytokines. To verify whether endothelial cells can produce serpinA3 we analyzed the mRNA expression of serpinA3 under different

stimuli. The result showed that the proinflammatory cytokine TNF- α induced serpinA3 expression more than 10-fold at 4 h, 3-fold at 24 h and 50% at 48 h as compared to non-stimulated cells (Fig. 5). IFN- γ slightly induced the expression of serpinA3 at 4 h. At later time points, IFN- γ reduced the expression of serpinA3 by 55% after 24 h and by 78% at 48 h compared to non-stimulated cells. IL-1 β did not have any dramatic effects on the serpinA3 regulation in the endothelial cells but resulted in a 55% induction at 48 h. LPS did not have any effects. SMCs from 2 different donors were also used without any dramatic effects by any of the used substances. TNF- α reduced the expression (data not shown).

Discussion

In the present study we investigated the expression and role of serpinA3 in atherosclerosis and AAA. The results suggest that AAA in humans is associated with a local decrease in serpinA3 expression in the aorta, whereas a local increase was found in atherosclerotic lesions. The presence of serpinA3 in the subendothelial layer of healthy aortas suggests that serpinA3 may be derived from endothelial and smooth muscle cells. The subendothelial protein expression of serpinA3 is not likely to be reflected by serpinA3 expression in plasma, since there was no difference in plasma levels between patients and controls. Cell culture experiments using vascular aortic SMCs and endothelial cells showed that these cells have the capacity to synthesize serpinA3.

The role of serpinA3 in atherosclerosis was investigated using mice transgenic for serpinA3 on an *Apoe* deficient background. To our surprise, we did not find any difference in lesion development in these mice compared to pure *Apoe* deficient mice at 20 weeks of age. Speculatively, serpinA3 could influence the formation of a more stable plaque phenotype in longer terms due to the protease inhibitory properties. Prolonged effects were however not investigated in this study.

In contrast to atherosclerosis, serpinA3 expression was decreased in AAA. Human AAA samples covered by thrombus are devoid of endothelial cells, and endothelial cell erosion is also increased in samples without thrombus (3,21). SMC apoptosis is one of the hallmarks of AAA and serpinA3 expression was absent in the major part of the media in AAA samples but increased in the media in carotid lesions. Conversely, it is unlikely that serpinA3 is expressed by leukocytes because these cells did not co-localize with areas of serpinA3 staining. No or very few inflammatory cells could be detected in the healthy aortas. Also, the increased leukocyte accumulation associated with aneurysm formation argues against inflammatory cells being responsible for the decreased serpinA3 expression in AAA. Taken together, these findings support that loss of endothelial cells and SMCs may be responsible for the decrease in serpinA3 expression in the intimal/medial part of aneurysmal tissue.

The role of serpinA3 in AAA was investigated using serpinA3n mice transgenic on a C57bl/6 background. To our surprise, we did not find any difference in AAA formation in transgenic mice compared to wild-type mice. A recent work by Ang *et al* (22) showed that administration of recombinant serpina3n reduced aortic rupture in Angiotensin II-induced AAA in Apoe^{-/-} mice by the inhibition of Granzyme B

mediated decorin degradation leading to enhanced collagen remodeling. Our experiments where primarily performed on serpinA3n transgenic mice on C57bl/6 background but also when crossing these mice with Apoe^{-/-} mice we obtained the same results (data not shown). In addition, rupture is very uncommon in the CaCl₂ induced aneurysm model.

There may be several mechanisms whereby low expression of serpinA3 is associated with aneurysm formation. The most plausible mechanism may be that proteases inhibited by serpinA3 are involved in the degradation of elastin fibers, since serpinA3 is known to inhibit a broad range of mast cell- and neutrophil-derived proteases, such as chymase, cathepsin G and elastase. Cathepsin G released from both neutrophils and mast cells is a potent elastinolytic protease with $\sim 20-30\%$ activity compared to elastase (23). We have previously found an increased expression of mast cell and neutrophil derived cathepsin G in human AAA as compared to control aorta (3) but its role in AAA formation has not yet been investigated. The role of chymase in AAA has been demonstrated by reduced aneurysm formation in mast cell deficient mice compared with wild-type mice (7,23). In the present study, we demonstrate a decreased chymase activity in serum from serpinA3n transgenic mice as well as a suppressed activity of cathepsin G and elastase in CaCl₂-induced transgenic mice compared to the CaCl₂treated wild-type mice. Taken together, this may explain the preserved elastin content in the CaCl₂-treated transgenic animals. Our results therefore suggest that serpinA3 could be involved in a phenotypic stabilization of the aorta that might inhibit rupture in longer perspectives, as seen in the study by Ang et al (22). A direct effect on elastinolytic activity could also have an effect on the recruitment of inflammatory cells. Decreased elastin degradation probably results in a decreased release of elastin-derived peptides (EDPs), which may influence the migration of inflammatory cells. Soluble EDPs released within human AAA tissue were proven to attract mononuclear phagocytes through ligand-receptor interactions with the elastin-binding protein, providing a plausible molecular mechanism explaining the inflammatory response that accompanies aneurysmal degeneration (24).

In summary, differential vascular expression of serpinA3 is clearly associated with atherosclerosis and AAA. SerpinA3 has no major effect on experimentally induced atherosclerosis or AAA development in mouse. However, serpinA3 might be involved in a phenotypic stabilization of the aorta that could prevent AAA rupture. These results further support the influence of neutrophils and mast cells in AAA.

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