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Changes in Endocan and Dermatan Sulfate Are Associated with Biomechanical Properties of Abdominal Aortic Wall during Aneurysm Expansion and Rupture

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Abstract

Background and Aims The study aimed to assess the potential of proteoglycans (PGs) and collagens as serological biomarkers in the abdominal aortic aneurysm (AAA). Furthermore, we investigated the underlying mechano-biological interactions and signaling pathways.

Methods Tissue and serum samples from patients with ruptured AAA (rAAA; $n = 29$), elective AAA (eAAA; $n = 78$), and healthy individuals ($n = 8$) were evaluated by histology, immunohistochemistry, and enzyme-linked immunosorbent assay, and mechanical properties were assessed by tensile tests. Regulatory pathways were determined by membrane-based sandwich immunoassay.

Results In AAA samples, collagen type I and III (Col1 and Col3), chondroitin sulfate, and dermatan sulfate (DS) were significantly increased compared with controls (3.0-, 3.2-, 1.3-, and 53-fold; $p < 0.01$). Col1 and endocan were also elevated in the serum of AAA patients (3.6- and 6.0-fold; $p < 0.01$), while DS was significantly decreased (2.5-fold; $p < 0.01$). Histological scoring showed increased total PGs and focal accumulation in rAAA compared with eAAA. Tissue β-stiffness was higher in rAAA compared with eAAA (2.0-fold, $p = 0.02$). Serum Col1 correlated with maximum tensile force and failure tension ($r = 0.448$ and 0.333; $p < 0.01$, and $r = 0.02$), tissue endocan correlated with α-stiffness ($r = 0.340$; $p < 0.01$). Signaling pathways in AAA were associated with extracellular matrix synthesis and vascular smooth muscle cell proliferation. In particular, Src family kinases and platelet-derived growth factor- and epidermal growth factor-related proteins seem to be involved.

Keywords
- abdominal aortic aneurysm
- biomarkers
- proteoglycans
- endocan
- dermatan sulfate

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Conclusion Our findings reveal a structural association between collagen and PGs and their response to changes in mechanical loads in AAA. Particularly Col1 and endocan reflect the mechano-biological conditions of the aortic wall also in the patient's serum and might serve for AAA risk stratification.

Introduction

According to the current standard of care, abdominal aortic aneurysms (AAAs) $\geq$5.5 cm in diameter are treated either by endovascular repair or by open surgical intervention to prevent aortic rupture.[1,2] Despite its limitations, the aortic diameter represents currently the most important diagnostic tool for surgical repair. The identification of potential biomarkers for AAA progression and risk of rupture could help to improve the decision for or against surgical intervention.[3,4] During the early stages of AAA development, loss of vessel wall integrity is caused in particular by alterations in vascular smooth muscle cell (VSMC) behavior, the main source of extracellular matrix (ECM). VSMC phenotypic switching and apoptosis, together with increased proteolytic degradation of the ECM, lead to the weakening of the aortic wall and increase the rupture risk over time.[5,6] Mechanical stress upon the aneurysm wall also plays a crucial role, as an artery will rupture when blood pressure exceeds the local wall strength, following the fundamental principles of material failure.[7]

The main ECM components of the aortic wall (approximately 60%) are collagen and elastin.[8] Furthermore, up to 5% consists of glycosaminoglycans (proteoglycans [PGs]), which are considered equally decisive for structural integrity.[9] Collagen types I and III (Col1 and Col3) are the most abundant and are responsible for the wall strength, whereas elastic fibers transmit the mechanical strain.[8] Much less is known about the role of PGs in AAA. They are characterized by central core proteins and covalently attached glycosaminoglycan chains, which define their specific groups, such as chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and keratan sulfate.[8,10,11] As CS and DS have already been described to provide mechanical stability to the vessel wall,[8,11] the current study focuses in particular on these PGs.[10,12] Endocan, a soluble DS, synthesized also by vascular endothelial cells (ECs), was of particular interest in our study, as its majority could be found in the bloodstream.[13] Mechanical properties of the aortic wall are closely linked to its physiological function. Our previous studies demonstrated already that altered biomechanical conditions in AAA are associated with the increased synthesis of various ECM components.[14,15]

Accordingly, two aspects of the vessel wall known to be decisive during aneurysm development were addressed in the current study: the biological composition of structural, load-bearing elements and the mechanical properties of these wall components at the protein level, especially the PGs. Particularly, we aimed to assess to what extent these wall components contribute to the mechanical stability and the resistance against aneurysm rupture. Furthermore, we evaluated their potential as serological surrogate parameters. In addition, we analyzed important regulatory pathways involved in these mechano-transduction processes.

Methods

Study Cohort and Sampling

Aortic tissue samples (total $n = 115$) were collected from patients with AAA during an open surgical repair. Of these samples, $n = 29$ were from patients with a ruptured AAA (rAAA) and $n = 78$ from patients who had an elective AAA (eAAA) repair. Healthy aortic samples ($n = 8$) were collected from kidney donors during transplantation and served as control. Patient characteristics and clinical data are summarized in ►**Tables 1** and **2**.

Tissue samples were divided according to further analyses. The central, largest part of the specimen was used for tensile tests performed within 24 hours after surgery. Adjacent pieces of the corresponding specimens were immediately frozen and stored at −80°C until further analyses. For histological studies, additional adjacent tissue pieces were fixed in 4% formalin and embedded in paraffin (FFPE). Corresponding serum samples ($n = 13$ for rAAA, $n = 72$ for rAAA) from the patients were obtained prior to surgery during routine blood collection. Serum samples ($n = 5$) from healthy individuals were used as a control. The study was approved by the ethical committee of the University Hospital Klinikum rechts der Isar, Technical University of Munich, Germany (2799/10). The study was performed in

Table 1 Patient characteristics

AAA patients (total $n = 107$)	
Sex (male)	94
Age (y)	69.1 ± 8.7
Max AAA diameter (mm)	67.2 ± 19.1
Ruptured AAA ($n = 29$)	
Sex (male)	27
Age (y)	72.3 ± 10.5
Max AAA diameter (mm)	83.9 ± 20.2
Elective AAA ($n = 78$)	
Sex (male)	67
Age (y)	67.9 ± 10.4
Max AAA diameter (mm)	61.4 ± 19.5

Abbreviation: AAA, abdominal aortic aneurysm.

Table 2 Patient characteristics

Comorbidities	(*n*/% of patients)	*p*-Value
Chronic kidney disease	29/27.1%	0.003
Hypertension	79/73.8%	1.000
Diabetes mellitus	11/10.3%	1.008
Coronary heart disease	35/32.7%	0.643
Smoking	65/60.7%	0.819
Peripheral artery disease	13/12.1%	1.000
Hyperlipidemia	53/49.5%	0.509
Medication	**(*n*/% of patients)**	***p*-Value**
Aspirin/clopidogrel	61/57.0%	0.792
Beta-blocker	47/43.9%	0.615
Statins	54/50.5%	0.296
ACE inhibitors	38/35.5%	0.609
Diuretics	33/30.8%	0.295
Antihypertensives	65/60.7%	0.151
Antidiabetics	5/4.7%	0.580

Abbreviation: ACE, angiotensin converting enzyme.

accordance with the World Medical Association Declaration of Helsinki.

Quantitative Protein and Proteoglycan Analysis

For extraction of total protein including PGs, lysis buffer (Tissue Extraction Reagent I, ThermoFisher Scientific, Waltham, Massachusetts, United States) was added to the tissue samples and a tissue homogenizer (Bio-Gen PRO200 with Multi-Gen 7XL probes, PRO Scientific, Oxford, Connecticut, United States) was used to homogenize the samples. The homogenate was centrifuged at 4°C and the supernatant was applied for subsequent determination of protein concentration via BCA assay (Pierce BCA Protein-Assay Kit, Thermo Fisher Scientific, Waltham, Massachusetts, United States). Quantitative evaluation of ECM components in tissues and serum samples was performed by appropriate enzyme-linked immunosorbent assay (ELISA) assays. Collagenous components were assessed by first determining hydroxyproline (HYP; Human Hydroxyproline ELISA Kit, BlueGene Biotech, Shanghai, China) as the main component of all types of fibrillar collagen. Furthermore, Col1 (ELISA Kit for Human Collagen Type I, Cloud-Clone Corp., Katy, Texas, United States) and Col3 (Human Collagen Type III ELISA Kit, Abbexa Ltd., Cambridge, United Kingdom) were measured as they are the most abundant collagens in the vessel wall and responsible for the force transmission.[8] Both collagen kits are designed to recognize the N-terminal end of the corresponding Col1 and Col3. To evaluate PGs, we focused on the individual specific glycoproteins already detected in the aortic wall such as CS (Chondroitin Sulfate ELISA Kit, Abbexa Ltd., Cambridge, United Kingdom), DS (Human Dermatan Sulfate ELISA Kit, Elabscience Biotechnology Inc., Wuhan, China), and HS (Human Heparan Sulfate Proteoglycan ELISA Kit, Abbexa Ltd., Cambridge, United Kingdom). To specifically address serological PG markers, endocan, a DS known to be secreted also by ECs,[13] was also measured (Human ESM1 ELISA Kit [Endocan], Abcam, Cambridge, United Kingdom). Regarding the epitopes and specificity of the PG ELISA kits, no further information is available.

Histological Analyses

Histological evaluation was performed for most samples ($n = 93$). Due to the limited specimen size and quality, histological analysis was not possible for all specimens. Sections of FFPE tissue samples (2–3 µm) were mounted on SuperFrost slides (ThermoFisher Scientific, Waltham, Massachusetts, United States), and stained according to the individual targeted tissue components. Hematoxylin–eosin staining was used for morphological evaluation focusing on cellular composition and inflammatory infiltration. The abundance and distribution of connective tissue components, in particular collagens, were evaluated using Elastica van Gieson staining. Alcian blue (alcian blue dye dissolved in 3% acetic acid, incubation time 10 minutes) was used to assess the score of PG components.

For immunohistochemistry, FFPE sections were mounted on precoated (0.1% poly-L-lysine, Sigma-Aldrich, St. Louis, Missouri, United States) SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, Massachusetts, United States), and antigen retrieval was performed by pressure cooking the slides in 10 nM citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 3% hydrogen peroxide and sections were incubated with anti-ESM1/endocan (ab224591, Abcam, Cambridge, United Kingdom) primary antibody, diluted with DAKO REAL Antibody Diluent (Dako, Glostrup, Denmark). Biotinylated secondary antibodies were applied and peroxidase-conjugated streptavidin and DAB chromogen (Dako REAL Detection System Peroxidase/DAB+, Rabbit/Mouse Kit; Dako, Glostrup, Denmark) were used for chromogenic detection. For nuclear counterstaining, Mayer's hematoxylin (Carl Roth, Karlsruhe, Germany) was applied. All slides were digitalized by a slide scanner (Aperio AT2, Leica, Wetzlar, Germany). Tissue characteristics were obtained by semi-quantitative scoring, ranging from "−" for 0% incidence of the specific histological characteristic to "++++" for 100% incidence and including intermediate stages (such as "+/++"). Concerning the characterization of PGs (Alcian blue), two different approaches were applied: (1) total amount of PGs, where the total extent of the staining (blue) was considered; (2) selective accumulation of PGs, where only the intact not-degraded PGs, characterized by visible structure and dark blue staining (see also arrows in ►**Fig. 1**), were taken into account. Scoring was conducted by two persons experienced in vessel wall histology and pathology, blinded for the study groups.

Mechanical Testing

To determine the mechanical properties of the collected aortic tissue samples, tensile tests were performed as described previously.[14,16] Of all collected samples, $n = 64$ were suitable for these experiments. Prior to testing, specimen

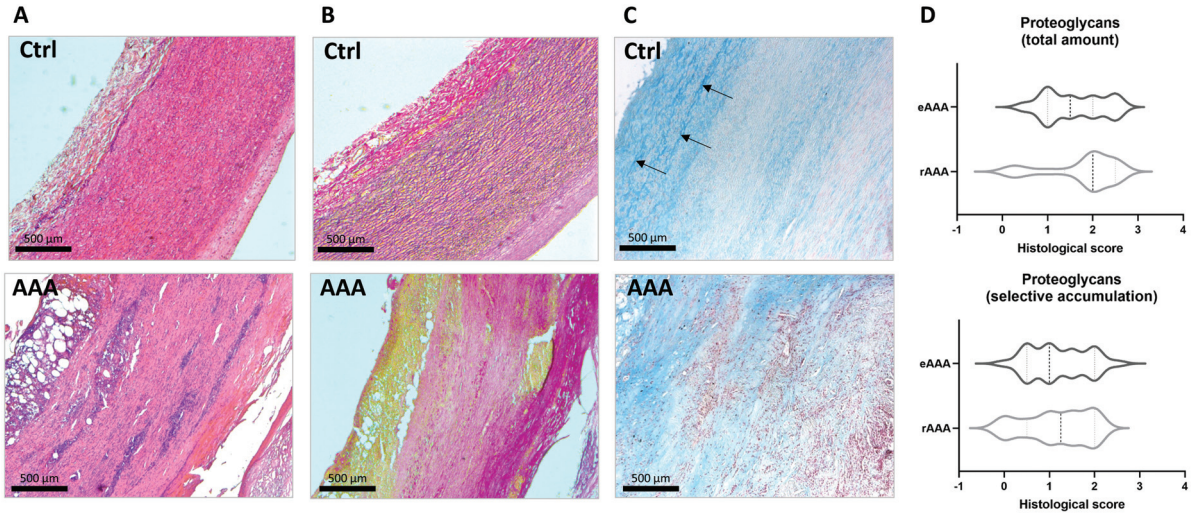

Fig. 1 Histological analysis of collagens, elastin, and proteoglycans in representative healthy (Ctrl) and aneurysmatic (AAA) human aortas. **(A)** Overview of vessel wall morphology with well-organized structures in the healthy aorta and large areas of inflammatory infiltration and wall thickening in the aneurysmatic section (hematoxylin–eosin staining). **(B)** Distribution of the collagen and elastin network. Increased collagenous fibers and decreased, fragmented elastin fibers are found in AAA sections (Elastica van Gieson staining). **(C)** Distribution of vessel wall proteoglycans (PGs). Total increase in the accumulation of PGs was observed in AAA sections (*Alcian blue*). **(D)** Semiquantitative scoring of total PG content (overall blue staining) and intact PG content (blue areas with clearly recognizable PG structure, see arrows) in eAAA and rAAA. Scale bars represent 500 µm. AAA, abdominal aortic aneurysm; eAAA, elective abdominal aortic aneurysm; rAAA, ruptured abdominal aortic aneurysm.

thickness was determined with a digital thickness gauge (Quick-Mini Series 700, Mitutoyo, Kawasaki, Japan). To measure the elastic material properties, cyclic loading was applied to all samples with a preconditioning phase of 19 cycles and a final (20th) cycle for data collection. Elastic material properties are described as α and β stiffness and represent the initial, load-free material stiffness (α-stiffness) as well as the material stiffness associated with higher stretch (β-stiffness). After the determination of the elastic properties, failure measures were evaluated by destructive testing. From the maximum measured force applied to the specimen (F_{max}) further failure parameters were derived. F_{max} per initial specimen width was defined as failure tension and F_{max} per cross-sectional specimen area was defined as maximum stress. Both elastic and destructive experiments were performed with a Zwick/Roell mediX0.1 (Messphysik Materials Testing, Fuürstenried, Austria).

Pathway Analyses

Profiling of potentially involved phosphokinases was performed using a membrane-based sandwich immunoassay (Proteome Profiler Human Phospho-Kinase Array Kit, R&D Systems, Minneapolis, Minnesota, United States). Aortic tissue samples ($n = 4$ rAAA, $n = 4$ eAAA, $n = 3$ healthy aortas) were homogenized (Bio-Gen PRO200 Homogenizer with Multi-Gen 7XL probes, PRO Scientific, Oxford, Connecticut, United States) and added to prespotted membranes. Phosphorylation of tissue proteins was determined using an infrared-dye labeled streptavidin (IRDye 800CW Streptavidin, LI-COR Biosciences, Lincoln, Nebraska, United States) and the fluorescence signal was detected with a digital imager (Azure c600, Azure Biosystems, Dublin, California, United States). Pixel densities were analyzed using the Fiji software[17] and were normalized to the reference proteins on each membrane, as well as to the healthy control samples.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism software (Version 8.4.2 for Windows, GraphPad Software, San Diego, California, United States). Data were tested for normal distribution with the D'Agostino–Pearson, Anderson–Darling, and Shapiro–Wilk tests and deviated from a Gaussian distribution in all three tests. For protein and PG amounts and mechanical data, a comparison of unpaired groups (rAAA vs. eAAA, rAAA vs. Ctrl, eAAA vs. Ctrl) was performed with the nonparametric Mann–Whitney U-test. The nonparametric Spearman correlation coefficients (r) and p-values for nonparametric correlation of biological and mechanical data were applied. All tests were two-sided and a p-value of ≤ 0.05 was considered significant.

Results

Tissue Collagen, Matrix-Associated PGs, and Endocan in AAA

First, we focused on the concentration of collagens and PGs in healthy versus eAAA and rAAA tissues (►**Fig. 2**). The analysis of HYP (component of all collagens) revealed significantly increased levels in eAAA (2.6-fold, $p < 0.001$) and rAAA (4.4-fold, $p > 0.001$) compared with healthy aorta. Specifically, collagens Col1 and Col3 were increased particularly in eAAA compared with healthy tissue (3.1- and 3.2-fold, $p = 0.001$ and $p = 0.016$, respectively) (►**Fig. 2**). Regarding PGs, CS and DS were significantly increased by 1.2-

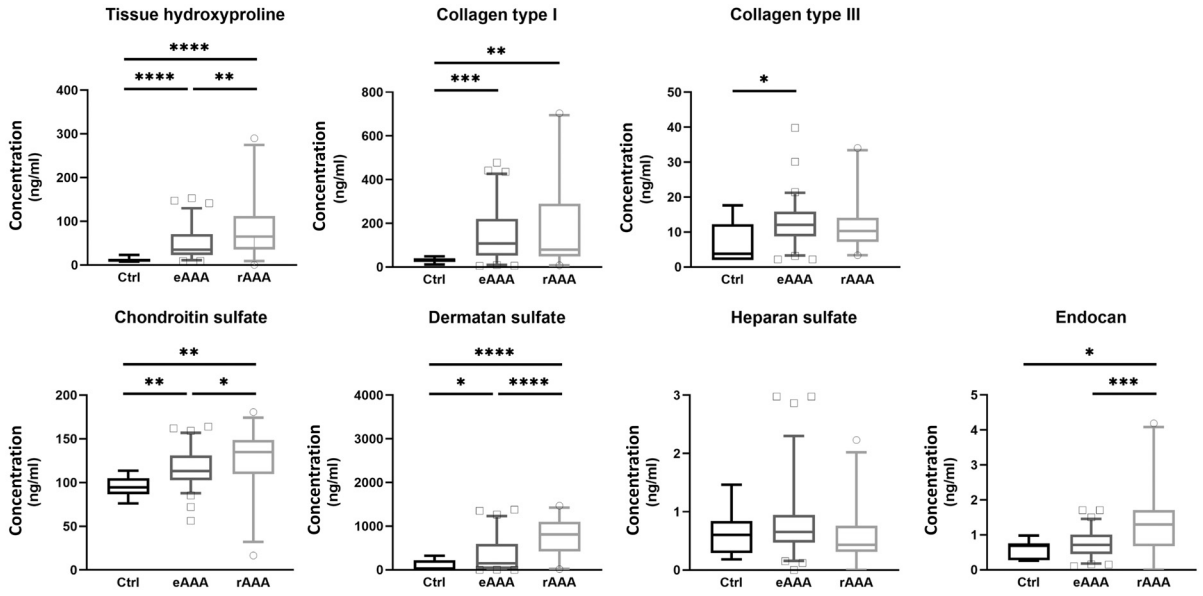

Fig. 2 Analysis of collagens and glycosaminoglycans in tissue lysate from elective (eAAA) and ruptured (rAAA) aortic aneurysm in comparison to healthy tissue (Ctrl). Results are displayed in ng/mL of total protein or glycosaminoglycan concentration. *p*-Values were determined with the Mann–Whitney U-test; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

and 19-fold ($p = 0.002$ and $p = 0.011$, respectively). In addition, CS, DS, and endocan were further increased in rAAA compared with eAAA (1.2-, 5.4-, and 1.8-fold; $p = 0.020$, $p < 0.001$, $p < 0.001$) (►**Fig. 2**). Furthermore, it is to mention that the expression of Col1 was approximately 20-fold higher than that of Col3 ($p < 0.001$). Concerning PGs, CS and DS showed comparable expression levels to Col1. In contrast, the abundance of HS and endocan was up to 100-fold lower than the other structural components ($p < 0.001$). The levels of HS did not alternate between the diseased tissue and controls.

Serum Collagen, Matrix-Associated PGs, and Endocan in AAA Progression

Similar to collagens and PGs in the tissue, we investigated potential changes in corresponding serum samples from AAA patients (eAAA and rAAA) versus healthy individuals (►**Fig. 3**). Interestingly, serum HYP did not show any significant differences between the study groups. Regarding

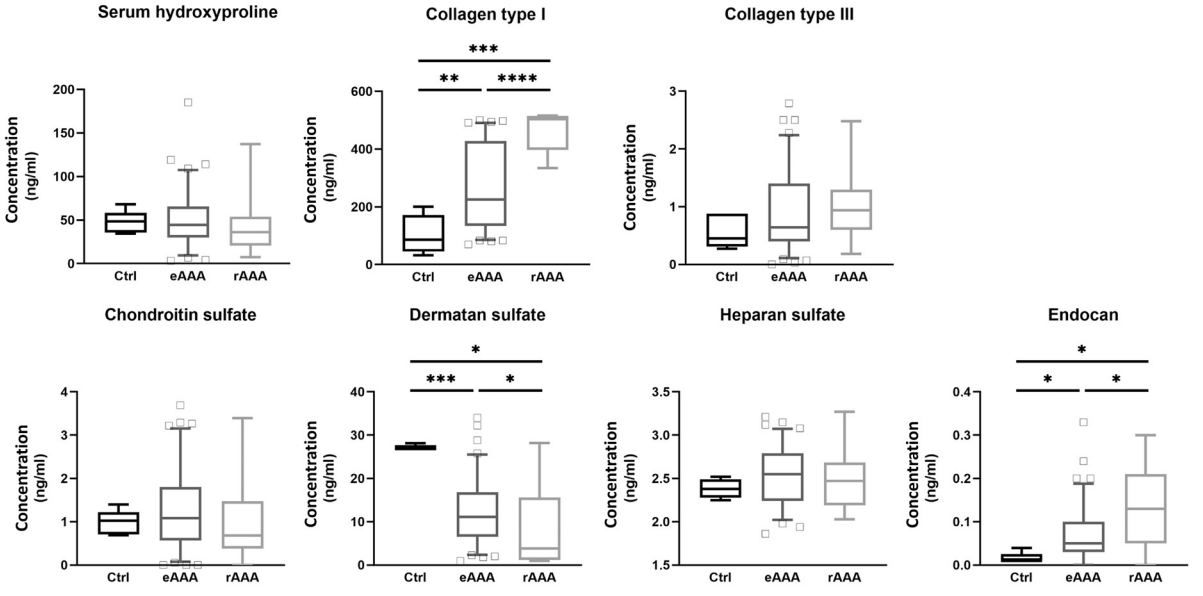

Fig. 3 Analysis of collagens and glycosaminoglycans in serum from elective (eAAA) and ruptured (rAAA) aortic aneurysm in comparison to healthy tissue (Ctrl). Results are displayed in ng/mL of total protein or glycosaminoglycan concentration. *p*-Values were determined with the Mann–Whitney U-test; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

collagens and AAA subcohorts (eAAA vs. rAAA), significantly increased amounts of Col1 and endocan were observed in rAAA (2.4- and 3.2-fold, $p < 0.001$ and 0.021, respectively). In contrast, DS was significantly decreased in eAAA compared with serum samples from healthy individuals (2.7-fold; $p = 0.001$) and was further reduced in rAAA (2.5-fold compared with eAAA and 6.8-fold compared with Ctrl; $p = 0.042$ and $p < 0.001$, respectively). Regarding the total serum levels, Col1 and Col3 showed similar results as in the tissue specimens, with Col1 being the most abundant structural protein. Of the PGs, DS showed the highest concentration in the corresponding blood samples and was interestingly significantly decreased in the serum of AAA patients compared with healthy controls (2.4-fold; $p = 0.001$). The concentration of endocan was at a low level but increased significantly in serum of the diseased patients ($p < 0.001$). It is to mention that the results represent a single time point from blood sampling within 24 hours prior to surgical intervention. No follow-up data were available.

Histological Evaluation of Collagens and PGs

To determine the distribution of structural proteins and PGs within the vessel wall, we performed also histological analysis of the tissue samples (►**Fig. 1**). Regarding the overall vessel morphology, AAAs showed progressively disrupted and disorganized wall structure with abundant inflammatory infiltrates (►**Fig. 1A**). Elevated amounts of collagen fibers were distributed in all aneurysmatic vessel wall layers while elastin fibers were scarce and highly fragmented (►**Fig. 1B**). In healthy tissue, PGs were found primarily in the medial, fiber-rich layer. Contrarily, in AAA specimens, PGs were detected in all aortic wall layers (►**Fig. 1C**). Semiquantitative scoring analysis showed increased total amounts as well as increased accumulation of intact PGs in rAAA compared with eAAA (►**Fig. 1D**).

Endocan Correlation with Matrix-Associated PGs and Collagens

To evaluate the interaction of the structural vessel wall components, circulating proteins, and PGs, we performed correlation analysis between tissue and serum datasets (►**Fig. 4**). Tissue endocan positively correlated with tissue CS ($r = 0.235$, $p = 0.04$), tissue DS ($r = 0.291$, $p < 0.01$), and serum levels of Col1 ($r = 0.388$, $p < 0.01$). Serum endocan correlated positively with serum CS ($r = 0.327$, $p < 0.01$) and serum Col3 ($r = 0.268$, $p = 0.02$). Serum CS and Col3 showed a strong positive correlation with each other ($r = 0.635$, $p < 0.01$).

In addition, to get a more detailed insight into the changes in the rAAA, we performed subgroup analysis comparing separately eAAA versus rAAA (►**Supplementary Fig. S1** [available in the online version], corresponding to the results from ►**Fig. 4**). All results from ►**Fig. 4** were confirmed also for eAAAs: tissue endocan versus tissue CS ($r = 0.231$, $p = 0.05$), tissue DS ($r = 0.274$, $p = 0.02$), and serum Col1 ($r = 0.391$, $p < 0.01$); serum endocan versus serum CS ($r = 0.430$, $p < 0.01$), serum Col3 ($r = 0.291$, $p = 0.01$); serum CS versus serum Col3 ($r = 0.680$, $p < 0.01$). Interestingly, no significant correlations were observed for rAAAs (►**Supplementary Fig. S1** [available in the online version]).

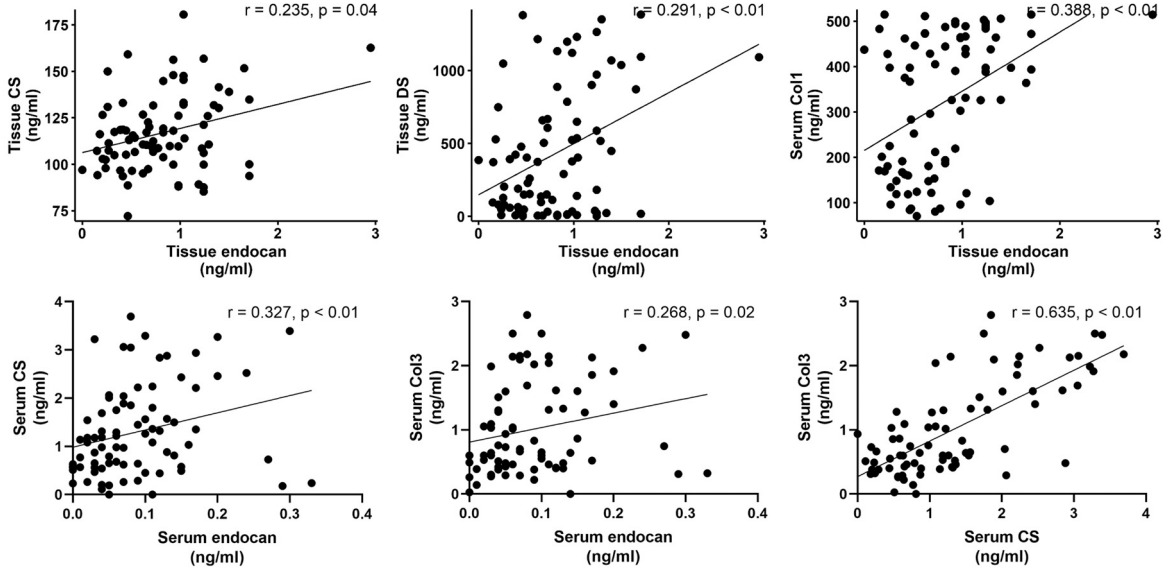

Fig. 4 Selected scatter plots with significant correlations in tissue and serum for collagens and proteoglycans. *Upper row*: tissue endocan with tissue chondroitin sulfate (CS), $n = 81$; tissue endocan with tissue dermatan sulfate (DS), $n = 81$; tissue endocan with serum collagen type I (Col1), $n = 81$. *Lower row*: serum endocan with serum CS, $n = 82$; serum endocan with serum collagen type III (Col3), $n = 78$; serum CS with serum Col3, $n = 78$. All measurements were performed using ELISA; total collagen and proteoglycan amounts are displayed in ng/mL. Spearman's rank correlation coefficient was used to determine *r*- and *p*-values. ELISA, enzyme-linked immunosorbent assay.

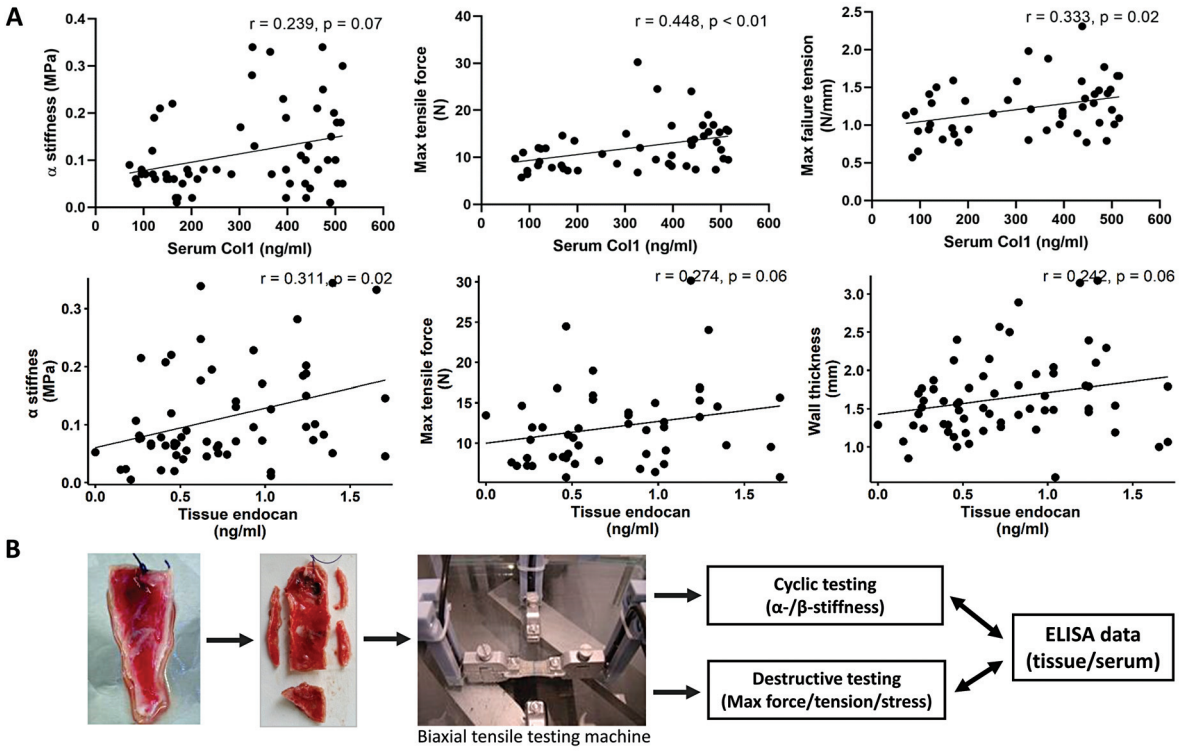

Fig. 5 Selected scatter plots with significant correlations in tissue, serum, and mechanical properties. **(A)** *Upper row*: serum collagen type I (Col1) with α-stiffness ($n = 59$); serum Col1 with maximum tensile force ($n = 48$); serum Col1 with maximum failure tension ($n = 48$). *Lower row*: tissue endocan with α-stiffness ($n = 60$); tissue endocan with maximum tensile force ($n = 49$); tissue endocan with wall thickness ($n = 63$). **(B)** Schematic diagram of aortic tissue sample processing: excision during open surgery (*left*), segmentation (*middle*), and biaxial testing machine (*right*). Adapted from Reeps et al[16]. Total collagen and proteoglycan amounts are displayed in ng/mL. Mechanical properties were derived from tensile tests: α-stiffness (MPa), maximum force (N), maximum failure tension (N/mm), wall thickness (mm). Spearman's rank correlation coefficient was used to determine *r*- and *p*-values.

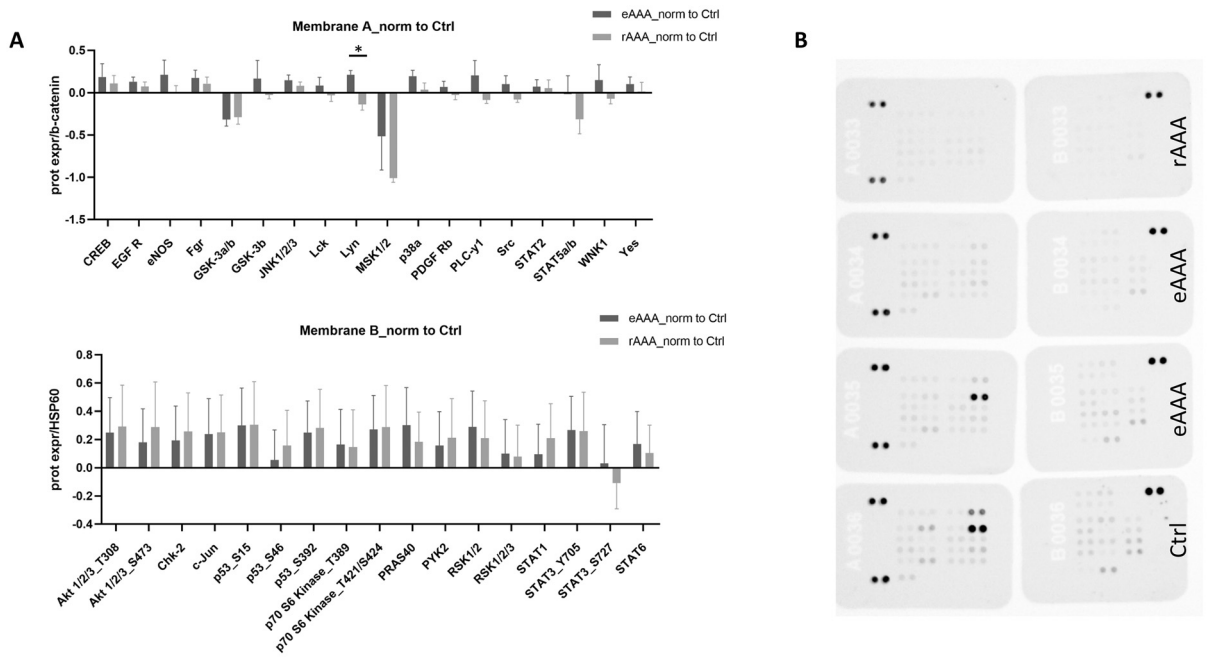

Fig. 6 Determination of signaling pathways in AAA and healthy aortas**. (A)** Phosphokinase profiling of ruptured (rAAA) and elective aneurysm (eAAA) samples normalized to healthy controls (Ctrl). **(B)** Representative membrane scans of the phosphokinase array (A). All proteins were measured by detection of fluorescence signal and calculation of pixel densities (normalized to reference proteins on each membrane). *p*-Values between groups determined with the Mann–Whitney U-test; $^{*}p \leq 0.05$. eAAA, elective abdominal aortic aneurysm; rAAA, ruptured abdominal aortic aneurysm.

Mechanical Properties of ECM Components in rAAA versus eAAA

Further correlation analyses comparing collagens and PGs with the mechanical properties in tissue and corresponding serum are shown in ►**Fig. 5**. Serum Col1 correlated positively with α-stiffness ($r = 0.239$, $p = 0.07$), maximum tensile force ($r = 0.448$, $p < 0.01$), and failure tension ($r = 0.333$, $p = 0.02$). Tissue endocan showed a similar pattern and correlated positively with α-stiffness ($r = 0.311$, $p = 0.02$), maximum tensile force ($r = 0.274$, $p = 0.06$), and wall thickness ($r = 0.242$, $p = 0.06$) (►**Fig. 5A**).

In addition, to gain further insight into the mechanical properties of the AAA tissue samples, tensile tests were performed focusing on the differences between rAAA and eAAA. The results were confirmed also for eAAA (►**Supplementary Fig. S2** [available in the online version ly], corresponding to the results from ►**Fig. 5**): serum Col1 correlated with α-stiffness ($r = 0.271$, $p = 0.05$), maximum tensile force ($r = 0.452$, $p < 0.01$), and failure tension ($r = 0.326$, $p = 0.04$); tissue endocan correlated with α-stiffness ($r = 0.328$, $p = 0.02$), maximum tensile force ($r = 0.309$, $p = 0.04$), and wall thickness ($r = 0.315$, $p = 0.02$). Furthermore, serum CS correlated significantly with α-stiffness, β-stiffness, and maximum tensile force both in total AAA and in eAAA (►**Supplementary Fig. S3A** [available in the online version]). Regarding elastic properties, β-stiffness was significantly increased in rAAA tissue (2.5-fold; $p = 0.022$) (►**Supplementary Fig. 3B** [available in the online version]). The α-stiffness was elevated as well, however, due to broad value distribution without statistical significance. Interestingly, no significant correlations were observed in the subgroup analysis from ►**Fig. 5** for rAAAs (►**Supplementary Fig. S2** [available in the online version]).

Signaling Transduction, ECM-Related and Proliferative Pathways in AAA Tissue

To identify possible relationships between the amounts of structural components and mechanical loads within the aneurysm wall, changes in phosphokinase activities were evaluated (►**Fig. 6** and ►**Supplementary Fig. S4** [available in the online version]). Phosphorylation of several signaling proteins was elevated in eAAA compared with rAAA samples, with tyrosine-protein kinase Lyn showing significant changes ($p = 0.029$) and lymphocyte-specific protein tyrosine kinase (Lck), Src, phospholipase C (PLC), signal transducer and activator of transcription proteins (STAT5, STAT3), epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptors (PDGFR) having also increased levels in eAAA (►**Fig. 6A**). Representative membrane scans show higher pixel densities in corresponding spots (►**Fig. 6B**). The achieved data indicate activation of signaling pathways related particularly to the regulation of the amounts of various ECM components and VSMC proliferation.

Discussion

Collagenous and elastic fibers, as well as PGs, play an important role in the maintenance of mechanical stability in the aortic wall.[10–14,18–22] The current results further confirm that besides collagens PG are also significantly affected by the altered mechanical loads in AAA.[14] In particular, a significant increase of CS and DS PGs has been observed in AAA tissue samples. These ECM-associated PGs are known to have collagen-binding capacities and to modulate the formation of collagenous fibrils.[23,24] Other matrix components like fibronectin and laminin were also described as binding partners of CS and DS and were shown to be altered under disease conditions.[25] Furthermore, DS has been shown to bind to transforming growth factor-β (TGF-β) and EGFR and therefore could exert a regulatory function in fibrosis.[26] In contrast to CS and DS, HS did not demonstrate any changes between the study groups. This particular PG is more associated with the cell surface and plasma membranes than with the extracellular space,[27] thus being less involved in providing mechanical stability of the aortic wall and less affected by AAA disease. Furthermore, under in vitro conditions, HS was described to modify elastin aggregation and assembly.[28] As the elastic fibers have a long half-life and their functions do not depend on continuous turnover,[29] the rather constant levels of HS in AAA could be also explained by its association to elastic fibers.

Our findings in the patient's serum suggest that, to a certain extent, ECM changes during AAA progression are reflected also in the blood and thus could provide a basis for potential circular biomarkers. Higher levels of both collagens and endocan in serum could represent increased ECM turnover in response to AAA growth. Similar results have already been described in AAA patients,[30] however, failed to serve as a reliable marker of AAA in a later study.[31] In the context of cardiovascular disease, increased serum endocan levels were found in patients with chronic kidney disease and were associated with cardiovascular events.[32] Endocan was also found to be elevated in septic patients and was described as a marker of endothelial dysfunction, neovascularization, and inflammation.[33] Our current findings support an essential role of endocan in AAA and its use as a potential biomarker. One conceivable approach would be the measurement of, e.g., endocan in blood in addition to the standard diagnostic tools to find patients at increased risk of AAA rupture. However, these data need to be handled with care, for instance, the association of such biomarkers with the aortic diameter and other routine imaging techniques. For such an approach, follow-up data on aneurysm growth and correlation with the clinical data are needed, which we cannot provide in this work.

PGs are secreted mainly from VSMCs into the surrounding ECM.[10] AAA-derived serum PGs could be explained either by being released into the blood circulation from granules or arising directly from the degraded ECM. Such assumption is conceivable because in advanced AAA, the endothelial layer is largely damaged and ECs are mainly dysfunctional. Consequently, an exchange of ECM components between the aortic wall and the blood is plausible. DS and its derivatives have already been identified as antithrombotic agents with therapeutic potential[34] and were also found to accelerate natural antithrombin activity.[35] A later study showed that AAA

tissue has a higher DS-related anticoagulant activity compared with normal aorta,[36] presumably due to the increased DS content, which is in line with our current findings. Interestingly, in contrast to the enhanced DS PG amount in AAA tissue, its concentration in serum was significantly reduced. These discrepancies can be explained by strong anticoagulant activation and DS consumption during AAA development. Our results show also strong associations between PG and collagen in the tissue as well as in the corresponding serum. Interrelated synthesis as well as binding activity could account for such correlated concentrations of endocan with Col1 and Col3 and might be an interesting cofactor in the context of a serum biomarker for AAA.[14,23]

Focusing on the mechanical properties of the diseased aorta, we found that serum Col1 and tissue endocan, a soluble PG derived from DS, were significantly associated with wall strength and stiffness and strongly increased in AAA tissue. Previous studies have already shown that AAAs have reduced mechanical compliance compared with non-aneurysmal segments and wall strength is significantly weaker compared with healthy aortas.[7] Col1 is the most abundant structural protein and one of the most important structural proteins stabilizing the aortic wall.[14] Growing aneurysms were already associated with increasing stress upon the vessel wall and ruptured tissue was significantly weaker than tissue from eAAAs.[37] Increased wall stiffness reflects the loss of elastic fibers during AAA development and the increased amount and turnover of collagen.[38] Consequently, the abundance of Col1 and DS endocan in AAA can be explained as an attempt to counteract the increased wall stress to stabilize the aortic wall.

Regarding the underlying functional mechanisms, we found increased activation of pathways related to the regulation of the expression of ECM components and VSMC proliferation. Especially Src family kinases (SFKs) as well as platelet-derived growth factor (PDGF)- and epidermal growth factor (EGF)-related proteins appear to be involved. The regulatory role of TGF-β in cellular proliferation and differentiation is well described and its effects are mediated through the activation of Src signaling molecules.[39] Moreover, TGF-β has been shown to induce expression of VSMC- and ECM-related genes via Src activation.[40] SFKs, including Src, Lyn, and Lck, regulate cell growth and survival and also proteolytic degradation of ECM components.[41] Lyn was shown to be expressed in VSMCs, along with decreased expression of collagen type I and α-smooth muscle actin.[42,43] Moreover, Col1 itself was demonstrated to enhance VSMC proliferation via Src-dependent crosstalk, suggesting a connection to the increased collagen levels in AAA.[44] PDGF and the downstream signaling protein PLC were described to stimulate VSMC proliferation through ECM components.[45,46] This would indicate the deregulation of PDGF and its downstream signaling factors in rAAA. Other potentially involved pathways in this context are the EGFR-induced PLC, STAT3, and the Src/EGFR/PI3K/Akt/JNK1/2.[47–49] However, the number of study samples was too small to draw any conclusions at this time point. Despite different levels of pathway activation in eAAA and rAAA and the small number of samples, our results indicate an overall increase in the synthesis of ECM components in the aneurysmatic tissue that seems to be associated in particular with the activation of the proliferative pathways to counteract the increasing mechanical loads.

Limitations of this study include the unequal number of samples in eAAA and rAAA groups, especially for serum samples, due to their restricted availability. Our results in AAA (in total) were confirmed also in the subgroup analysis of eAAA. Surprisingly, however, no significant correlations were observed in the rAAA study group. The reason is the small number of tissue samples, particularly blood. Open surgeries of rAAAs have a decreasing caseload and are usually emergency procedures, where samples could not always be obtained. Furthermore, the rAAA specimens are very heterogeneous and the distance from the ruptured site is variable. Control groups for tissue and serum samples were relatively small due to the limited availability of human samples and could not be matched to the diseased samples regarding age and gender. Moreover, further patient characteristics and clinical data could not be obtained from control patients undergoing kidney transplantation because of the legal restrictions. Another limitation was that mechanical properties of healthy control tissue on principle strongly deviate from the properties of AAA disease, as different material characteristics are presumed. Concerning the potential role of, e.g., endocan or collagens as biomarkers for AAA risk stratification, further important aspects need to be stressed. Follow-up data, such as computed tomography imaging, continued patient records, or repetitive blood sampling were not available for this study. Therefore, our serum data cannot be set in context with the actual patient outcome and AAA development over time. Further studies with longitudinal patient data are required for a comprehensive assessment of the biomarker value. In addition, correlation analyses between biological and mechanical data can only describe possible associations between the obtained values, but no conclusions can be drawn on underlying causalities. Finally, it is to mention that the experiments concerning signaling pathways were performed only with a small number of samples. Thus, the corresponding results should be treated with caution. Further experiments are necessary to confirm these data.

Conclusion

The current study confirmed that not only collagen and elastin but also PGs are playing an important role in the maintenance of mechanical stability of the aortic wall. In particular, changes in CS and DS PGs mirror the state of AAA development. Furthermore, DS has been shown to bind to TGF-β and EGFR and therefore could exert a regulatory function in fibrosis. Our findings suggest that ECM changes during AAA progression are reflected to a certain extent also in the blood and thus provide a basis for potential serum markers. Increasing levels of collagen and endocan could represent increased ECM turnover or degradation in response to AAA progression. Especially endocan, having various functions in the context of vascular inflammation and

cardiovascular disease, might be a promising marker of AAA risk stratification. Further studies, particularly including follow-up data associated with patient outcome, are needed for a comprehensive evaluation of the diagnostic potential and usefulness of such biomarkers in clinical praxis. Furthermore, endocan was significantly associated with wall strength and stiffness and could reflect also the weakness of the AAA wall. Regarding underlying functional mechanisms, it seems that pathways related to the regulation of ECM components and VSMC proliferation are activated. Especially SFKs, PDGF- and EGF-related proteins appear to be involved. The role of Lyn and Lck in AAA is almost unknown and has to be further elucidated. With AAA progression, proliferation seems to be downregulated and eventually associated with an increased risk of rupture.

What is known about the topic?

- The extracellular matrix (ECM) proteins play a crucial role in the maintenance of the aortic wall integrity and vascular smooth muscle cells (VSMCs) are the main source of their synthesis.
- Little is known about the role of proteoglycans, in particular regarding the mechanical properties and corresponding changes in abdominal aortic aneurysm (AAA).
- Furthermore, little is known about the underlying functional mechanisms, leading to the activation of pathways related to the regulation of ECM components and VSMC proliferation.

What does this paper add?

- Changes in chondroitin and dermatan sulfate proteoglycans mirror the state of AAA development and are significantly associated with changes in mechanical loads in AAA.
- In particular, endocan reflects the mechano-biological conditions not only in the aortic wall but also in the patient's serum and might serve as a marker of AAA risk stratification.
- Regarding underlying signaling pathways related to the regulation of the expression of the ECM components and mechanical properties of the aortic wall in AAA, factors associated with VSMC proliferation seem to be involved, especially Src family kinases (SFKs), PDGF- and EGF-related signaling proteins.

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Conflict of Interest
None declared.

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