The composition of collagen in the aneurysm wall of men and women

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Background: **Loss of vessel wall integrity by degradation is essential for the development of abdominal aortic aneurysm (AAA) and ultimately its rupture. The observed greater rupture rate in women with AAA might be related to gender differences in the biomechanical properties of the aneurysm wall. The aim of the study was to compare the biomechanically important structure of collagen between men and women with AAA.**

Methods: **Biopsies of the aneurysm walls were obtained during elective open repair of men (n = 14) and women (n = 14) treated for AAA. High-performance liquid chromatography (HPLC), Western blot, messenger RNA expression, and histochemical analyses were performed to assess the cross-linking and the amount and the composition of collagen.**

Results: **There was neither a difference in the thickness of the aneurysm wall, nor in the histological evaluation of the collagen composition between the sexes. Relative collagen content in the aneurysm wall was similar in men and women, as assessed by messenger RNA expression and HPLC. Collagen cross-linking differed between the sexes; women had more lysyl pyridinoline (LP) than men (0.140 vs 0.07; $P = .005$), resulting in a lower hydroxyl pyridinoline (HP):LP ratio (3.28 vs 8.41; $P = .003$). There was no difference in messenger RNA and protein expressions of lysyl hydroxylase and lysyl oxidase to associate with the lower HP:LP ratio in women.**

Conclusions: **The composition of collagen in the aneurysm wall of men and women are in several aspects similar, with the exception of collagen cross-linking, suggesting that the difference in rupture rate between the sexes rather depend on the composition of other vessel wall structures. (J Vasc Surg 2017;66:579-85.)**

Clinical Relevance: **The marked differences in prevalence and rupture risk of abdominal aortic aneurysm between men and women suggest gender to be of importance for both aneurysm development and progression. To study the amount and composition of collagen in men and women is of great importance for the understanding of the degenerative process occurring in aneurysms in both sexes and how it potentially differs, in regards to the increased rupture rate observed in women.**

Loss of vessel wall integrity by degradation is essential for the development of abdominal aortic aneurysm (AAA) and ultimately its rupture.[1] The observed greater rupture rate in women with AAA might be related to gender differences in the biomechanical properties of the aneurysm wall.[2,3]

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The biomechanical properties of the aortic vessel wall can be ascribed to elastin and collagen.[4,5] With elastin fragmentation, the aortic wall loses its elasticity. Yet, it is the failure of collagen that deprives the aorta of its principal load-bearing capacity and ultimately leads to aneurysm rupture.[6]

A triplet of amino acids, most common glycine together with the stabilizing proline and hydroxyproline, builds the collagen molecule. The capacity to form covalent cross-linking within and in between adjacent collagen molecules is essential for the mechanical stability of collagen.[7] The formation of the stable cross-linking depends on a series of post-transcriptional modifications on the α-chains of the collagen triple helix.[8] The hydroxylation of lysine residues in the collagen α-chains, by the enzyme lysyl hydroxylase (PLOD), is one of the necessary reactions for the subsequent formation of stable collagen. The mature collagen cross-linking is ultimately formed in the extracellular space. Lysyl oxidase (LOX) enables the last enzymatic catalysis required by oxidating peptidyl lysine residues into aldehydes.[9] The reactive aldehydes can then form the stable collagen cross-linking; hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP).[8]

An impaired cross-linking affects the tensile strength of collagen and its role in maintaining vascular integrity.[10] In animal models, PLOD1- and LOX-deficient mice develop aortic aneurysms and suffer sudden aortic rupture.[10,11] In humans, a mutation in PLOD1 causes Ehlers-Danlos syndrome VI and aneurysm development in patients with bicuspid aortic valve disease has been associated with a reduced expression of PLOD1.[12,13]

An intraluminal thrombus is present in most AAA and has been shown to influence the biomechanical properties of the underlying aneurysm wall, but its potential role in aneurysm growth and rupture is controversial.[14-16] Some studies have shown a protective effect of the intraluminal thrombus on the underlying aneurysm wall, whereas others have illustrated an increased deterioration with hypoxia and vascular smooth muscle cell apoptosis beneath the intraluminal thrombus.[14,15,17]

Little is known of the structures providing vascular integrity in the aneurysm wall of women.[18] We have recently reported similar expressions of elastin and elastolytic enzymes in the thrombus-covered aneurysm wall in men and women.[19] A difference in the amount of collagen and its cross-linking in the thrombus-covered aneurysm wall between men and women could help explain why AAAs in women are more prone to rupture. We hypothesized that there might be a difference in the collagen composition between men and women with AAA and therefore aimed to investigate collagen and its cross-linking in men and women with AAA.

METHODS

Study population and tissue handling. All women treated electively with open repair (OR) at Karolinska University Hospital, Stockholm, Sweden, from November 2008 to December 2012, with infrarenal AAA (n = 13) and juxtarenal AAA (n = 1) were included. Male patients treated during the same time period (n = 14) were chosen to match the ages and aneurysm diameters of the participating women to exclude those parameters as confounding factors. The patients were treated with OR, either because they were unsuitable for endovascular aneurysm repair or because of their relatively young age. During the operation, biopsies of the ventral, infrarenal aneurysm walls at the maximum diameter were obtained. Only thrombus-covered aneurysm walls were used in this study because nonthrombus-covered walls could not be obtained from all participants, because very few patients undergoing OR have nonthrombus-covered aneurysm walls. The majority of patients with aneurysms, as large as 5.5 cm in diameter, have at least a thin circumferential thrombus but most likely a thick thrombus. Patient characteristics were obtained from hospital charts. Body surface area (BSA) was calculated according to DuBois: $(\text{weight}^{0.425} \times \text{height}^{0.725}) \times 0.007184$.[20] Aortic size index (ASI) was calculated as aneurysm diameter (cm)/BSA (m^2).[21] Aneurysm growth rate was obtained by collecting information from the last two ultrasound- and/or computed tomography examinations that the participants underwent before the OR of AAA. The potential underestimation and overestimation of aneurysm diameter with the different modalities were considered and the measurements converted accordingly.[22] All patients had signed an informed consent before the operative procedure. The study was approved by the local ethics committee.

Histochemical analysis. The 5-μm sections of thrombus-covered aneurysm walls were deparaffinized in TissueClear (Sakura, Torrance, Calif) and rehydrated in ethanol. Masson trichrome/Lillie's trichrome staining was performed using Mason Trichrome Stain Kit (Sigma-Aldrich, St. Louis, Mo) by immersing the sections in the various solutions according to manufacturer's instructions. Muscle fibers turned red and collagen turned blue.

Sirius red staining was performed by immersing the sections in Weigert's hematoxylin and Picro-sirus red solution according to a standardized protocol, in Leiden, Netherlands, as has been described recently.[23] The thickness of the medial and adventitial layers were measured at an interval of 50 mm.

Movat's pentachrome staining was performed in Leiden, Netherlands, and has been described recently.[24] Nuclei and elastic fibers turned *black*; collagen turned *yellow*; proteoglycans turned *black*; and muscle fibers turned *red*. The composition of collagen and its relation to other vessel wall structures was assessed by spectrophotometry and presented in proportions and per cent of the medial layer.

Messenger RNA expression analysis. Frozen thrombus-covered medial layers were homogenized with Fastprep. RNA was isolated with Trizol (Invitrogen, Carlsbad, Calif), RLT buffer (from Rneasy Mini kit, Qiagen, Valencia, Calif), and Dnase1 (Rnase free Dnase Set, Qiagen) according to a standardized protocol. RNA was quantified by a Nanodrop (NanoDrop Products, Wilmington, Del). RNA quality and integrity were verified using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, Calif). For quantification of gene expression, total RNA was reversely transcribed to cDNA using Superscript II according to the manufacturer's protocol (Invitrogen). Real-time polymerase chain reaction (PCR) was performed on the Applied Biosystems 7000 Real-Time PCR System with TaqMan Assays-on-Demand Gene Expression Probes for collagen1α1, collagen1α2, collagen3α1, PLOD1-3, and LOX. Robust multiarray average normalization was performed and gene expression data were log2-transformed. The housekeeping gene Ribosomal Protein Large P0 (RPLP0) was used for normalization.

Western blot analysis. Thrombus-covered medial layers were shred and mixed with a lysis buffer containing 50 μL of protease inhibitor and 30 μL 1 mol/L Tris-HCl, pH 8.0. The mixture of samples and lysis buffer samples were then granulated with a Tissuelyzer, according to manufacturer's protocol, and centrifuged for 5 minutes at 220 rpm. The supernatants were sonicated for 5 minutes at a high level followed by centrifugation for 10 minutes at 12,000 rpm. The protein content in the supernatants was determined using Bradford protein assay. The samples were

diluted with lysis buffer before being loaded on a 4% to 12% SDS gel (Novex NuPAGE 4-12% Bis-Trisgel 15 well, Invitrogen) in MOPS-SDS running buffer. Electrophoresis was run for 90 minutes at 100V, in a cold room. The gel and membrane (Hybond PVDF transfer membrane, GE Healthcare, Little Chalfont, UK) were equilibrated in transfer buffer before transfer by electroblotting for 90 minutes at 400 mA, in a cold room. For blocking, the membrane was suspended in blocking buffer (3% bovine serum albumin/Tris-buffered saline, 0.1% Tween 20) for 60 minutes. The membrane was incubated over night with PLOD1 (Santa Cruz Biotechnology, Santa Cruz, Calif), PLOD2 (Abcam, Cambridge, Mass), LOX (Millipore, Billerica, Mass), glyceraldehyde-3-phosphate dehydrogenase (Abcam), and beta-tubulin (Santa Cruz) followed by the second antibody (antimouse and antirabbit horseradish peroxidase; Bio-Rad, Berkeley, Calif) for 45 minutes. Finally, the developing solution from ECL Prime Western Blotting Detection Reagent kit (GE Healthcare) and film (Amersham Hyperfilm ECL; GE Healthcare) were used for chemiluminescent detection. Densitometry was performed using ImageJ analysis software.

High-performance liquid chromatography. Thrombus-covered medial layers were available from 16 participants. The method has been described recently.[23] In short, 2 mg of each sample were put in Sarstedt-tubes containing 70% ethanol followed by hydrolyzation for 20 hours in 1 mL 6 mol/L HCL. The samples were then dried and redissolved in pyridoxine (10 μmol/L) and homoarginine (2.4 mmol/L) followed by a 5-fold dilution with heptafluorobutic acid (0.5%; Sigma-Aldrich) in acetonitrile (10%) for cross-linking analysis. For amino acid analysis the samples were further diluted 50-fold with 0.1 mol/L sodium borate buffer, pH 8.0. Derivatization of the amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography (HPLC) of amino acids and cross-links were performed on a Micropak ODS-80 TM column (Varian, Palo Alto, Calif). The quantities of the cross-link HP and LP were expressed as the number of residues per collagen molecule. Relative collagen content was depicted as hydroxyproline:proline ratio.

Statistical analysis. The statistical analysis was performed with SPSS 21.0 (SPSS, Inc, Chicago, Ill). The independent *t*-test was used for gender comparisons of normally distributed variables and Mann *U* test for not normally distributed variables. Pearson's χ^2 test and Fisher's exact test were used for parametric and nonparametric variables, respectively. Statistical significance was defined as $P < .05$.

RESULTS

Men and women, matched for age and aneurysm diameter, had similar body mass index and smoking habits. Women with AAA had lower BSA than men with AAA, yet the absolute diameter relative to BSA; that is, the ASI did not differ between the sexes (Table I). Growth rate could only be calculated for 13 out of 28 patients and was therefore considered inconclusive. The lack of prior examinations was owing to the fact that more than one-half of the patients, mostly women, were diagnosed with an AAA en passant and the aneurysms were at that time large enough to require treatment.

Table I. Patient characteristics

	Men with AAA (n = 14)	*Women with AAA (n = 14)*	P
Age	72 ± 6.0	72 ± 6.7	.905
AAA diameter, mm	62.7 ± 10.4	60.0 ± 6.2	.398
BMI	24.5 ± 3.6	25.0 ± 3.0	.635
BSA	1.9 ± .2	1.7 ± .2	<.001
ASI	3.3 ± .5	3.5 ± .3	.118

AAA, Abdominal aortic aneurysm; *ASI,* aortic size index = aneurysm diameter(cm)/BSA(m^2); *BMI,* body mass index; *BSA,* body surface area = $(\text{weight}^{0.425} \times \text{height}^{0.725}) \times 0.007184$.
Values are presented as mean ± standard deviation for normally distributed data. Significance calculated by independent *t*-test.

Table II. Thickness of the vessel wall layers in the aneurysm wall

	Men with AAA	*Women with AAA*	P
Media, mm	.73 ± .62	.41 ± .11	.202
Adventitia, mm	.55 ± .21	.55 ± .20	.987
Whole wall, mm	2.24 ± 1.19	1.50 ± .38	.149
Proportion of media in whole wall	.27 ± .16	.29 ± .07	.847

AAA, Abdominal aortic aneurysm.
Values are presented as mean ± standard deviation for normally distributed data. Significance calculated by independent *t*-test.

Table III. Proportions of collagen, muscle and proteoglycan, in the aneurysmal medial layer, presented in percent

	Men with AAA	*Women with AAA*	P
Collagen	83 (24)	87 (20)	.897
Muscle	15 (22)	12 (13)	.897
Proteoglycan	44 ± 29	40 ± 26	.747
Collagen within muscle	37 (24)	47 (21)	.633
Collagen within proteoglycan	57 ± 19	51 ± 13	.529

Values are presented as mean ± standard deviation for normally distributed data and median (interquartile range) for not normally distributed data. Significance calculated by independent *t*-test and Mann Whitney *U* test.

Thickness of the aneurysm wall and its layers. There was neither a difference in the thickness of the aneurysm wall nor in the proportion of the medial layer in relation to the whole aneurysm wall between the sexes (Table II).

Collagen composition. There was no difference between men and women in the histological evaluation of collagen and its relation to muscle and proteoglycans in the medial layer of the aneurysm wall (Table III; Fig 1).

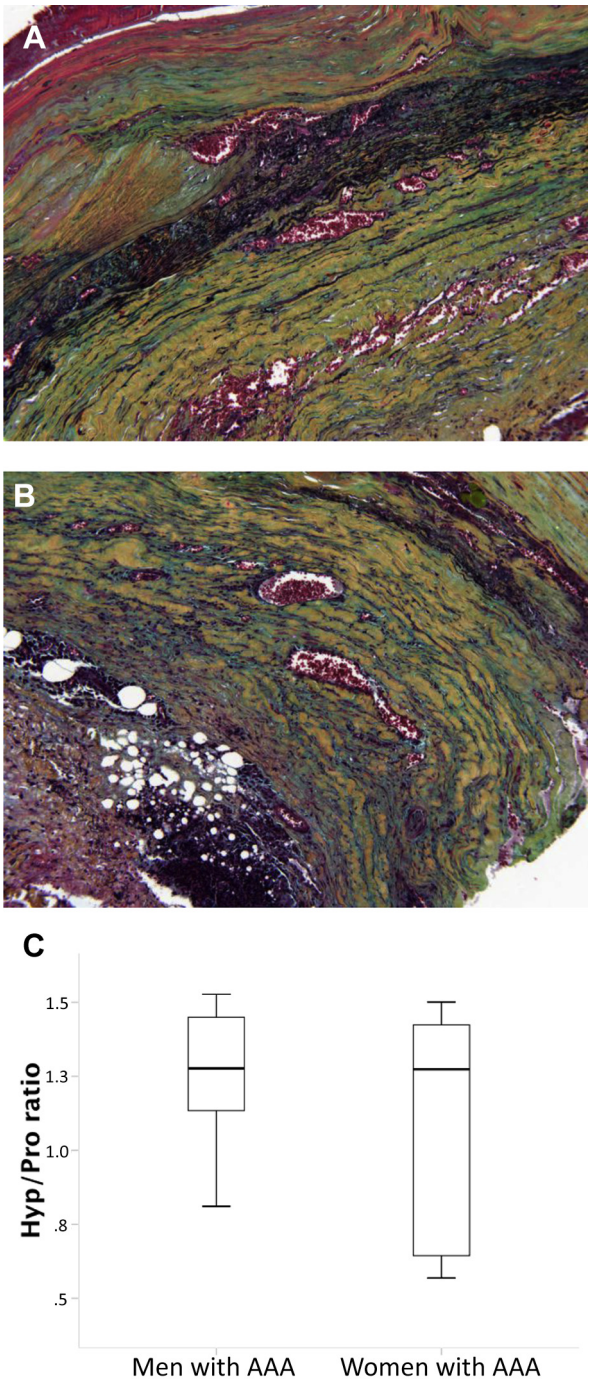

Fig 1. A, Movat's pentachrome staining of thrombus-covered media in man and **(B)** woman. Collagen, *yellow*; muscle, *red*; proteoglycan, *blue*; elastin, *black*. **C,** Relative collagen content, depicted as ratio of hydroxyproline:proline (*Hyp:Pro*; $P = .645$).

Relative collagen content. Relative collagen content in the medial layer of the aneurysm wall, as assessed by HPLC and depicted as hydroxyproline:proline ratio, was similar in men and women with AAA (Fig 1). The messenger RNA expressions of the procollagens, collagen1α1, collagen1α2, and collagen3α1, were similar in men and women with AAA (Table IV).

Table IV. Messenger RNA expression analysis of collagen, lysyl hydroxylase (*PLOD*), and lysyl oxidase (*LOX*) in the thrombus-covered aneurysm wall of men and women

	Men with AAA	*Women with AAA*	P
Collagen1α1	1.18 (1.10)	1.81 (.64)	.094
Collagen1α2	−1.14 ± .70	−.76 ± .90	.223
Collagen3α1	.39 ± .87	.81 ± .94	.231
LOX	4.43 (3.20)	5.04 (3.20)	.184
PLOD1	5.22 ± .85	5.32 ±1.50	.827
PLOD2	5.58 ± .89	6.00 ± .66	.173
PLOD3	4.21 ± .72	4.30 ± .68	.740

Messenger RNA expressions were normalized to the housekeeping gene: Ribosomal Protein Large P0 (RPLP0). Values presented log2-transformed and as arbitrary units. Values are presented as mean ± standard deviation for normally distributed data and median (interquartile range) for not normally distributed data. Significance calculated by independent *t*-test and Mann Whitney *U* test.

Collagen cross-linking. The amount of mature cross-linking was assessed by HPLC and presented as HP and LP per triple helix. There was no difference in HP per triple helix (0.582 vs 0.462; $P = .139$). Women had higher LP per triple helix than men (0.139 vs 0.072; $P = .005$), resulting in a lower HP:LP ratio in women (3.282 vs 8.414; $P = .003$; Fig 2). There were no gender differences in messenger RNA and protein expressions of PLOD1-2 and LOX (Table IV; Supplementary Fig, online only).

DISCUSSION

The collagen composition in the aneurysm wall of men and women are in several aspects similar, with the exception of the collagen cross-linking.

Changes in collagen content in AAA are of interest as it is the ultimate structural component preventing rupture.[6] There is little knowledge of the collagen composition in women with AAA, which might be of interest owing to their greater risk for rupture.[2] In this study, we found men and women to have equally thick aneurysm walls and that collagen makes up an equally large part of the medial layer of the aneurysm wall. There was neither a difference in the composition of collagen in relation to other vessel wall structures nor a difference in relative collagen content between men and women, assessed by histological evaluation and HPLC. Tong et al[18] recently published data illustrating less collagen content in the aneurysm wall of women compared with men as assessed by a modified hydroxyproline assay. They also studied the medial layer of the aneurysm wall. However, the two studies may not be comparable, because there are differences in the ages and the aneurysm diameters of the participating men and women, as well as the fact that the two studies use different analyses, biomechanical vs biochemical. In our study, men and women were matched according to age, whereas in the

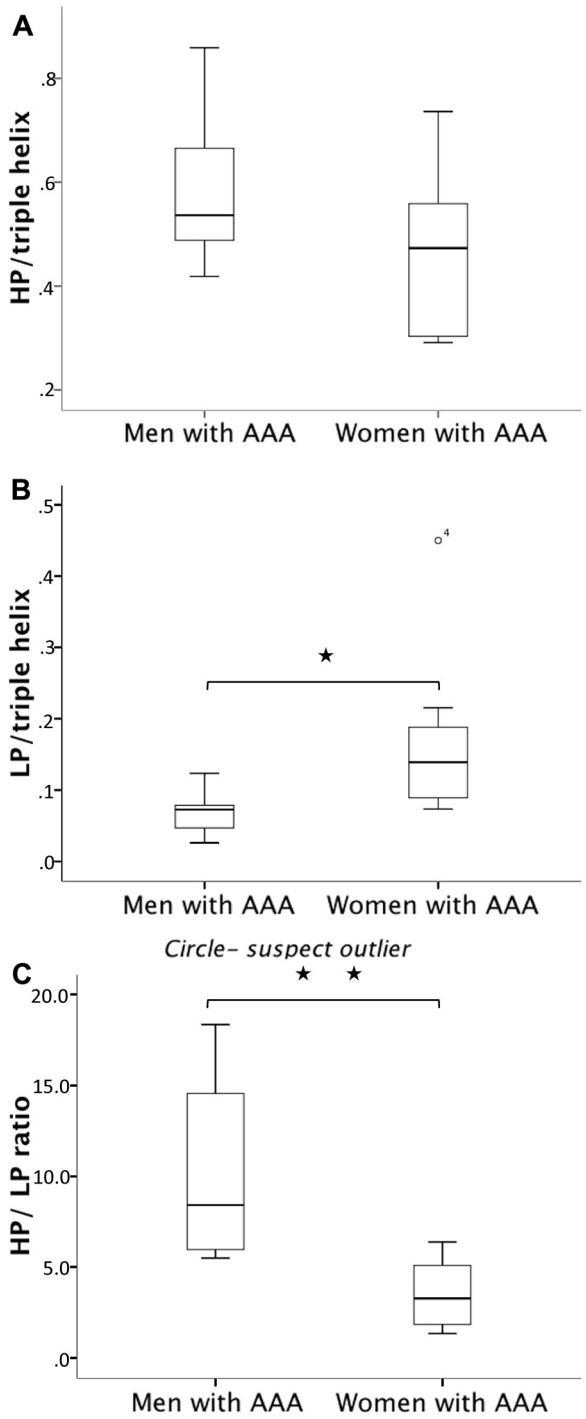

Fig 2. Cross-linking of collagen in thrombus-covered aneurysm wall of men and women, assessed by high-performance liquid chromatography and depicted as **(A)** hydroxyl pyridinoline (*HP*) per triple helix ($P = .139$). **B,** Lysyl pyridinoline (*LP*) per triple helix ($^{\star}P = .005$) and **(C)** ratio of HP:LP ($^{\star\star}P = .003$). *AAA,* Abdominal aortic aneurysm.

study by Tong et al,[18] the men were younger than the women, which could have implications for the observed gender difference in collagen content between the two studies.

Collagen cross-linking enables the mechanical stability of collagen.[25] The two forms of mature cross-linking, HP and LP, link three collagen α-chains in separate collagen molecules; three hydroxylysine side chains in HP and two hydroxylysine and one lysine side chain in LP.[8,26] There is no known apparent difference in the physiological effect of LP and HP, yet the amount of LP in relation to HP has been shown to influence the stability of the collagen structure.[27] The higher the HP:LP ratio the greater is the stability of the structure.[27,28] In patients with AAA, intermolecular collagen cross-links are higher compared with controls.[23,29] In this study, we found a greater LP content in women compared with the one in men and consequently a lower HP:LP ratio in women. The lower HP:LP ratio in the thrombus-covered aneurysm wall of women suggests that the post-transcriptional modification of collagen differs in women with AAA; whether it might affect the mechanical properties of the aneurysm wall remains to be shown.

Two of the essential enzymatic steps in the formation of stable collagen are the hydroxylation of lysine residues by PLOD and the formation of reactive aldehydes by LOX. PLOD1 hydroxylates lysine side chains in the helical domain of the collagen α-chain, whereas PLOD2 hydroxylates lysine residues in the telopeptide.[10,28,30] The greater amount of LP in women and consequently lower HP:LP ratio suggests a difference in the formation of HP between the sexes. A reduced amount of PLOD1 could lead to a decreased formation of HP and a weaker mechanical structure. In this study, we found no difference in the expression of PLOD1 between the sexes to be associated with the lower HP:LP ratio observed in the aneurysm wall of women. One plausible explanation is that very few cells are involved in tissue repair in the end stage of AAA.

Women have smaller aortas than men, rendering the relative aneurysm enlargement in women to exceed that of men at any given diameter.[31] The ASI is used to value the relative enlargement and is determined by dividing the aneurysm diameter by the BSA.[32] ASI has been shown to be a potential determinant for rupture risk in women with AAA.[21] In this study, women were found to have significantly lower BSA, but the ASI between men and women did not differ. We do not rule out the possible effect of women's proportionally larger AAAs to the higher rupture rate observed in women, but cannot conclude that the potential differences in collagen cross-linking found in this study are related to such an effect.

The role of sex hormones in AAA development is mostly studied using animal models, illustrating a protective role of estrogen by inhibition of the proteolytic activity in the aneurysm wall.[33,34] In humans, sex hormones alter the elastin

and collagen composition of the aorta. Estrogen decreases collagen deposition and prevents its accumulation, whereas testosterone has shown less effective or rather mediating an opposite effect.[35,36] Estrogen has been shown to affect the cross-linking capacity of collagen. In skin and bone of mice, estrogen stimulates the activity of LOX and accelerates the maturation of collagen whereas estrogen deficiency, by ovariectomy, reduces collagen cross-linking in bone of ewes.[37,38] The vasculature is, as skin and bone, composed of fibrillar collagen but little is known of how sex hormones specifically affect the collagen composition in the aorta. The results of this study, which show similar collagen composition in men and women, could be due to an inherent difference in how sex hormones affect the vessel wall in women developing AAA or an acquired one during the course of the aneurysm disease.

There are limitations to this study such as the limited sample size, yet it is similar to other studies within this field.[23,39] Another limitation is the location in the aneurysm wall from where the biopsies were obtained. The biopsies were from the anterior wall, owing to surgical availability. Consequently, the results of this study may not be applicable to other parts of the aneurysm wall. Another limitation is the lack of a control material to establish the collagen composition in elderly men and women without disease. The study would have benefitted from a mechanical analysis, yet the biopsies were unfortunately not large enough, nor handled in a manor appropriate for a mechanical analysis.

CONCLUSIONS

The collagen composition in the aneurysm wall of men and women are in several aspects similar, with the exception of collagen cross-linking, suggesting that the difference in rupture rate between the sexes rather depend on the composition of other vessel wall structures.

AUTHOR CONTRIBUTIONS

Conception and design: CV, PE, RoH, JL, ReH
Analysis and interpretation: CV, PE, RoH, JL, ReH
Data collection: CV
Writing the article: CV, PE, ReH
Critical revision of the article: CV, PE, RoH, JL, ReH
Final approval of the article: CV, PE, RoH, JL, ReH
Statistical analysis: CV, PE, ReH
Obtained funding: CV, PE, ReH
Overall responsibility: ReH

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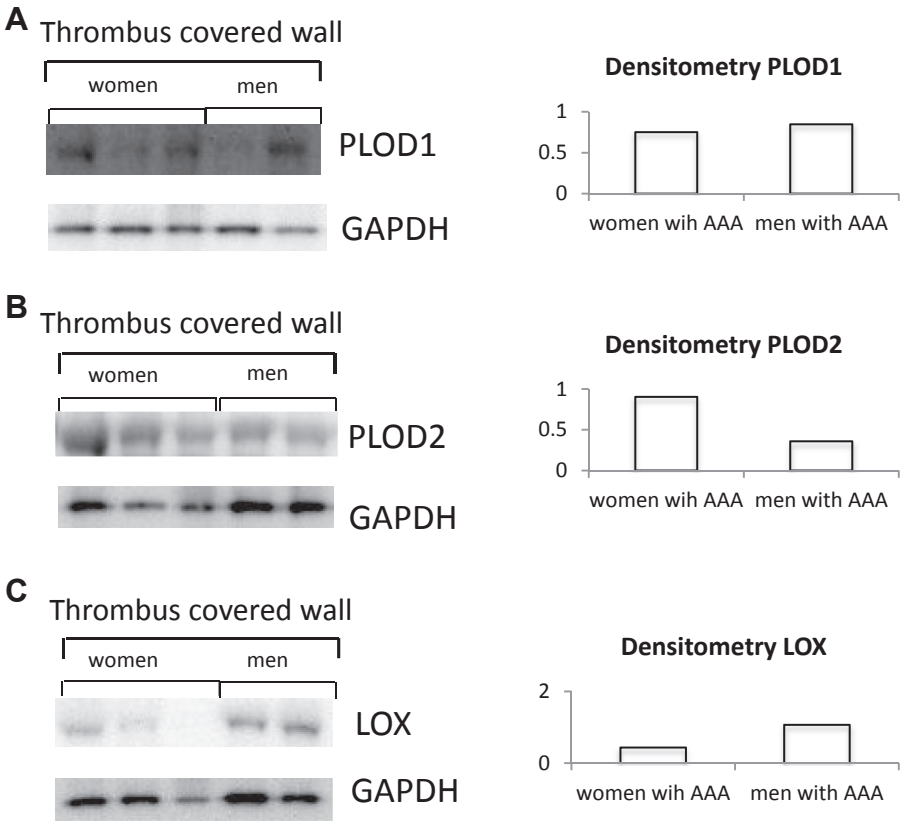

Supplementary Fig (online only). Western blot and densitometry of **(A)** lysyl hydroxylase (*PLOD1*) in 3 women and 2 men (0.75 vs 0.85; $P = .8$), **(B)** PLOD2 (0.92 vs 0.36; $P = .2$), and **(C)** lysyl oxidase (*LOX*; 0.43 vs 1.1; $P = .4$). Loading control: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).