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Abdominal aortic aneurysm in women

-risk factor profile and aneurysm wall

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To Gillis

Abstract

Abdominal aortic aneurysm (AAA) in women is rare; the prevalence is approximately 0.5% in elderly women, to compare with 2-4% in aging men. The few women that suffer from AAA are older and have a higher rupture risk. A preventive effect of estrogen on AAA formation in animal models implies that the lower prevalence of AAA in women can depend on a protective effect of female sex hormones. The knowledge is scarce of how the aneurysm wall of women differs from that of men. Altogether, it is likely that biological gender differences could influence the risk of AAA development, growth rate and rupture risk. This thesis, based on five papers, focuses on risk factors for AAA in women and gender differences in the aneurysm wall.

In *the first paper*, a case-control study, the reproductive history in women with AAA and a control group of women with peripheral artery disease was investigated. 280 women were invited to answer a questionnaire about their reproductive history and general health. The response rate was 70% and the results showed a lower mean menopausal age in women with larger AAA compared with women with smaller AAA and women with peripheral artery disease. *The second paper*, a case-control study, investigated previously reported, potential biomarkers for AAA. These had never been analysed in women with AAA and thus it was uncertain if they would apply to a female cohort. Men and women were in many aspects similar in biomarker profile, with the exception of matrix metalloproteinase 9 (MMP9). The levels of MMP9 were higher in women compared with men, with equally large AAA. In *the third paper* elastin content and elastolytic proteins in the aneurysm wall of men and women were analysed. 37 patients were included. The results suggest a more pronounced elastolysis and a lower elastin content in women compared with men. In *the fourth paper* collagen and its cross-linking were studied in 28 patients with AAA. The results showed no difference in the relative collagen content between men and women but a different collagen cross-linking in women compared with that in men. The findings might have implications for the biomechanical properties of the aneurysm wall in women, yet further analyses are required to clarify the mechanisms. *The fifth paper* was a study of the degree of apoptosis and inflammation, in relation to smooth muscle cells in the aneurysm wall of men and women. 40 patients with AAA were included. The findings suggest a more pronounced apoptosis in the aneurysm wall of women compared with men, which might be related to a greater infiltration of inflammatory cells.

In conclusion, women with larger AAA have an altered reproductive history with a lower mean menopausal age, suggesting hormonal changes to be of importance for AAA development in women. The observed gender differences in the aortic wall described in this thesis, contribute to the presently poorly understood biological and morphological processes that trigger aneurysm development, progression and rupture.

List of scientific papers

I. **Reproductive history in women with abdominal aortic aneurysms**
Villard C, Swedenborg J, Eriksson P, Hultgren R
J Vasc Surg. 2011 Aug;54(2):341-5

II. **Biomarkers for abdominal aortic aneurysms with a gender perspective**
Villard C, Wågsäter D, Swedenborg J, Eriksson P, Hultgren R
Gend Med. 2012 Aug;9(4):259-266

III. **Differences in elastin and elastolytic enzymes between men and women with abdominal aortic aneurysm**
Villard C, Eriksson P, Swedenborg J, Hultgren R
Accepted for publication in AORTA

IV. **Collagen cross-linking in men and women with abdominal aortic aneurysm**
Villard C, Eriksson P, Hanemaaijer R, Lindeman J H, Swedenborg J, Hultgren R
Manuscript

V. **More apoptosis in women with abdominal aortic aneurysm compared with men**
Villard C, Eriksson P, Jorns C, Swedenborg J, Hultgren R, Roy J
Manuscript

Populärvetenskaplig sammanfattning

Bråck på stora kroppspulsådern i buken kallas för abdominellt aortaaneurysm (AAA) vilket uppstår till följd av att de proteiner som ger kärlväggen dess stadga och elasticitet bryts ned. Detta i sin tur leder till att kärlväggen försvagas och därmed vidgas. Allteftersom bråcket växer ökar belastningen på kärlväggen och därmed även risken för att bråcket spricker, s.k. ruptur. Rupturerat AAA är ett tillstånd som är förenligt med hög dödlighet. Den underliggande mekanismen bakom nedbrytningen av kärlväggen och därmed uppkomsten av AAA är ännu inte påvisad. Man har emellertid kunnat påvisa att manligt kön utgör en riskfaktor. Förekomsten av AAA hos äldre män är 4-6 gånger högre än den för kvinnor i samma åldersgrupp. Det finns andra skillnader i utveckling, prognos och behandling mellan de två könen såsom att risken för ruptur är högre för kvinnor jämfört med män. Det övergripande syftet med denna avhandling var att undersöka kvinnor med AAA; deras riskfaktorprofil och hur den aneurysmatiska kärlväggen skiljer sig mellan könen.

Det första delarbetet kartlade den gynekologiska sjukhistorien hos kvinnor med och utan AAA. Resultaten visade att kvinnor med stora AAA har en lägre menopausålder jämfört med kvinnor med mindre AAA och kvinnor utan.

I det andra delarbetet uppmättes nivåer av kärlväggsförsvagande proteiner, som skulle kunna utgöra diagnostiska och prognostiska markörer för AAA, i cirkulerande plasma hos kvinnor och män med AAA. Resultaten visade att män och kvinnor är i många avseende lika, med undantag av en biomarkör. Nivåerna av denna biomarkör var högre hos kvinnor jämfört med män, med lika stora aneurysm.

I det tredje delarbetet undersöktes mängden av elastin, som ger kärlväggen dess elasticitet och proteiner som bryter ned det, i aneurysmvägg hos män och kvinnor. Mindre elastin och en större mängd kärlväggsnedbrytande protein sågs i kvinnors aneurysmvägg jämfört med mäns, vilket tyder på en större nedbrytning av kärlväggen hos kvinnor.

Det fjärde delarbetet syftade till att jämföra mängden kollagen, det protein som ger kärlväggen dess styrka, och dess uppbyggnad hos män och kvinnor med AAA. Resultaten kunde inte påvisa någon skillnad i kollagenmängd men i dess uppbyggnad mellan könen. Vidare studier krävs för att klarlägga mekanismen bakom skillnaden i kollagenets uppbyggnad mellan könen och dess beydelse för aneurysmväggens styrka.

I det femte delarbetet undersöktes graden av celldöd,och mängden glatta muskelceller och inflammatoriska celler i aneurysmväggen hos män och kvinnor. Resultaten visade på en mer uttalad celldöd i kvinnors kärlvägg tillsammans med lägre uttryck av markörer för glatta muskelceller och högre uttryck av markörer för inflammatoriska celler. Detta talar för en mer uttalad nedbrytning, i form av celldöd, i kvinnors aneurysmvägg jämfört med mäns.

Sammantaget visar studierna att kvinnor med större AAA har en förändrad reproduktiv anamnes med en lägre menopausålder, vilket skulle kunna vara av betydelse för aneurysmutveckling hos kvinnor. De biologiska och morfologiska processer, som ger upphov till utveckling, tillväxt och ruptur av AAA hos män och kvinnor är väsentligen okända. De beskrivna analyserna av könsskillnader i aneurysmväggen i denna avhandling, bidrar till att utöka det idag begränsade kunskapsläget.

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List of abbreviations

AAA	Abdominal aortic aneurysm
AP	alkaline phosphatase
ASI	aortic size index
BAX	BCL2-associated X protein
BMI	body mass index
BSA	body surface area
CD	Cluster of differentiation
CT	computer tomography
CVD	Cardiovascular disease
DM	Diabetes mellitus
ECM	extracellular matrix
ELISA	Enzyme-linked immunoassay
ER	Endoplasmatic reticulum
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
EVAR	Endovascular aneurysm repair
FAS	Fas cell surface death receptor
HP	Hydroxylysyl pyrdinoline
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
ICU	Intensive care unit
IFU	Instructions for use
ILT	Intraluminal thrombus
IR	interquartile range
LH	Lysyl hydroxylase
LOX	Lysyl oxidase
LP	Lysyl pyridinoline
MMP	Matrix metalloproteinase
MS4A1	Membrane-spanning 4 domains subfamily A member 1

MYH11	Myosin, heavy chain 11, smooth muscle
NIH	National Institutes of Health
OR	open repair
PAA	Popliteal artery aneurysm
PAD	Peripheral artery disease
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
PLOD	Procollagen-lysine-2-oxoglutarate-5-dioxygenase
PR	Progesterone receptor
p53	Tumor protein p53
RCT	Randomized controlled trial
RPLP0	Ribosomal protein large P0
PCR	Polymerase chain reaction
SD	standard deviation
SHBG	Sex hormone-binding-globulin
TAA	Thoracic aortic aneurysm
TUNEL	Terminal deoxynucleotidyl transferase mediated 2'-deoxyuridine 5'-Triphosphate nick-end labeling
VSMC	Vascular smooth muscle cell
WHI	Women's Health Initiative

Introduction

Aneurysms in history

"If thou examinest a swelling of vessels in any limb of a man, and thou findest that it is hemispherical and grows under thy fingers, at every going (i.e. pulsation) but if it is separated from his body, it cannot an account of that become big and not give out (diminish), then thou shalt say concerning it: it is a swelling of a vessel. It is vessels that cause it and it arises through injury to a vessel.

Citation from Ebers Papyrus dated 1550 B.C.

The Ebers Papyrus is the first document describing aneurysms and their characteristics. Surgical treatment was not considered a possible remedy: "Thou shalt not put thy hand on any likeness of this" in favour of the prescribed spell: "Flow out, thou vessel that jumps (i.e. pulsates)".(1)

The treatment for aneurysms has come a long way since the recommended ancient spell. The use of synthetic grafts for aortic aneurysms, thoracic and abdominal, was first introduced in the 1950s and the minimally invasive endovascular approach, in the 1990s. (2-4) They are both today, in refined forms, the surgical treatments considered when treating aortic aneurysms.

Definition

An aneurysm is a widening of a blood vessel and it arises as a result of a weakened vessel wall. Arterial aneurysms can be classified based on their location, form and etiology. Arterial aneurysms can arise at any location but are most prevalent in the infrarenal abdominal aorta. (5-8) Based on the form of the aneurysm it can be categorised as fusiform or less common, saccular.(9) Figure I. For the majority of arterial aneurysms the etiology is unknown. Aneurysms with a clear etiology such as congenital, infectious and those related to connective tissue disorders are rare.(9-13) This thesis focuses on aneurysms in the abdominal aorta, i.e. abdominal aortic aneurysms (AAAs).

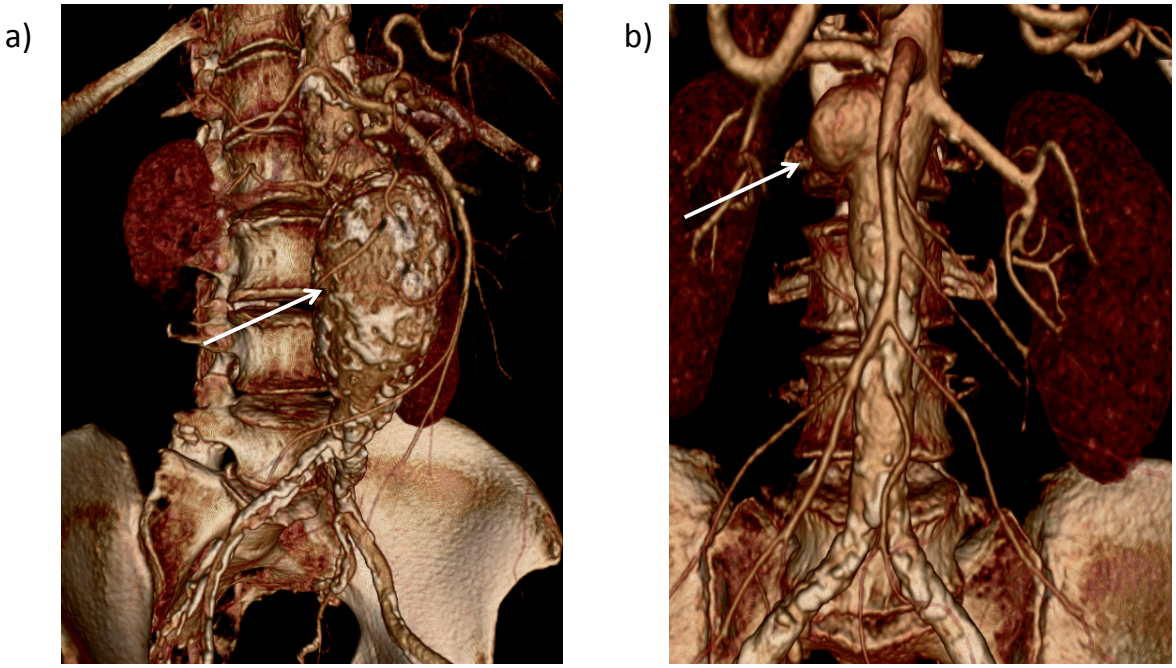


Figure I. *Shape of AAA: a) fusiform and b) saccular.*

The abdominal aorta is defined as aneurysmal if its diameter measures at least 1.5 times to that of the adjacent or expected normal aortic diameter.(14, 15) In general practice an AAA is considered present if the abdominal aortic diameter measures 3.0 cm or more.(16)

The aortic vessel wall

The aorta is composed of three layers. Tunica intima, which is the innermost layer, faces the arterial lumen and is made up of endotelial cells attached to the basal lamina. Tunica media, the middle layer, is a network of vascular smooth muscle cells (VSMCs) fenestrated by elastic and collagen fibres. Tunica adventitia, the outermost layer, is mainly composed of fibroblasts and loose connective tissue. Figure II. The vasa vasorum, the blood supply of the vessel wall, nourishes the tunica media and adventitia, whereas the innermost layer is provided for by the vessel lumen.(9)

The aortic wall depend on the biomechanical properties of elastin, collagen and VMSCs to withstand the pulsatile arterial blood flow.(17, 18)

Elastin

The elastic fibres provide elasticity to the aortic vessel wall.(17) The elastic fibers form layers between VSMCs in the medial layer of the aortic wall. Figure II and Figure III c.(19) The principal components of elastic fibres are elastin and microfibrils.(20) Elastin is formed from its precursor tropoelastin and is cross-linked by the enzyme lyxyl oxidase (LOX) into an amourphorus core, mantled by microfibrils.(20-22) The elastin fibres are composed to impart resilience for a lifetime, with limited synthesis in adulthood.(23)

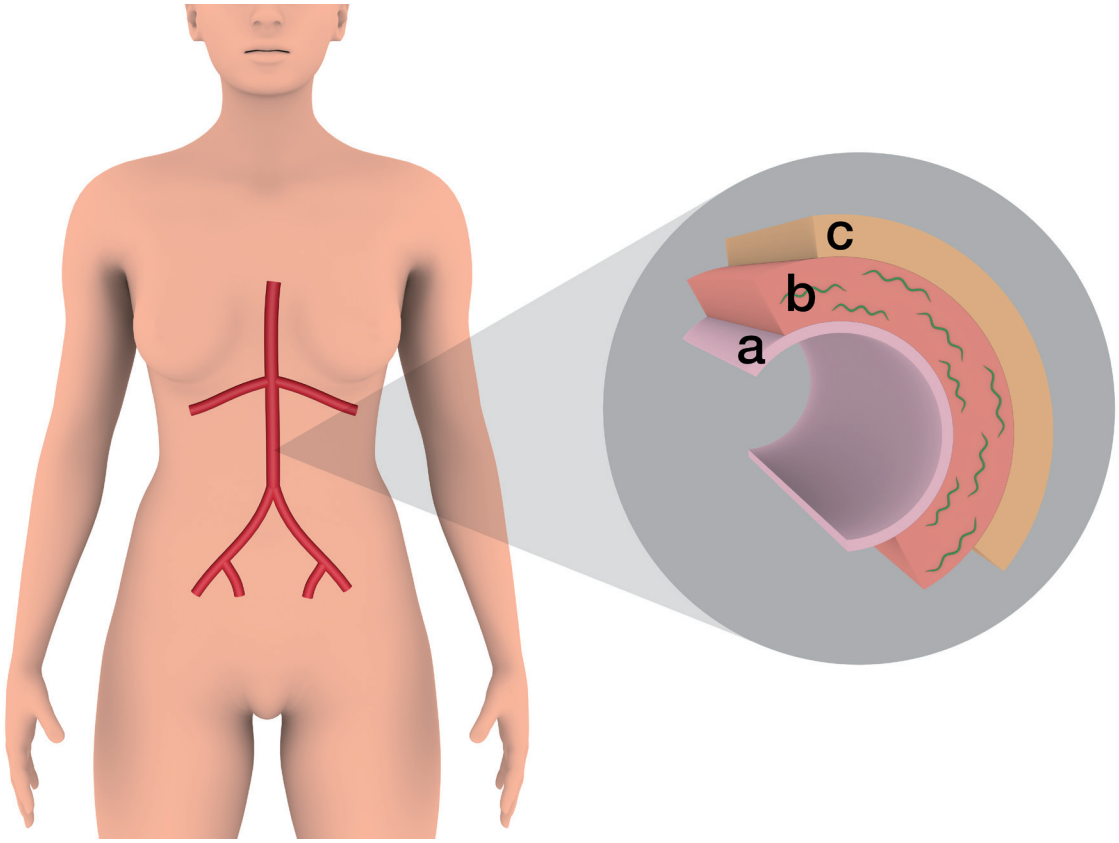


Figure II. *Aorta and magnification of the three layers of the aortic wall: a) tunica intima, b) tunica media and c) tunica adventitia.*

Collagen

The strength of the aortic wall can be ascribed to collagen. Figure III a.(17, 24) Collagen type I and III are the predominant collagens of the aorta.(25) The biosynthesis of the unique molecular structure of collagen is an intricate process with several critical steps of modification, to ensure its mechanical stability.(26, 27) The triple helix is the fundamental unit of the collagen fibre. It is composed of three polypeptide chains, linked toghether by disulfid bonds in the endoplasmatic reticulum (ER).(24) Besides the solid stucture of the collagen triple helix, the stability of collagen is dependent on cross-linking, which bind adjacent collagen molecules to one another. The collagen cross-linking is inititated in the ER by lysyl hydroxylase (LH), also referred to as: procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD). LH hydroxylates lysine side chains in the polypeptide chain.(28) In the extracellular space, triple helices cross-link to one another, partly by LOX and partly by self-assembly.(29) The mature collagen cross-linking ultimatelly formed are hydroxylysyl pyrdinoline (HP) and lysyl pyridinoline (LP).(30)

Vascular smooth muscle cells

VSMC is the main celltype of the tunica media. Figure III e. The principal function of mature VSMCs is to control vessel tone and diameter through the contractile proteins that they express.(31) VSMCs also have an important role in synthetizising and repairing the structural elements of the extracellular matrix (ECM), such as elastin and collagen.(32-35) It is the

remarkable placticity of VSMCs that makes them capable of switching phenotype, from contractile to synthetic. By phenotype modification VSMCs can adapt to different stages of development and environmental changes.(31)

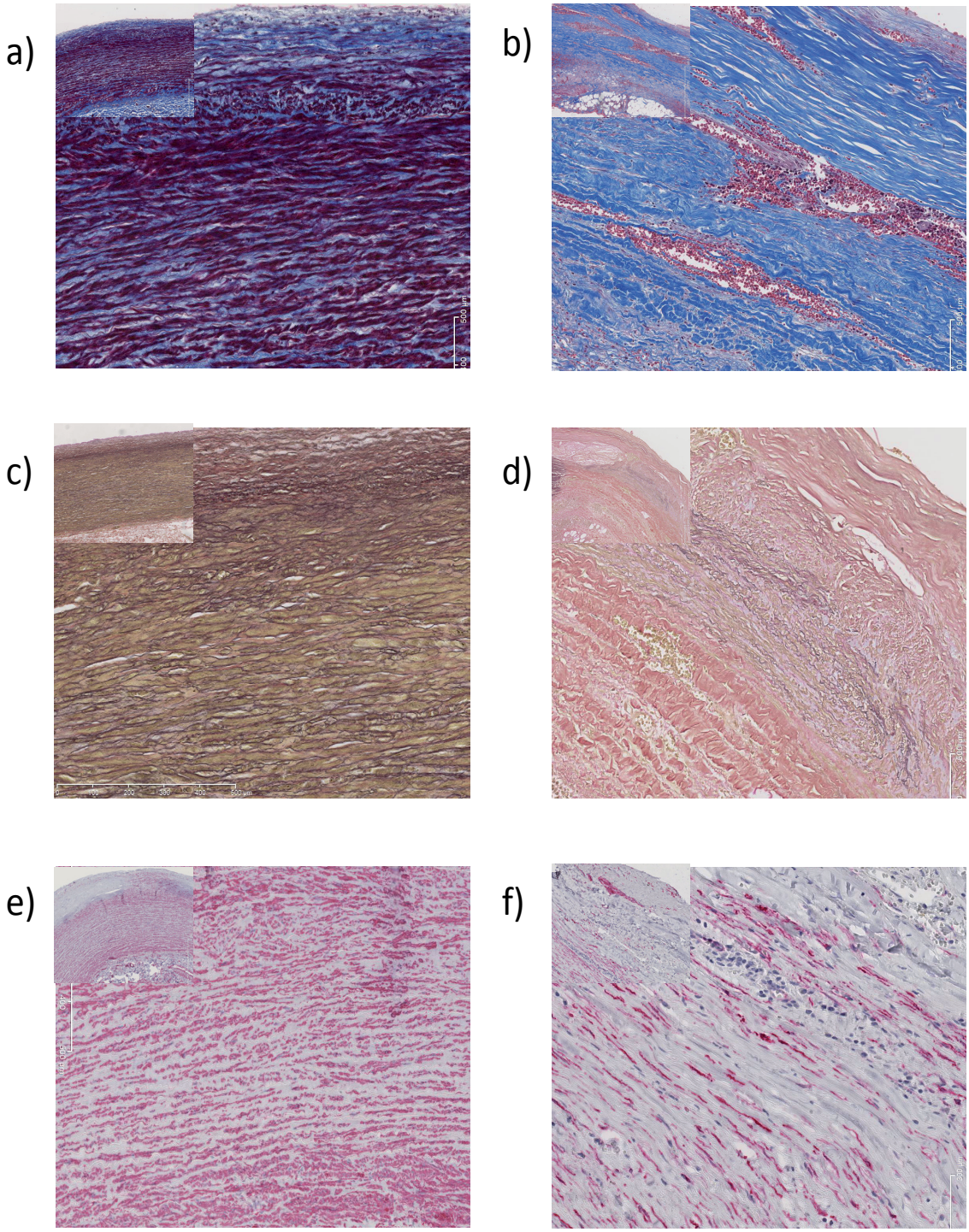


Figure III. *a) Masson trichome staining of healthy aortic wall and b) aneurysm wall. c) Weigert's- van Gieson's staining of healthy aortic wall and d) aneurysm wall. e) Staining of smooth muscle α-actin in healthy aortic wall and f) aneurysm wall.*
Masson trichome staining: muscle fibres turned red and collagen turn blue. Weigert's- van Gieson's staining: elastic fibres turn dark purple, muscle turn yellow, nuclei turn brown and connective tissue turn red. Smooth muscle α-actin staining: smooth muscle α-actin turn red.

Development of AAA

Pathogenesis

AAA develops due to a weakening of the aortic wall, resulting in the characteristic dilatation and elongation of the aorta. The mechanisms by which aneurysms are initiated are poorly understood. Present knowledge suggests inflammation to play a pivotal role, yet it remains unknown if the inflammatory process is in fact the triggering event.(36-39)

The aortic wall is weakened by proteolytic enzymes, which have the capacity to degrade the structural elements of the ECM.(40-42) One of the hallmarks of AAA is the fragmentation of elastin, causing the initial dilatation in AAA formation.(43) In the absence of elastin the strength of the vessel wall resides in collagen, with its ultimate load-bearing capacity.(43, 44) It is the continued deterioration of collagen and ultimately its failure in sustaining the pulsatile pressure, that results in aneurysm rupture.(43) Another important element in AAA pathogenesis is the loss of VSMCs, by apoptosis.(45, 46) With the depletion of VSMCs the vessel wall loses its principal cell type involved in maintaining vessel wall integrity.(17, 47)

The proteolytic enzymes involved in aneurysm formation are produced by infiltrating inflammatory cells and modified VSMCs, in the tunica media and adventitia.(36, 48, 49) The protoelytic enzymes most associated with AAA development are members of the family of matrix metalloproteinases (MMP).(50)

Biomarkers for AAA

The identification of biomarkers for AAA could be of importance both as a diagnostic tool to differ healthy from diseased but also to single out AAAs at risk of growth and rupture. All molecules involved in the degenerative process are hypothetical candidates in the pursuit of finding biomarkers for AAA.(51) There are today, despite numerous studies, no universal biomarkers for AAA, illustrating the complexity of the pathogenic processes involved in AAA development.(51)

The intraluminal thrombus

An intraluminal thrombus (ILT) is present in most AAAs.(52, 53) The ILT is a bioactive fibrin clot with intercalated hematopoietic cells and inflammatory cells, and it arises as a result of altered blood flow in the dilated aorta.(54) The ILT, was initially perceived as a by-product of the aneurysm disease, but at present contradictory results are reported regarding the effect of the ILT on aneurysm progression.(55) On one hand, the ILT has been shown to promote the degradation of the underlying aneurysm wall and thereby contribute to aneurysm progression, whereas other studies suggest the ILT to mediate a protective effect on aneurysm wall stress.(53, 56-61)

Risk factors for AAA

Although the pathogenesis of AAA is still unknown there are some important risk factors associated with its development and progression:

Age is an important risk factor for both development of AAA and aneurysm rupture risk.(62, 63) AAA primarily affects an elderly population and is rare beneath 50 years of age.(64)

Male gender is strongly associated with AAA development, with a male:female prevalence ratio of 4-6:1.(63, 64) Wherein the risk of being male lies is not fully understood but it has been associated with differences in the endogenous production of sex hormones, anatomy and behavioural health in men compared with women.(65, 66)

Family history of AAA increases the risk of AAA formation. The genetic influence is illustrated by the higher prevalence in siblings to patients with AAA compared with that in the general population.(67, 68) In a first-degree relative to a patient with AAA, the relative risk of developing an AAA is approximately doubled compared with that in the general population.(68)

Smoking is an important risk factor for AAA and is the only modifiable one.(7, 69) Besides being linked to its formation, smoking has been shown to increase the growth and rupture rate of AAAs.(62) The mechanism by which smoking promote AAA formation is not fully understood but animal models suggest an effect of nicotine on elastin degradation, expressions of MMPs and inflammatory activity.(70-72)

Hypertension and cardiovascular disease are strongly associated with AAA.(7, 64) Atherosclerotic disease, i.e. cerebrovascular, coronary artery and peripheral artery disease (PAD) have all been observed in a greater extent in patients with AAA.(7) On the other hand, diabetes mellitus (DM), another important risk factor for atherosclerotic disease, has been negatively associated with AAA occurrence.(7, 73)

Gender aspects on presentation and progression of AAA

Prevalence and screening

AAA predominantly affects an elderly male population.(63) When using the threshold of 3.0 cm for defining an AAA, the prevalence in an elderly Swedish population is approximately 0.5% in women and 2% in men.(74, 75) Men are recommended screening for AAA at the age of 65 years, according to several guidelines.(76) The screening recommendations for women are more controversial.(76) Some guidelines do not recommend screening in women, probably due to the low prevalence rate and the late development of disease, which limit the number of preventable deaths with a screening a programme.(77, 78) Other guidelines however, do recommend screening of women with risk factors, such as smoking, cardiovascular disease (CVD) and familial history of AAA.(79, 80)

Anatomy and aneurysm wall strength

The diameter of the aorta correlates with height, weight and body surface area (BSA). Women are in general smaller than men and consequently they have smaller vessels.(81, 82) The proportionally smaller aortas in women have implications for aneurysm progression. Because women's aortas are smaller than men's, the relative enlargement of women's AAA exceed that of men's, at any given diameter.(83, 84) Aortic size index (ASI) is a measurement, which takes the relative enlargement into account. It has shown to be a determinant for rupture risk in women.(85) Thereto, women have smaller aneurysm neck diameter, shorter neck length, increased neck angulation and smaller iliac arteries.(81, 86) These anatomical components are all of importance for the choice of surgical treatment.(81)

In finite element models, aimed to assess aneurysm wall strength, women have been reported to have a lower aneurysm wall strength compared with men.(87, 88) The lower wall strength in women has been associated with the increased rupture risk in women and as suggested, could be related to differences in biomechanical properties in the aneurysm wall between men and women.(87, 88)

Risk factor - smoking

The decline of male smoking is believed to have contributed to the decline in AAA prevalence observed in the 21th century, yet smoking trends differ in women.(89, 90) The incidence of smoking in women is increasing in several countries and fewer women than men quit smoking. (90-92) Thereto the effects of smoking in women may be more detrimental, illustrated by the higher risk for AAA in smoking women compared with smoking men.(69, 93, 94)

Growth rate

The aneurysm growth rate varies widely in different reports (1 mm /year - 8 mm/year).(95, 96) A mean aneurysm growth rate of 2.2 mm/year, independent of gender, was reported in a recent meta-analysis.(62)

Different factors have been shown to influence aneurysm growth rate, such as aneurysm size, smoking and more controversial, intraluminal thrombus and female sex (62, 97-100). A few studies have reported a greater expansion rate in women.(97, 101)

Rupture risk

A higher proportion of women with AAA presents with rupture, 21% of women to compare with 16% of men.(102) Women with AAA have a fourfold risk of rupture compared to men and it occurs much earlier in the course of the disease.(62, 95) The average diameter preceding rupture is 6.0 cm in men while it is 5.0 cm in women.(95) Potential explanations are, as mentioned above, a greater relative aneurysm enlargement in women and/or a reduced wall strength in the aneurysm wall of women.(83, 87, 88)

Concurrent aneurysms

A higher proportion of women have concurrent thoracic aortic aneurysms (TAAs) but rarely femoral and popliteal artery aneurysms (PAAs), which are almost exclusively observed in men. (103, 104)

Biomarkers

An ideal biomarker is involved in a pathogenic pathway prevalent in all affected patients. The difference in prevalence and progression of AAA in women suggest that the pathogenic pathways might differ in some aspects between the sexes.(7, 62) Despite these observed gender differences, the studies performed on potential biomarkers for AAA are all primarily based on male cohorts.(51, 62) Biomarkers for AAA in women have not been studied.

Gender differences in treatment of AAA

Intact AAA

The medical treatment prescribed to patients with AAA is similar to other patients with cardiovascular risk factors, i.e. antiplatelet treatment and statins.(16, 79) The current guidelines recommend surgical treatment in men at an aneurysm diameter of 5.5 cm.(16, 79) For women the threshold is set lower since they, due to their increased rupture rate, might benefit from early repair, i.e. at an aneurysm diameter of 5.0 - 5.4 cm.(16, 79) The extent of secondary prevention, prescription of aspirin and statins, has been shown inadequate for both men and women, yet more pronounced in women. Women are less likely to be prescribed aspirin as secondary prevention, compared with men.(105)

Two vascular interventions are presently used to prevent sudden death from aneurysm rupture in patients with AAA; open repair (OR) and Endovascular aneurysm repair (EVAR). EVAR is a less invasive treatment, enabling treatment without the risks associated with open surgery. However, it cannot be used in all patients due to aneurysm morphology.(106-110) An increasing number of patients with AAA are treated with EVAR rather than OR, but this trend is predominantly found in men.(102, 111, 112) One potential explanation for the lower number of women receiving EVAR might be a less favourable morphology. The smaller diameter of the aneurysm neck, shorter aneurysm neck length, increased aneurysm neck angulation and smaller iliac arteries in women most likely contribute to fewer women meeting the Instructions for Use (IFU) criterions for the devices used in EVAR.(81) It was recently reported that only 12% of women receiving EVAR meet the criterions for length, diameter and angulation of the aneurysm neck.(81) Thereto, women are more often treated outside IFU criterions.(113)

Outcome after surgery of intact AAA

Several randomized controlled trials (RCT) have been performed in order to identify the optimal treatment alternative for patients with AAA.(106-110) The limited amount of women in these trials makes it difficult to generalize the findings of these RCTs to female patients. In registry studies including women, a worse outcome after surgery have been reported in women compared with men, regardless of surgical treatment.(102, 114)

Women treated electively with EVAR suffer higher 30-day mortality and more postoperative complications compared with men.(114, 115) The more complex vascular anatomy in women render them less favourable for EVAR and may contribute to the greater occurrence of procedural-related complications.(111) Women treated with EVAR have an estimated four times higher rate of complications compared with men.(116)

Women treated electively with OR suffer higher 30-day mortality, more postoperative-complications and have a worse long-term outcome.(114, 117) Women suffer from cardiac and pulmonary complications as well as renal failure to a greater extent compared with men, leading to longer stays in the Intensive care unit (ICU).(111, 117) Proposed explanations to the worse outcome and greater degree of complications in women are their older age at treatment and more comorbid conditions.(111)

Ruptured AAA

Women who present with rupture are older than men.(118, 119) Women's AAA rupture at smaller diameter and more women present with aneurysm rupture compared with men, yet women are less likely to be admitted to hospital and receive treatment.(102, 111, 120, 121)

Outcome after surgery of ruptured AAA

Even though outcome results for women treated for ruptured AAA have varied the majority have reported women to fare worse.(102, 118-120, 122) Women with AAA are more likely to die from their ruptured AAA, as illustrated by the higher 30-days case fatality rate in women compared with men.(102, 118, 122)

The effect of gender on aneurysm formation

The gender differences observed in CVD, being more prevalent in men than in women and with an increasing incidence in women after menopause, point towards an effect of sex and sex hormones on CVD pathogenesis.(123, 124) Estrogen has been shown to have an antiatherogenic effect, which is mediated both directly and through the actions of the estrogen receptors, estrogen receptor α (ER α) and estrogen receptor β (ER β).(125) However, the antiatherogenic effects of exogenous estrogen and progesterone are not universal, as has been illustrated by the adverse events observed in the Women's Health Initiative (WHI) trials.

Women in the treatment group, receiving hormone therapy, suffered more cardiovascular events compared with the placebo group.(126)

A similar pattern of gender disparity is observed in AAA, predominantly affecting men and developing later in women, which suggest gender to be of importance for AAA formation. The effect of gender on AAA development has been demonstrated in several animal models.

In the beginning of the 21 century, estradiol treatment was reported to limit aneurysm development in an angiotensin-II-induced aneurysm model on apolipoprotein E-deficient mice.(127) In an elastase perfusion model, male rats developed larger AAA than females, associated with increased macrophage infiltration and higher levels of MMP-9. It was also shown that female rat aortas, when transplanted into males, became more susceptible to aneurysm development.(128) A protective effect of estrogen was further illustrated by the observed increased growth rate of AAA in ovariectomized female rats, compared with sham-operated females and male rats that received estradiol pellets. The growth rate was associated with increased levels of MMP-2 and -9.(129) The protective effect of estrogen was in female mice associated with an increased protein and mRNA expression of ERα compared with males.(130)

An effect of androgens on AAA development was observed in an angiotensin-II-induced aneurysm model on apolipoprotein E-deficient mice, where orchiectomy was shown to decrease aneurysm growth.(131, 132) In an attempt to elucidate the effect of both androgens and estrogens on aneurysm formation, rats were orchidectomized and oophorectomized. As a result, lower aneurysm growth rates were observed in orchidectomized males compared to male controls, whereas oophorectomy did not affect growth rates in females.(133) However, in the same study oophorectomized female rats given estrogen exhibited decreased aneurysm growth, which was instead increased in male rats that underwent orchiectomy and received testosterone pellets.(133) Most animal models illustrate a preventive effect of estrogen on aneurysm formation by inhibiting the production of proteolytic enzymes involved in the degradation of the ECM. The role of androgens has been less investigated and consequently its potential role in AAA formation is less elucidated.

The scarce numbers of studies focused on the potential effect of sex hormones on AAA development in men and women are not without discrepancy. In women, the association between HRT and aneurysm occurrence is controversial.(73, 134, 135) A negative association between AAA occurrence and hormone therapy for more than five years in women has been observed.(73) Although, two other studies, partly looking at the same study population as the latter study found no association between HRT and even more AAA events in women with AAA than controls, respectively.(135, 136)

In men, lower levels of testosterone have been associated with AAA.(137) Noteworthy, elderly men without AAA have higher levels of circulating estradiol compared with women in the same age. As opposed to women, in whom the production of estrogen ceases in menopause, the levels in men do not decrease with age due to peripheral aromatization and continued synthesis in the testicles.(138, 139)

Aims of the thesis

The overall aim of this thesis was to study women with AAA

The specific aims were:

STUDY I. To evaluate the reproductive history in women with AAA in comparison to women with an expected similar risk factor profile and secondary to study if women with larger AAA differ in their reproductive history from women with smaller AAA.

STUDY II. To investigate if potential biomarkers for AAAs differ between men and women with equally large AAAs and to compare biomarker levels in women and without AAA.

STUDY III. To compare elastin content and some of the elastolytic enzymes mostly associated with AAA development, between men and women with equally large AAA.

STUDY IV. To study collagen and collagen cross-linking in the aneurysm wall of men and women with AAA.

STUDY V. To investigate the degree of apoptosis and inflammation, in relation to VSMC content in the aneurysm wall of men and women.

Patients and methods

Study cohort

Study I. Female patients treated for AAA or PAD consecutively monitored at the Department of Vascular Surgery at Karolinska University Hospital, between 2007 and 2010 were included. The choice of control group, women with PAD, was based on their expected similar risk factor profile for atherosclerotic disease.(7, 140) An a priori power analysis was performed to detect the minimum number of participants required to detect a significant difference of 2 years in menopausal age, at a power of 80%. An additional number of patients were added, based on the assumption of a missing case frequency of at least 40%, resulting in a number of 140 patients in each group.

Study II. Women treated for AAA and women consecutively controlled for AAA at the outpatient clinic during the time period January 2009 to October 2011, were invited to participate. The participating men were included prior to their elective aneurysm repair. A control group of age-matched women with PAD, consecutively controlled for their condition at the outpatient clinic during the same time period, was also included. The threshold for large AAA was set at 5.5 cm, in accordance with practice guidelines for considering treatment. (79) This rendered 4 groups; women with PAD (n=18), women with AAA < 5.5 cm (n=16), women with AAA > 5.5 cm (n=20) and men with AAA > 5.5 cm (n=18).

Plasma samples were collected from the participating women at a scheduled appointment. The aneurysm diameter of the patients with AAA ≥ 5.5 cm had been measured by computer tomography (CT), within the last 6 months. The aortic diameter of patients with AAA < 5.5 cm had been measured with either ultrasound or CT no later than 12 months earlier. The age-matched control group of women with PAD underwent an ultrasound examination, where the maximal aortic antero-posterior diameter was measured. Aortic diameter above or equal to 2.7 cm was considered aneurysmal.(141) Data from patients' hospital charts were extracted regarding risk factors and comorbid conditions.

Study III-V. In 2008, the Department of Vascular Surgery, Karolinska University Hospital, initiated a bio bank of tissue obtained during elective OR. By late February 2012 the number of women amounted to 14 and in June 2014 to 19. All treated women were included together with male patients chosen to match the age and aneurysm diameter of the participating women.

The biopsies were obtained from the ventral infrarenal aneurysm wall, often in midline and at the maximum diameter. If possible, two biopsies were taken from each patient, thrombus covered and non-thrombus covered aneurysm wall. The thrombus was removed and stored separately. The samples were stored at -80°C until further preparation. Patient characteristics were obtained from hospital charts.

Questionnaire

To access information of the general health and the reproductive history of the participating women in Study I, a validated questionnaire was used.(142) The self-completion questionnaires, including 45 questions, were delivered and returned through post. The reliability of the questionnaire was confirmed by comparing 20% of the answered questionnaires, about general health, to data from the women's hospital charts. The validity of the questionnaire had been established by its use in a prior study of reproductive history in women with lower limb ischemia in 2004.(142)

Laboratory procedures

ELISA. For the detection and quantification of biomarkers in plasma samples Enzyme-linked immunoassay (ELISA) was used. ELISA is a method based on the concept of a stationary antibody onto which a sample of unidentified amounts of target antigen is added, i.e. a pre-coated microplate. A secondary antibody, targeting the antigen, is then added followed by a substrate solution. As these components react colour is formed, which can be measured with a spectrophotometer.(143) ELISA was used in Study II.

Immunohistochemistry. To detect proteins and identify their location in the aneurysmal wall immunohistochemistry was performed. Immunohistochemistry is a technique used to detect proteins in a section. A primary antibody, designed to attach to a specific antigen in the tissue, is added to the tissue section. The section is then incubated with a biotin-free combination of a probe and polymer, which detect the primary antibody and probe, respectively. The polymer is labelled with alkaline phosphatase (AP). The probe and polymer form a complex that when adding chromogen, give rise to a red colour signal. The signal is the result of a reaction between the chromogen and the AP of the probe-polymer complex.(144-146)

Terminal deoxynucleotidyl transferase mediated 2'-deoxyuridine 5'-Triphosphate nick-end labeling (TUNEL) staining detects apoptotic cell death in tissues. The technique is designed to identify the oligonucleosome fragments in the apoptotic, disintegrated nucleus.(147) Terminal doexynucleotidyl transferase catalyses the polymerization of free 3'-OH DNA ends and labelled nucleotides. The complex can be visualised using the AP-labelled conjugate and chromogen described above.(148)

Enzyme labels for immunohistochemistry was used in Study III-V. All sections were paraffin embedded and prepared by vacuum steaming. TUNEL staining was used in Study V. The sections were pre-treated with Proteinase K. The investigator was blinded for sex when valuing the staining, using ImageJ analysis software, National Institutes of Health (NIH).

mRNA expression analysis. Real-time quantitative reverse transcription polymerase chain reaction (PCR) was used to quantify the expression of mRNA in the non-thrombus and thrombus covered mediae of the aneurysm wall of men and women. The principle of the

technique is to firstly convert RNA to a DNA by reverse transcriptase and secondly to amplify a precise DNA fragment and quantify its expression. The amplification is regulated by temperature and is a result of repeated cycles of DNA denaturation, annealing and synthesis of DNA strands. Fluorescent markers bind to the DNA-strands and when they are degraded in the DNA synthesis they omit a signal. The omitted fluorescent signaling can be summarised and thereby quantified. In quantification, the emissions of the samples are compared to a threshold value, set at a given cycle, called the threshold cycle (C_t) of the amplification. For normalization the C_t value for the gene of interest is compared to the C_t value of a housekeeping gene that is expressed at a constant level.(149)

mRNA expression analysis by real-time reverse transcription PCR was used in Study III-V. The housekeeping gene Ribosomal Protein Large P0 (RPLP0) was used for normalization and samples were run in duplicates.

Western blot. Western blot analysis was used to quantify protein expression in the thrombus and non-thrombus covered media of the aneurysms. Western blotting is a technique used for detection and analysis of individual proteins. Tissue samples are prepared by protein extraction and the proteins are separated from one another by gel electrophoresis. By electroblotting the separated proteins are transferred to a membrane of polyvinylidene fluoride (PVDF). Unspecific binding of antibodies to the surface of the membrane is prevented by incubation with a buffer and protein mixture. Thereafter, the membrane is incubated overnight, with an enzyme-labelled antibody, i.e. the primary antibody. The primary antibody complex binds to the transfected protein of interest. A secondary antibody, binding to the first antibody, is then added before the membrane is incubated with a chemiluminescent substrate. Together they form light as an end product. The light product can be captured on a film or with the use of a CCD camera. The intensity of the signal correlates with the abundance of the antigen on the blotting membrane.(150)

Western blot analysis was performed in Study III-V. Tris 4-12% SDS-PAGE gels were used and densitometry was performed to value the protein expressions.

High performance liquid chromatography. High performance liquid chromatography (HPLC) was used for quantification of collagen and its cross-linking compounds in the thrombus covered aneurysm wall of men and women. HPLC is a separation technique where the amount of the separated compounds ultimately can be measured.(30) The sample of interest is injected with a liquid of water and organic solvent, mobile phase, into a tube packed with small porous non-polar particles, called the stationary phase. High pressure, administered by a pump, forces the liquid of the mobile phase including the sample of interest through the stationary phase. The packing of the stationary phase and the chemical and physical interactions, occurring between the molecules of the sample and stationary phase separate the compounds from one another. These separated components are detected at the exit of the tube by a fluorescence detector connected to a computer, that measures their amount. The separation mode using water and organic solvent as liquid phase and non-polar particles in the stationary phase is called reverse-phased HPLC.

HPLC was used in Study IV and the analysis was performed in Leiden, Netherlands.

Statistical analysis

The independent sample t-test was applied for comparisons between groups with normally distributed, continuous variables and Mann-Whitney or Kruskal-Wallis test were applied for comparisons between groups where the data that did not fulfil the assumption of normality. Differences for categorical variables were assessed with $\chi 2$ test and Fischer's exact test. Differences were considered significant at the level $<.05$. SPSS software 20 and 21 was used for the statistical analysis.

Results and discussion

Reproductive history in women with AAA

The difference in prevalence rate and later development of disease in women suggest gender and sex hormones to be of importance for AAA development.(7, 63)

The main result of Study I was the two years lower mean menopausal age in women with large aneurysms compared with women with PAD and women with small AAA. Table I. As a result, the duration of endogenous production of female sex hormones (years between menarche and menopause) was lower in women with AAA ≥5.0 cm than in women with AAA <5.0 cm or PAD. Table I. There were no differences between the groups in relation to the use of contraceptives, parity, HRT, prior gynecological surgery or breast cancer.

Regarding risk factor for AAA, i.e. hypertension, hyperlipidemia, heredity for AAA and CVD, the two groups of women were in many aspects similar, with one exception. Angina pectoris, one of many manifestations of CVD differed between women with AAA and women with PAD. The occurrence of angina pectoris was greater in women with AAA compared with women with PAD, 26% vs.11%, P=.026. These results are in accordance with the prior studies on risk factors for AAA in both men and women.(7) DM has been negatively associated with AAA in women.(73) The results of this study are in accordance with these observations, illustrated by the lower occurrence of DM in women with AAA compared with women with PAD, 15% vs. 28%, P=.034.

Table I. Reproductive history in women with PAD, AAA < 5.0cm or AAA ≥ 5.0cm.

	PAD N=98	AAA<5.0cm n=44	AAA≥ 5.0cm n=54	P-value
Menarcheal age (range)	13,5 (10-17)	13,5 (10-16)	13,8 (11-18)	.597
Menopausal age (range)	49,7 (31-67)	49,9 (30-57)	47,7 (39-64)	.011
Duration of endogenous hormone production (range)	36,7 (19-54)	36,4 (20-45)	33,9 (24-49)	.003

Data presented as mean (range). Significance calculated with Independent t-test.

Smoking has been shown more deleterious to AAA formation in women compared with men, in that sense that there is a higher risk of AAA development in smoking women.(69, 93) In this study women with AAA were previous or current smokers to a greater extent than women with PAD: previous smokers, 52% vs. 46% and current smokers, 46% vs. 34%, P= .001.

One way by which smoking affects women is by reducing the menopausal age.(151) The mean age for when women in Sweden reach natural menopause is at 51 years of age.(152) Smoking reduces the menopausal age by approximately 1.44 years, possibly by affecting ovarian function and thereby lowering estrogen levels.(151, 153, 154) Other factors known to influence menopausal age are: BMI, parity and menarcheal age.(154) A menopausal age of approximately 49.8 years in both women with PAD and women with smaller AAA, as observed in this study, is similar to the observed menopausal age of female smokers in Sweden.(154) The smoking habits in women with larger and smaller AAA were similar, as were BMI, parity and menarcheal age. Consequently, the observed lower menopausal age in women with larger AAA could not, in this study, be associated with any of the known determinants for menopausal age.

The effect of female sex hormones on AAA development has been illustrated most distinctly in animal models, by an inhibiting effect of estrogen on the protoelytic enzymes involved in AAA pathogenesis.(128, 133) The mechanisms observed in animal models have their limitations. There are apparent difficulties in mimicking a multifactorial and complex disease, such as AAA, in an animal model. In order to value the potential effect of gender on AAA development in humans, studies on how hormonal changes in men and women are related to AAA occurrence are an important complement to the mechanisms studied in animal models.

Hypothetically, the susceptibility of aneurysm formation in women might be associated with an altered hormone profile as has been observed for CVD. Women with early artificial menopause and thereby lower menopausal age have an increased risk for CVD, compared with women with natural menopause.(155, 156)

If a lower menopausal age influences AAA formation one could hypothetically argue for hormone therapy to postpone menopause and thereby prevent AAA development. However, based on the results of the WHI trials, where postponing menopause with hormone therapy failed to prevent coronary heart disease and potentially even increased its risk, prescribing hormone therapy to women in order to prevent AAA would not be safe.(157)

In conclusion, women with larger AAA reach menopause earlier than women with smaller AAA and women PAD and this could influence an earlier onset of aneurysmatic disease or an increase in aneurysm growth.

Methodological considerations

One of the strengths of a self-completion questionnaire with several closed-ended questions is the exclusion of interviewer bias. A weakness on the other hand is an assumption of literacy and comprehension of the Swedish language. Another limitation is the potential of recall bias, since several years had passed since some of the events asked about took place, but it is unlikely that such a recall bias would influence the groups differently. The questionnaire had been validated and its reliability confirmed by an earlier study and by comparing the provided data to the patients' hospital charts.(142) In retrospect the use of a pre-testing and conformational interviews could have improved the study.(142)

The distinction of large and small aneurysms at 5.0 cm could be questioned but was chosen since 5.0 cm is the recommended diameter, according to current guidelines, for considering treatment in women.(79)

Biomarkers for AAA in women

The lack of studies on biomarker for AAA in women initiated this study.(51) Study II is the first one to describe biomarker levels for AAA in women. The results showed, in many aspects, similar biomarker profiles in men and women, with the exception of MMP-9. Women with AAAs ≥ 5.5 cm had higher levels of MMP-9 compared with men with AAAs ≥ 5.5 cm, 43 ηg/ml vs. 36 ηg/ml, P=.036. Figure IV. On the other hand, women with larger AAA ≥ 5.5 cm had lower levels of MMP-9 compared with women with AAA < 5.5 cm and women with no AAA, 43 ηg/ml vs. 117 ηg/mL vs. 133 ηg/mL, P =.003. Figure IV. MMP-9 is the biomarker most associated with AAA and has been implicated in AAA formation, growth and rupture. (158-160) The finding of lower levels of MMP-9 in women with larger AAA compared with women with smaller AAA and women without AAA could theoretically be explained by a lower production or a greater consumption in the aneurysm wall of women. In either way the difference in MMP-9 concentration suggests that the pathogenic pathway involving MMP-9 differs in men and women with AAA.

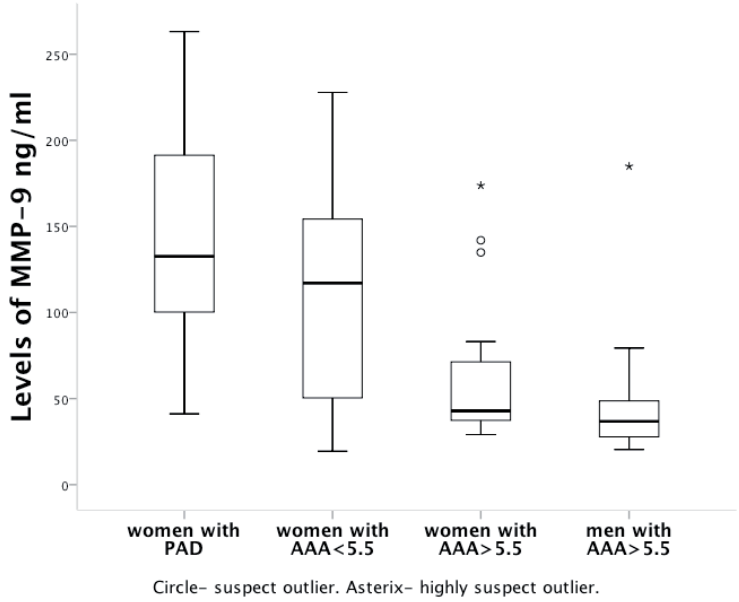


Figure IV. *Circulating levels of MMP-9 in women with PAD, AAA<5.5cm and women with AAA≥5.5cm.*

Prior studies on biomarkers for AAA present different results.(51) It might be related to the difficulty in identifying a biomarker for such a complex disease as AAA but it might also be related to the inconsistencies in used methodology. It is hard to interpret the results of studies, which use different media, forms of proteins, controls and aneurysm size.(159, 161-164)

The results of animal models illustrating a protective effect of estrogen on aneurysm formation could, if applied to men and women, suggest a reduced female sex hormone production to be contributing to AAA pathogenesis.(128, 129) In Study II, it was evaluated by measurements of estrogen levels in women with and without AAA, as well as in men with AAA. The results showed higher levels of estradiol in men with AAAs ≥ 5.5 cm compared with women with AAAs ≥ 5.5 cm, 87 ρmol/l vs. 30 ρmol/l, P<.001. The observed lower level of estradiol in women compared with men is consistent with prior studies.(138, 139) The higher levels of estradiol in men compared with women and the similar levels in women with and without AAA render estradiol a less suitable biomarker for AAA in women. If estrogen has a protective effect on aneurysm formation and the development of AAA in women is in fact related to its loss, can be neither confirmed nor denied with this one-time analysis of estradiol.

In conclusion, the higher levels of MMP-9 in women compared with men suggest that MMP-9 could be a biomarker related to sex differences in AAA development. The lower levels of estradiol in women with AAA compared with men suggest that the possible protective effect of endogenous estrogen cannot be explained by a difference in circulating levels of estradiol between the sexes. The results illustrate the need for further in-depth analyses of true biological sex differences in AAA patients.

Methodological considerations

The study included a control cohort of patients with PAD. It excluded the potential confounding factor of elevated levels as a result of occlusive disease, yet with a group of healthy controls even more information could have been obtained. Another group of patients, men with smaller AAA, could have further strengthened the study. Retrospectively, the choice of some of the biomarkers used in this study can be questioned.(158, 165) The study could have benefited from the analysis of additional sex hormones, for instance: testosterone, estrone and sex hormone-binding-globulin (SHBG), thereby providing a more complete hormone profile of the participating men and women.

The distinction of large and small aneurysms, which was set at 5.5 cm, was chosen based on the treatment recommendations for men with AAA.(79) We do recognise that the threshold for recommending treatment to women is lower, yet in order to avoid making comparisons between different sized aneurysms the threshold diameter was set based on the treatment recommendations for men.(79)

Elastin and elastolysis in the aneurysm wall

The loss of elastin is one of the hallmarks of AAA development.(18) There is scarce knowledge of the elastolytic process and elastin degradation in the aneurysm wall of women, which might be of importance for the observed gender differences in aneurysm progression. (97, 166, 167)

In Study III, a lower protein expression of elastin in women compared with men, in the non-thrombus covered aneurysm wall, was observed. Figure V. There was no difference in mRNA expression of elastin between men and women. Table II. We found no difference in the expression of MMP-9 between men and women in the thrombus covered aneurysm wall.

The amount of elastin in the aneurysm wall of men and women have been studied recently, by Tong et al, reporting a greater dry weight of elastin in women than in men in the thrombus covered aneurysm wall.(167) The contradicting findings in this study might be related to the use of different methods or differences in patient demographics, i.e. smoking habits, age and aortic diameter between the participating men and women.(167) The men were older in our study and thoroughly matched to the participating women in relation to age, aneurysm diameter and smoking habits.

Table II. Gene expression analysis of elastin, MMP-2, -9 and cathepsin K in the non-thrombus covered aneurysmal wall of men and women.

	Non-thrombus covered wall		P-value
	Men with AAA	Women with AAA	
elastin	4.55 ± 3.21	5.01 ± 3.31	.722
MMP-2	1.52 ± 1.22	1.74 ± 1.51	.744
MMP-9	-.83 ± 1.08	.90 ± 2.59	.041
cathepsin K	1.68 ± 1.06	2.63 ± 1.01	.063

Values presented as mean±SD for normally distributed data. Significance calculated by Independent t-test. Values presented log2-transformed and as arbitrary units. MMP- matrix metalloproteinase.

MMP-2 and -9 are the proteolytic enzymes most associated with AAA.(42, 49, 160, 168, 169) MMP-2 has primarily been associated with early aneurysm formation whereas MMP-9 has been related to later stages of aneurysm disease.(36) MMP-2 and -9 have both elastolytic and collagenolytic qualities.(170, 171) We found higher mRNA and protein expressions of MMP-9 in women compared with men, in the non-thrombus covered aneurysm wall. Table II and Figure V. There were no differences in mRNA expressions of MMP-2 and cathepsin K between men and women. Table II. There were neither differences in the mRNA expressions of elastin, MMP-2, -9 and cathepsin K between prior and current smokers, nor between smoking and non-smoking men and women.

The differences in elastin and elastolytic enzymes were observed in the non-thrombus covered aneurysm wall, as opposed to the thrombus covered wall. The impact of a difference in the non-thrombus covered aneurysm wall as opposed to the more prevalent thrombus covered aneurysm wall is unknown and requires further studies.

Based on a report from two decades ago, the degradation of elastin leads to moderate dilatation. (18) Theoretically, if applied to the results of this study, it could suggest the lower elastin content in women to have implications on aneurysm enlargement. The observed similar ASI between men and women in this study suggest that the lower expression of elastin and higher

expression of MMP-9 cannot be explained by proportionally larger AAAs in women. It rather points towards inherent differences in the aneurysm wall between the sexes. The studies on a potential effect of sex hormones on the composition of elastin in the vasculature are scarce.

Two of these in number few studies, have suggested an impact of female sex hormones on elastin composition. One study reported that female sex hormones increase the elastin deposition of human aortic VSMCs and another that gender could be of importance for the observed greater aortic stiffness in elderly men compared with elderly women.(173, 174)

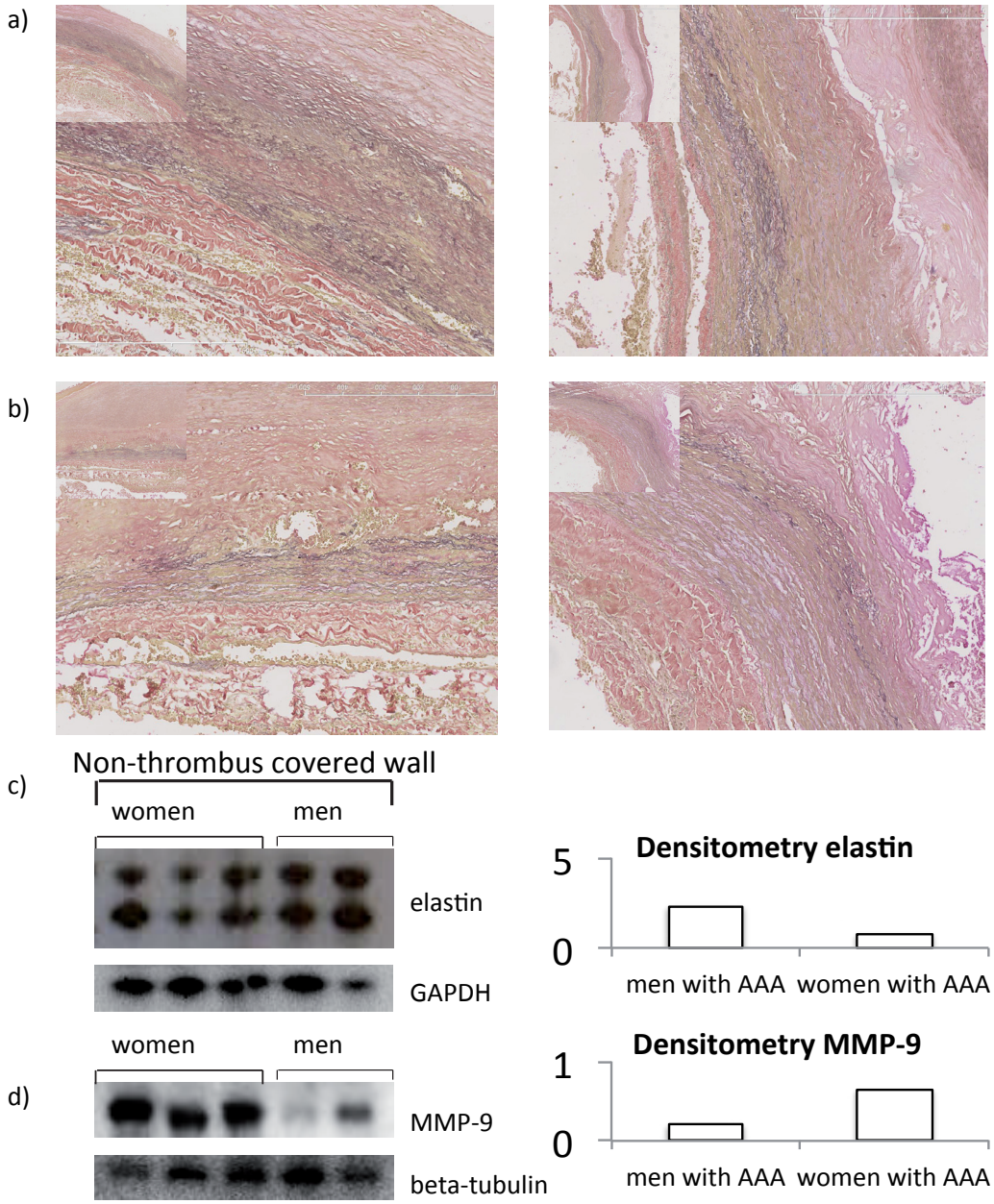


***Figure V**. Weigert's-van Gieson's staining of elastin in the non-thrombus covered aneurysm wall of two men. b) Weigert's-van Gieson's staining of elastin in the non-thrombus covered aneurysm wall of two women. c) Western blot of and densitometry of elastin in the non-thrombus covered aneurysm wall of three women and two men, .81 vs. 2.30, P=.200. d) Western blot and densitometry of MMP-9 in the non-thrombus covered aneurysm wall of three women and two men, .65 vs. .22, P=.200.*

In conclusion, a greater expression of MMP-9 in the non-thrombus covered aneurysm wall in women with a concurrent lower expression if elastin suggest that the proteolytic process differs in women in that part of the aneurysm wall. A weaker non-thrombus covered aneurysm wall in women, as a result of greater elastolysis could be of importance for the enlargement of AAA in women.

Methodological considerations

All aneurysm tissue in Study III-V was obtained from patients treated electively with OR for AAA and consequently the results illustrate an end-stage disease. Little can therefore be said of the potential gender differences in earlier stages of disease. Information of the earlier stages of aneurysm disease would be even more valuable from a gender perspective and have clinical implications. The reducing number of OR, in favour of EVAR, limits the number of attainable biopsies. Nevertheless, the sample sizes of Study III-V are consistent with prior studies within the research field.(172, 175)

An ILT is present in almost all larger AAAs.(53) One could argue that an ILT is present in all larger aneurysms, although thin, thereby eliminating the concept of non-thrombus covered aneurysm wall.

Collagen and its cross-linking in the aneurysm wall

The greater aneurysm rupture risk observed in women makes the biosynthesis of the load-bearing collagen of special interest to study from a gender perspective.(62, 95)

In Study IV, we found the relative collagen content in the aneurysm media, assed by HPLC and depicted as Hyp/Pro ratio, to be similar in men and women. Figure VI. Thereto, the mRNA expressions of the procollagens: collagen1α1, collagen1α2 and collagen3α1, were similar in men and women with AAA. Table III.

Collagen cross-linking is essential for its stability. An impaired collagen cross-linking affects its role in maintaining vascular integrity, as observed in human and animal models with defective collagen cross-linking.(30, 176-178) HP/LP ratio is an estimate of the stability of collagen tissue. The greater the ratio the greater is the stability.(30, 179) The results of collagen cross-linking analysis showed no difference in HP per triple helix, .58 vs. .46, P=.139 and higher LP per triple helix in women, .14 vs. .07, P=.005. As a result, the HP/LP ratio was lower in women, 3.28 vs. 8.41, P=.003. Figure VII.

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.(30) However, we found no difference in expressions of PLOD1-2 and LOX to associate with the observed gender difference in HP/LP ratio, data not shown.

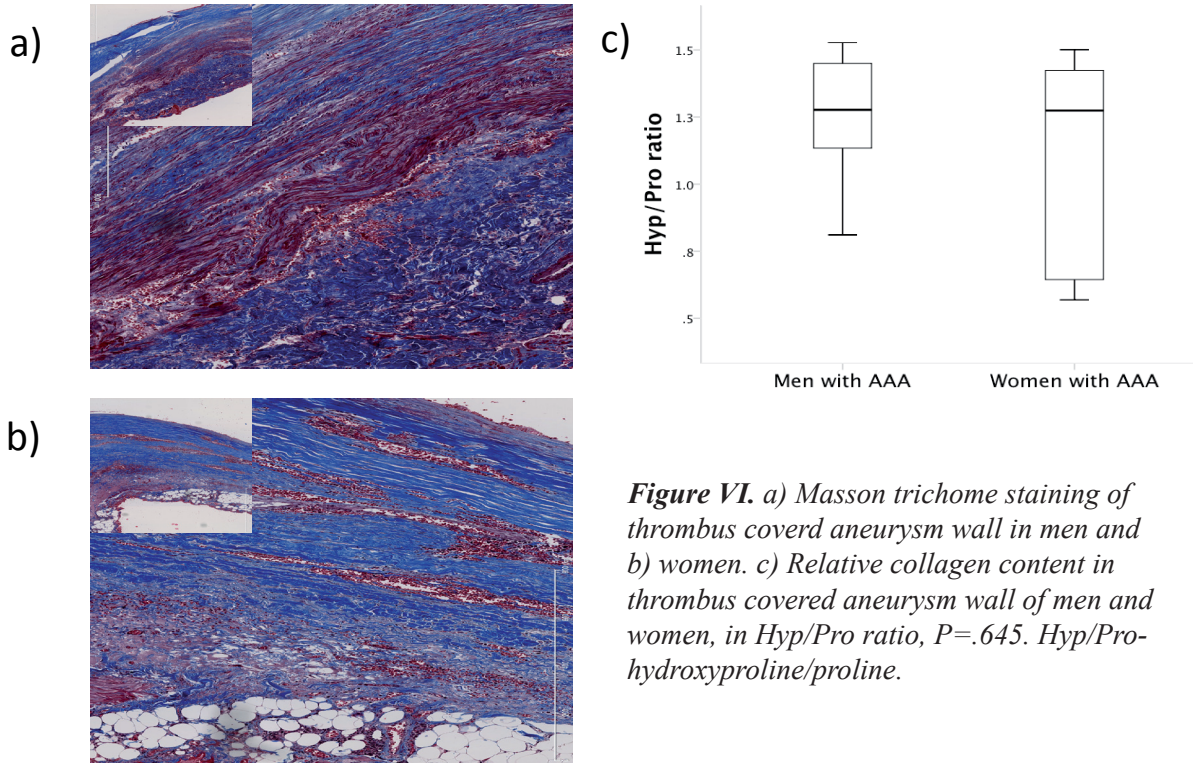


Figure VI. *a) Masson trichome staining of thrombus coverd aneurysm wall in men and b) women. c) Relative collagen content in thrombus covered aneurysm wall of men and women, in Hyp/Pro ratio, P=.645. Hyp/Pro-hydroxyproline/proline.*

Table III. Gene expression analysis of collagen in the thrombus covered aneurysm wall of men and women.

	Men with AAA N=14	**Women with AAA N=14**	**P-value**
collagen1α1	1.18 (1.10)	1.81 (.64)	.094
collagen1α2	-1.14 ± .70	-.76 ± .90	.223
collagen3α1	.39 ± .87	.81 ± .94	.231

Values presented log2-transformed and as arbitrary units. Values are presented as median(IR) for not normally distributed data and mean±SD for normally distributed data. Significance calculated by Mann Whitney-U test and Independent t-test.

Collagen is the ultimate load-bearing structure in the aneurysm wall, as illustrated two decades ago when vessels were treated with collagenase and elastase, respectively.(18) Collagenase-treated vessel ruptured, whereas elastase-treated vessels merely dilated.(18)

The results of this study suggest that either there is no difference in collagen between men and women and thereby no relation to the gender differences observed in aneurysm progression, or there is a difference but this study failed to address it, by for instance not comparing the amount of collagen between the sexes.(95)

The findings of a lower HP/LP ratio suggest a different collagen cross-linking in the aneurysm wall of men and women but it is difficult to value the difference without a plausible explanation as to how it might have come about. One plausible explanation to the lack of difference in the expression of the enzymes essential for collagen cross-linking is that few cells are involved in tissue repair in the end-stage of aneurysm disease.

In conclusion, the lack of difference in relative collagen content between the sexes together with a difference in collagen cross-linking between the sexes in the aneurysm wall, suggest that there might be biomechanical differences between the sexes. Further analyses are required to assess the role of gender on collagen biosynthesis and its potential association to women's higher rupture risk.

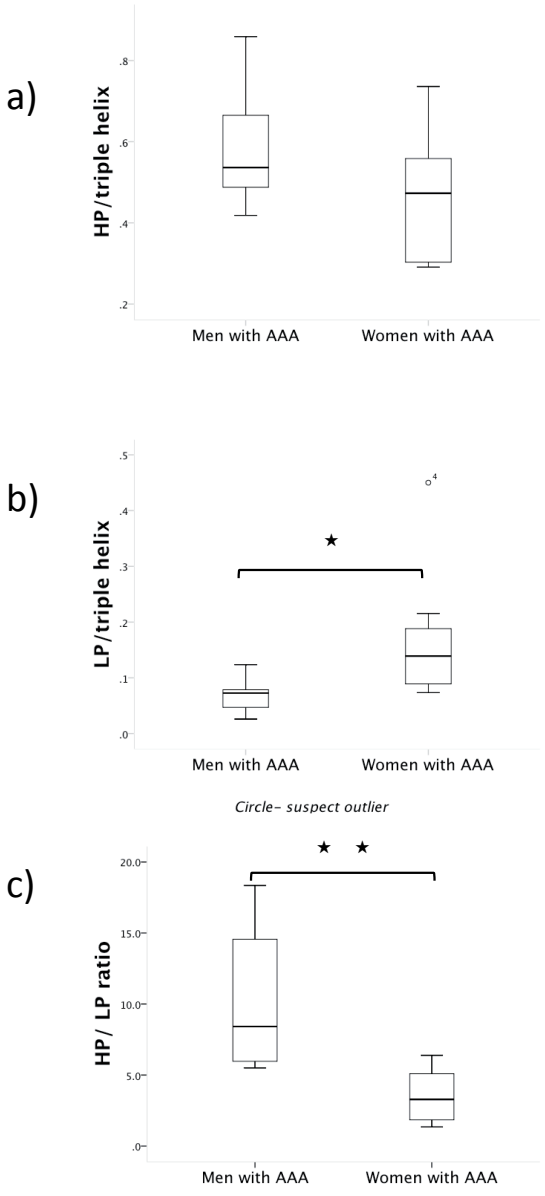


Figure VII. *a) Cross-linking of collagen in thrombus covered aneurysm wall of men and women. HP per triple helix, P=.139, b) LP per triple helix, ★P=.005 and c) HP/LP ratio, ★★P=.003.*

Methodological considerations

The main limitation of study IV is the analysis of collagen content. As opposed to relative collagen content, collagen content was not analysed. The study would have benefited from the analysis.

VSMCs, apoptosis and inflammatory cells in the aneurysm wall

The loss of vascular smooth muscle cells by apoptosis is an important element in the pathogenesis of AAA, but there is little knowledge of its extent in the aneurysm wall of women.(180)

In Study V, we investigated the expressions of smooth muscle cell markers in the aneurysm wall of men and women and found a lower protein expression of smooth muscle α–actin in women, assessed with immunohistochemistry and western blot analysies. Figure VIII and Figure X. This difference could not be observed in mRNA expression analysis of smooth muscle α–actin. Table IV. There were no differences in the protein and mRNA expressions of myocardin and MYH11 between the sexes. Table IV. The mRNA expression of smoothelin was higher in women compared with men, yet no differences were observed in protein expression analyses.

The lower expression of smooth muscle α–actin in women compared with men suggest a lower amount of VSMCs in the aneurysm wall of women. A plausible explanation, to the lack of difference in mRNA expression analysis of most smooth muscle markers, might be that there are few cells in both men and women that express and synthesise the contractile proteins in the end-stage of aneurysm disease.

This study is the first to evaluate the apoptotic process in the aneurysm wall with a gender

Table IV. Gene expression analysis of markers of smooth muscle cells, apoptosis and inflammation in men and women with AAA

	Men with AAA n=21	Women with AAA n=19	P-value
Markers of smooth muscle cells			
smooth muscle α-actin	-.73 (1.87)	-.09 (2.33)	.136
myocardin	7.67 (3.70)	8.51 (3.22)	.581
MYH11[a]	.13 ± 1.54	.88 ± 2.35	.267
smoothelin	4.63 (1.01)	5.41 (1.94)	.045
Markers of apoptosis			
caspase 3	5.53 (.58)	5.69 (.86)	.970
p53[b]	4.47 (.60)	4.83 (.82)	.045
BAX[c]	4.11 ± .51	4.55 ± .74	.045
Fas[d]	3.86 (.42)	3.89 (.92)	.407
Markers of inflammation			
CD247[e]	6.38 (1.14)	6.84 (1.49)	.068
CD4	4.01 (1.06)	4.95 (1.19)	.012
CD8	6.60 (1.32)	7.34 (1.96)	.271
CD68	.63 (1.20)	1.67 (1.20)	.011
MS4A1[f]	4.81 (3.87)	4.21 (3.69)	.510

Values presented log2-transformed and as arbitrary units. Values are presented as mean ± SD for normally distributed data, and median (IR) for not normally distributed data. Significance calculated by independent t-test, and Mann Whitney-U test. [a]MYH11- myosin, heavy chain 11, smooth muscle. [b]p53- tumor protein p53. [c]BAX- BCL2-associated X protein. [d]FAS- Fas cell surface death receptor. [e]CD- cluster of differentiation [f]MS4A1- membrane-spanning 4 domains subfamily A member 1.

perspective. The observed greater proportion of apoptotic cells in the medial layer of women compared with that in men, .92 vs. .66, P=.005, suggest a more pronounced apoptosis in the aneurysm wall of women compared with men. Figure VIII and Figure IX. In addition the expressions of p53 and BAX, both markers of apoptosis, were higher in women compared with men. Table IV and Figure X.

The more pronounced apoptosis in the aneurysm wall of women could be related to a more pronounced inflammation. In order to value the amount of inflammatory cells, we analysed the expression of inflammatory markers in men and women. We found a greater expression of CD68, marker for macrophages, and CD4, a marker for $CD4^{+}$lymphocytes. Table IV, Figure VIII and Figure X. There was no significant difference in the mRNA expression of CD247, a marker of T-lymphocytes, CD8, a maker or $CD8^{+}$lymphocytes and MS4A1, a marker or B-cells between the sexes. Table IV.

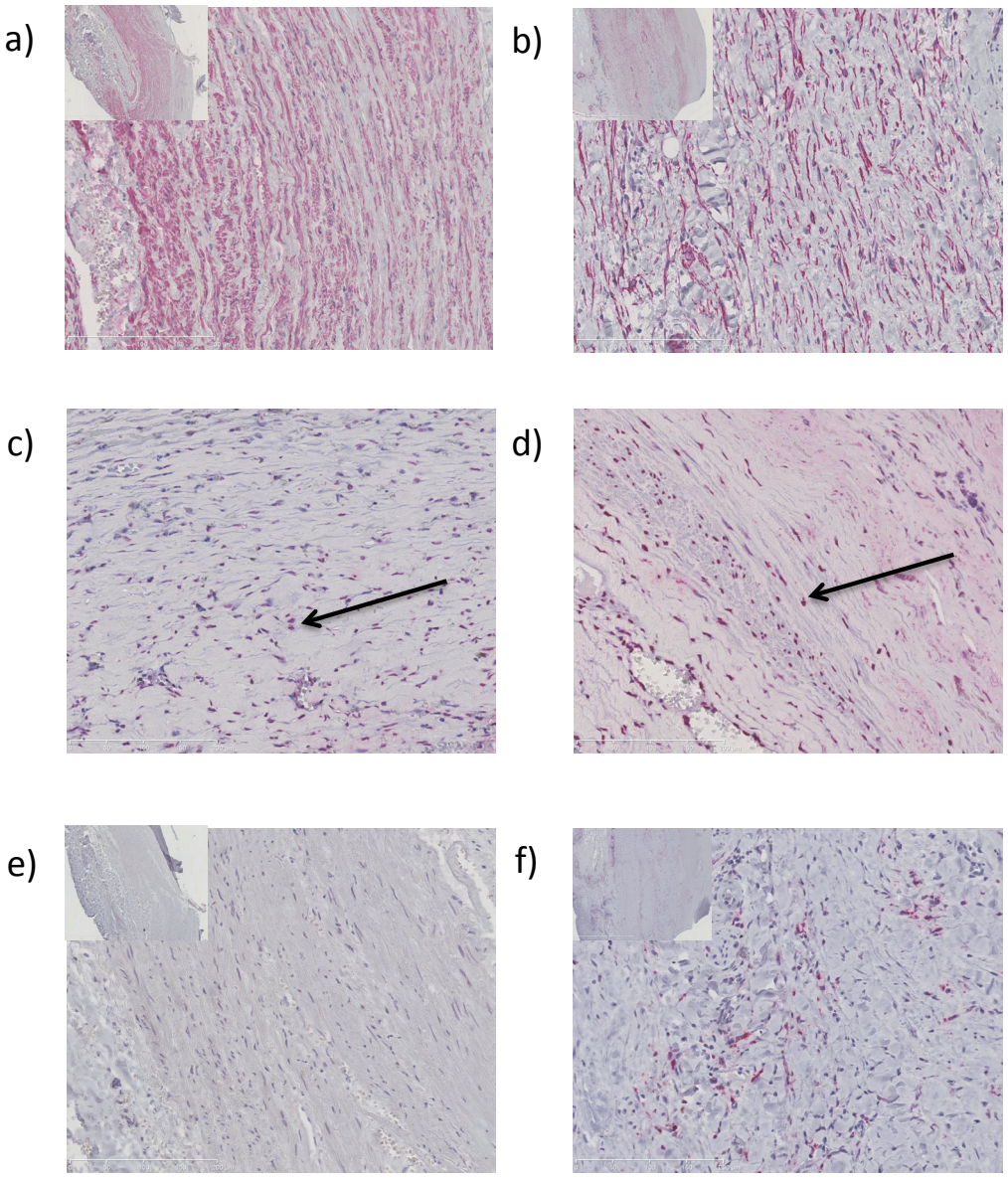


Figure VIII. *a) α -actin staining of the thrombus covered aneurysm wall of men and b) women. c) TUNEL staining of thrombus covered aneurysm wall of men and d) women. e) Staining of CD68 in men and f) women.*

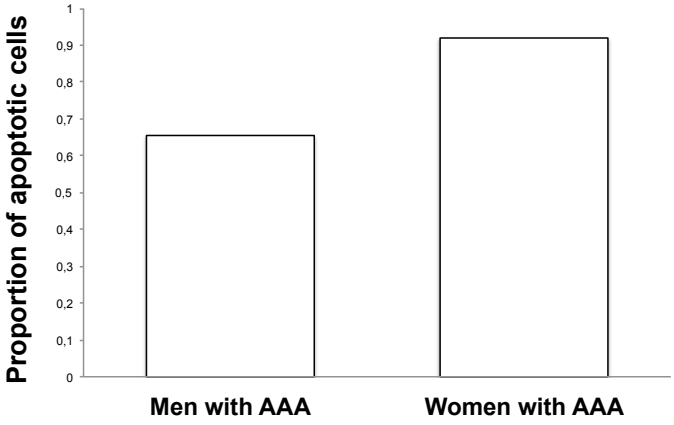
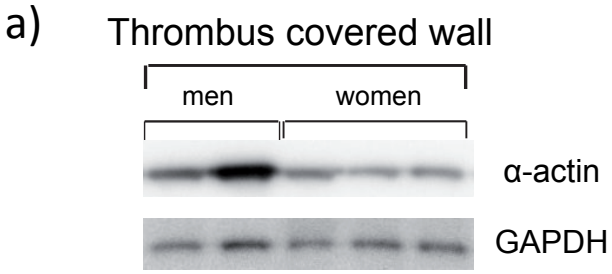
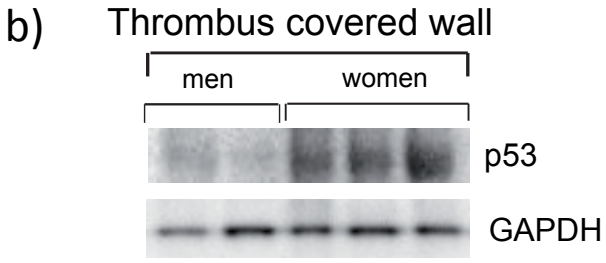


Figure IX. *Proportion of apoptotic cells in the thrombus covered aneurysm wall of men and women, ⋆P=.005*





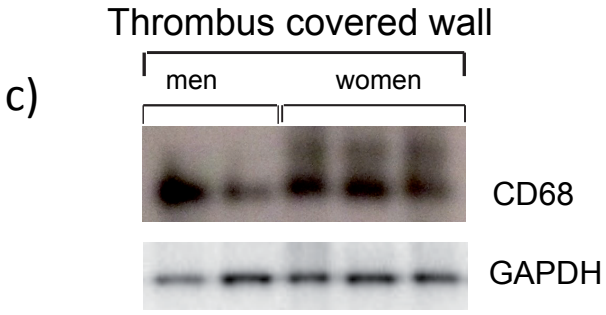


Figure X. *Western blot of α-actin in the thrombus covered aneurysm wall of 2 men and 3 women. b) Western blot of p53 in the thrombus covered aneurysm wall of 2 men and 3 women. c) Western blot of CD68 in the thrombus covered aneurysm wall of 2 men and 3 women.*

VSMC is the primary cell type of the aortic wall.(9) The role of VSMCs in aneurysm formation is dual, with the loss of VSMC by apoptosis the vessel wall loses its capacity to oppose degradation, but VSMCs have also been shown to promote aneurysm development by producing proteolytic enzymes.(48, 181) The observed lower expression of smooth muscle α–actin could suggest a lower amount of VSMCs in the aneurysm wall of women. This study was not designed to value phenotype modification in men and women with end-stage aneurysm disease and consequently no conclusions can be drawn from the results of mRNA expression analysis.

The observed greater proportion of apoptotic cells in the aneurysm wall of men and women suggest that the aneurysm wall has a heightened sensitivity for apoptotic stimuli, which might be related to an amplification of the death-promoting pathway of p53 and BAX.

The higher expressions of the inflammatory markers of macrophages and $CD4^+$lymphocytes in the aneurysm wall of women could be explained by a more pronounced infiltration of these cells in the aneurysm wall of women. A more pronounced inflammation in the aneurysm wall of women could hypothetically affect the observed findings of apoptosis and smooth muscle cell markers. However, without analyses on how they potentially interact little can be said of their relation to one another. Thereto we found a difference in ASI between the sexes, to relate to the above mentioned findings. Women had a higher ASI compared with men, 3.4 vs. 3.1, P=.013.

In conclusion, a more pronounced apoptosis in the aneurysm wall of women, potentially related to an abundance of inflammatory cells, could be of importance for the gender differences in AAA development and progression.

Methodological considerations

The observations in Study V suggest an association between the amounts of infiltrating inflammatory cells, VSMCs and apoptosis but cannot provide information on how the association is mediated. Analysis of relevant cytokines might be a first step. Furthermore there are limitations to the TUNEL analysis. We cannot conclude that it is in fact apoptosis of VSMCs that we have observed even though it is likely so. Another limitation is the use smooth-muscle α-actin as a marker of VSMCs, since smooth-muscle α-actin is not exclusive for VSMCs but can also be expressed by for instance myofibroblasts.(182) Thereto it could be of value to perform western blot analysis on all the smooth muscle cell markers that was analysed by mRNA expression analysis.

Implementations and Future perspectives

The finding of a lower menopausal age in women with large AAA corresponds with a protective effect of female sex hormones on the development and growth of AAA. Theoretically, women who develop AAA are fundamentally different from women in the population, with a vascular susceptibility to disease, possibly related to sex hormones. Together with a disease-provoking event, such as for instance smoking, AAA formation might be triggered.

Considering hormone therapy to postpone menopause and thereby prevent aneurysm development in women is an appealing idea but could not be considered safe due to the adverse events observed in the WHI trails. The results of these trials illustrate the complex and opposing effects that sex hormones mediate on the vasculature in different stages of life.

It would be interesting to pursue the studying of a potential effect of gender on aneurysm development, which could have implications for both men and women. One way to further approach this area is to specifically study groups of patients with either premature artificial menopause or treatments including hormones and hormone antagonists and the potential effect of these therapies on the aortic wall and aortic diameter. Another way to deepen the understanding of the aneurysm wall and triggers for aneurysm development could be to study the aneurysm wall in relation to the aortic wall of healthy persons in different age groups. This wall analysis could then be linked to circulating hormone levels, giving better estimations of the influence of sex hormones on the aging aortic wall and what is altered in the aneurysm wall.

The observations from our study on biomarkers showed that even though men and women do not have identical biomarker profiles, they are in many aspects similar. The results do emphasize that future analysis should consider gender differences and efforts should be made to include women in reports on AAA. It seems particularly important to seek different biomarkers for different risk groups; it is probable that these are dependant on the "trigger" mechanisms for disease and progression. Maybe focus should be on identifying a biomarker able to single out patients with risk of growth and rupture, rather than a diagnostic marker, due to the present difficulty in estimating which patients are at risk for progression.

AAA behaves differently in women, illustrated by the higher rupture rate in women compared with men. The findings of Study III-V suggest that the aneurysm wall differ in men and women. The observed differences in the structural elements and degenerative markers of the aneurysm wall in men and women imply that there is a more pronounced degradation in the aneurysm wall of women. It could have implications for the observed lower wall strength in the aneurysm wall of women. A greater relative enlargement in women cannot however, solely explain the suggested more pronounced degradation in the aneurysm wall of women. Instead there are also most likely essential differences in the vessel wall between

men and women and between women who develop AAA and those who do not. The potential differences in the composition of the aortic wall between men and women might become especially important as an AAA evolves, resulting in, as suggested, a weaker aneurysm wall in women.

A lower expression of elastin and higher expression of MMP-9 in the aneurysm wall of women could have implications for aneurysm enlargement. It would be interesting to study women and men without disease to value if there are any inherent gender differences in the elastin content to associate with our findings.

It is appealing to study collagen and its cross-linking with a gender perspective, based on the load-bearing qualities of collagen and women's higher rupture risk. However, the results from our study are inconclusive regarding a potential effect of gender on collagen biosynthesis. Further analyses are required in order to reach a more accurate conclusion of the role of collagen in relation to women's higher rupture risk.

In order to value the observed differences in apoptosis, VSMC content and inflammatory cells in the aneurysm wall of men and women, further analyses are required. It would be of interest to study potential mechanisms through which the greater proportion of apoptotic cells, higher expression of inflammatory cells and lower expression of smooth muscle α–actin possibly relate to one another. It could potentially increase the understanding of AAA development in both men and women.

In conclusion, women with larger AAA have an altered reproductive history with a lower mean menopausal age, suggesting hormonal changes to be of importance for AAA development in women. The observed gender differences in the aortic wall described in the thesis, contributes to the presently poorly understood biological and morphological processes that triggers aneurysm development, progression and rupture.

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