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

Christina Villard, MD, PhD,[a,b] **Per Eriksson, PhD,**[c] **Roeland Hanemaaijer, PhD,**[d] **Jan H. Lindeman, MD, PhD,**[e] and **Rebecka Hultgren, MD, PhD,**[a,b] *Stockholm, Sweden; and Leiden, Netherlands*

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 2016;■:1-7.)**

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]

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]

From the Department of Molecular Medicine and Surgery, Karolinska Institutet,[a] the Department of Vascular Surgery, Karolinska University Hospital,[b] and the Atherosclerosis Research Unit, Center for Molecular Medicine, Department of Medicine, Karolinska Institutet,[c] Stockholm; the TNO Metabolic Health Research,[d] and the Department of Vascular Surgery, Leiden University Medical Center,[e] Leiden.

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Correspondence: Christina Villard, MD, PhD, Department of Vascular Surgery, Karolinska University Hospital, 171 76 Stockholm, Sweden (e-mail: christina.villard@ki.se).

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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