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# Differential expression of sex hormone receptors in abdominal aortic aneurysms

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ABSTRACT

*Objective:* Male sex is a significant risk factor for abdominal aortic aneurysm (AAA). Female sex hormones have been reported to prevent aneurysm formation in animal models. The study aims to describe the expression profile of sex hormone receptors in the aneurysm wall of men and women with AAA and compare with unaffected controls.
*Methods:* Aneurysm wall biopsies were obtained during elective open repair of AAA in men and women (n = 16 + 16). Aortic vessel wall from controls were obtained at organ donation (n = 6). Western blot-, mRNA expression- and immunohistochemical analyses were performed to assess the expression profile of the sex hormone receptors – androgen receptor (AR), progesterone receptor (PR), estrogen receptor α (ERα) and β (ERβ).
*Results:* The mRNA- and protein expression levels of AR were higher in AAA compared with control aorta (7.26 vs. 5.14, P = 0.001). mRNA- and protein expression levels of ERβ were lower in AAA compared with control aorta (9.15 vs. 12.29, P < 0.001). mRNA expression levels of PR were higher in AAA compared with control aorta (8.73 vs. 6.21, P = 0.003), but could not be confirmed on protein level. The expression profile of sex hormone receptors in men and women with AAA was similar.
*Conclusion:* Expression of sex hormone receptors differs in the aneurysmal aorta compared with unaffected aorta in men and women. A higher expression of AR and a lower expression of ERβ suggest that sex hormone activity could be associated with aneurysm development.
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## 1. Introduction

Male gender is an important risk factor for abdominal aortic aneurysm (AAA) [1]. The prevalence in elderly men is approximately 2% compared with 0.5% in aging women [2,3]. Albeit the prevalence of AAA in women is lower than in men, the risk of rupture is higher, suggesting gender to be of importance for both the development and the progression of AAA [4,5].

The lower prevalence of AAA and later onset of disease in women resemble the gender differences observed in the presentation of cardiovascular disease (CVD), which have been ascribed to changes in the levels of female sex hormones [1,6–8]. The protective effects of estrogens on the vasculature are mediated, both directly and indirectly, by the estrogen receptor α (ERα) and β (ERβ) [9]. However, the antiatherogenic effects of estrogens were seriously challenged, as hormone replacement therapy was found to be associated with prothrombotic events in the Women Health Initiative (WHI) trial and the Heart and Estrogen/Progestin Replacement Study (HERS) [10,11].

An effect of sex hormones on aneurysm formation has been demonstrated in animal models. In both angiotensin-II- and elastase-induced aneurysms, estradiol attenuated aneurysm formation by inhibiting the proteolytic activity [12,13]. In a rodent model, orchidectomy and treatment with estradiol prevented aneurysm enlargement, whereas no effect on aneurysm growth was observed in females given testosterone or undergoing oophorectomy [14]. An effect of gender on aneurysm formation was again illustrated when rat aorta was transplanted from different sexed rats. The observed resistance to aneurysm formation

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observed when the recipient was female was lost if the recipient instead was male [15].

Studies on the potential effect of gender on aneurysm development in humans are scarce. The observed negative association between AAA occurrence and long-term hormone therapy and the lower menopausal age in women with larger AAA support the theory of a protective effect of female sex hormones [16,17]. On the other hand, lower levels of testosterone are associated with AAA occurrence in elderly men [18].

Studies on gender aspects on aneurysm formation primarily focus on an effect of endogenous as well as exogenous sex hormones and little is known about the expression profile of sex hormone receptors in the aneurysm wall [12,15,18,19]. The aim of this study was to examine the expression profile of the sex hormone receptors: ERα, ERβ, progesterone receptor (PR) and androgen receptor (AR) in the aneurysmal walls of men and women and compare with the expression profile in non-aneurysmal aortic walls. The study focused on the medial layer of the aortic vessel wall as it is most affected by degradation in aneurysm development [20].

## 2. Material and method

### 2.1. Study population and tissue handling

All women treated electively for AAA with open repair (OR) at Karolinska University Hospital, Stockholm Sweden, November 2008–June 2014, were included (n = 16). Male patients treated during the same time period (n = 16), were chosen to match the age and aneurysm diameter of the participating women. Mycotic aneurysms were not included. The patients were treated with OR, as they were considered unsuitable for endovascular aneurysm repair (EVAR). Biopsies of the ventral, infrarenal aneurysm wall, at the maximum diameter, were obtained during the surgical procedure. Only thrombus covered aneurysm walls were used since non-thrombus covered aneurysm walls could not be obtained from all participants, which is probably due to the low occurrence of thrombus free walls in such large aneurysms. Patient characteristics were obtained from hospital charts. Body mass index (BMI) was defined as the ratio of weight/square of the height [21]. Body surface area (BSA) was predicted using DuBois formula: $(\text{weight}^{0.425} \text{x} \text{height}^{0.725}) \times 0.007184$ [22]. Aortic size index (ASI) was defined as the ratio of aneurysm diameter/BSA [23]. All patients had signed an informed consent prior to the surgical procedure.

The control group consisted of 6 organ donors, male and female. The aortic diameters of the organ donors were not measured, but defined non-aneurysmal by the transplant surgeon. During the organ donation biopsies of the infrarenal aorta were obtained. The aortic biopsies from organ donors and patients with AAA were handled according to the same protocol and stored during transport in RNAlater and formalin. The aortic walls, both aneurysmal and non-aneurysmal, were divided into initimal, medial and advential layers. The biopsies from organ donors could not be transported freshly frozen, which limited the protein expression analysis. All donors, or their close relatives, had signed an informed consent regarding donation of the tissue for research purposes. Patient characteristics were obtained from a form filled out by the organ coordinator in charge and based on information from hospital charts and from the patients' relatives. The study was approved by the local Ethics Committee.

### 2.2. Immunohistochemical analysis

5 μm sections of aortic walls were deparaffinised in Tissue-Clear (Sakura) and rehydrated in ethanol. The sections were boiled under high pressure in DIVA-buffer (BioCare Medical) and Background Sniper Solution (BioCare Medical) was used for background blocking. Antibodies ERβ (Thermo), PR (Dako) and AR (Dako) were diluted in DaVinci Green Solution (BioCare Medical) for 60 min in room temperature, followed by short incubations with Probe and –Polymer Kit specific for mouse/rat (BioCare Medical). Vulcan Fast Red Solution (BioCare Medical) was used for detection and counterstaining was performed with Mayer's hematoxillin (Vector Laboratories). Double staining with ERβ and smooth muscle α-actin (Abcam) were performed in a similar manner but with a double staining probe and polymer Kit (BioCare Medical) and Warp Red Solution together with Vine Green (Biocare Medical) for detection. Double stainings with AR and smooth muscle α-actin and PR and smooth muscle α-actin were unfortunately not possible and therefore the stainings were performed on consecutive sections.

### 2.3. mRNA expression analysis

Frozen medial layers of aortic walls were homogenized with tissue lyser (Qiagen). RNA was isolated with Qiazol (Qiagen), RLT buffer (from Rneasy Mini kit, Qiagen) and Dnase1 (Rnase free Dnase Set, Qiagen) according to a standardized protocol. RNA was quantified by a Nanodrop (NanoDrop Products). RNA quality and integrity were verified using the Agilent 2100 Bioanalyzer System (Agilent Technologies). For quantification of gene expression, total RNA was reversely transcribed to cDNA using High capacity RNA to cDNA kit (Life technologies) the manufacturers protocol. Real time PCR was performed on the Applied Biosystems 7000 Real-Time PCR System with TaqMan Assays-on-Demand Gene Expression Probes for ERα, ERβ, PR and AR. 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.

### 2.4. Western blot analysis

The medial layers of the aneurysm samples were shred and mixed with a lysis buffer containing 50 μl protease inhibitor and 30 μl 1 M 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 min at 220 rpm. The supernatants were sonicated for 5 min at high level followed by centrifugation for 10 min at 12000 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 4–12% SDS gel (Novex NuPAGE 4–12% Bis-Trisgel 15well, Invitrogen) in MOPS-SDS running buffer. Electrophoresis was run for 90 min at 120 V, in a cold room. The gel and membrane (Hybond PVDF transfer membrane, GE Healthcare) were equilibrated in transfer buffer before transfer by electroblotting for 90 min at 400 mA, in a cold room. For blocking, the membrane was suspended in blocking buffer (3% bovine serum albumin/TTBS) for 60 min. The membrane was incubated over night with ERβ (Thermo), AR (Abcam) and GAPDH (Abcam) followed by the second antibody (anti-Mouse and anti-Rabbit HRP, BioRab) for 45 min. Finally, the developing solution from ECL Prime Western Blotting Detection Reagent kit (GE Healthcare) and CCD camera (Fujifilm LAS-1000) were used for chemiluminescent detection.

### 2.5. Statistical analysis

Statistical analysis was performed with SPSS 21.0. Independent *t*-test was used for gender comparisons of normally distributed data and Mann *U* test for not normally distributed data. Pearson's chi-square test and Fischer's exact test were used for normally and not normally distributed categorical variables, respectively. AAA occurrence and AAA diameter were estimated by multivariate logistic

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