Actions of androgens and estrogens in experimental models of cardiovascular disease

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ABSTRACT

Men are at higher risk of developing both atherosclerotic cardiovascular disease and abdominal aortic aneurysm (AAA). Actions of sex steroids are hypothesized to underlie these gender differences. Testosterone, the major androgen, reduces atherosclerosis in male animal models but is suggested to promote AAA formation. However, the role of the androgen receptor (AR) in mediating these effects of androgens is unknown. Further, the physiological metabolic actions of androgens in females are unclear. Estradiol, the major estrogen in females, reduces atherosclerosis in female animal models and can be metabolized to 2-methoxyestradiol, a biologically active metabolite, in the vascular wall.

This thesis aimed 1) to determine the role of the AR in the atheroprotection by testosterone in male mice, and 2) to investigate the physiological, AR-dependent actions of androgens in the development of atherosclerosis in female mice, and 3) to investigate the role of the AR in the development of AAA in male mice, and 4) to examine whether 2-methoxyestradiol affects the development of atherosclerosis in female mice.

Male and female AR-deficient mice (AR^{-} and $AR^{-/-}$) on apolipoprotein E-deficient background were generated using Cre/loxP technology. Male AR^{-} mice fed a high-fat diet displayed accelerated atherosclerosis and reduced atheroprotection by testosterone. Female $AR^{-/-}$ mice fed a high-fat diet displayed accelerated atherosclerosis associated with several features of the metabolic syndrome including obesity, insulin resistance and dyslipidemia. In an angiotensin II-induced model of AAA formation, male AR^{-} mice were protected from the development of AAA while displaying increased atherosclerosis, and testosterone increased AAA formation in controls, but not in AR^{-} mice. In addition, 2-methoxyestradiol treatment reduced atherosclerotic lesion formation in female apolipoprotein E-deficient mice.

In conclusion, AR-mediated actions of androgens play important roles in both male and female mice. In males, AR-mediated actions of testosterone reduce atherosclerosis and promote AAA formation. In females, AR-mediated effects of androgens are important for metabolism and protects against atherosclerosis. Further, the estradiol metabolite 2-metoxyestradiol may hold promise as an atheroprotective drug.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. **Androgen Receptor-Dependent and -Independent Atheroprotection by Testosterone in Male Mice**
Bourghardt J, Wilhelmson ASK, Alexanderson C, De Gendt K, Verhoeven G, Krettek A, Ohlsson C, Tivesten Å.
Endocrinology 2010, *in press*

II. **Accelerated Atherosclerosis Associated with Features of the Metabolic Syndrome in Female Mice Lacking the Androgen Receptor**
Bourghardt J, Wilhelmson ASK, Alexanderson C, De Gendt K, Verhoeven G, Holmäng A, Krettek A, Ohlsson C, Tivesten Å.
Manuscript

III. **Protection Against the Development of Abdominal Aortic Aneurysms in Male Androgen Receptor Deficient Mice**
Bourghardt J*, Alexanderson C*, Wilhelmson ASK, Alexanderson C, De Gendt K, Verhoeven G, Tivesten Å.
Manuscript
*These authors contributed equally

IV. **The Endogenous Estradiol Metabolite 2-Methoxyestradiol Reduces Atherosclerotic Lesion Formation in Female Apolipoprotein E-Deficient Mice**
Bourghardt J, Bergström G, Krettek A, Sjöberg S, Borén J, Tivesten Å.
Endocrinology 2007 Sep; 148(9):4128-4132

ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ApoE	Apolipoprotein E
AR	Androgen receptor
CHD	Coronary heart disease
COMT	Catechol-*O*-methyltransferase
CVD	Cardiovascular disease
DHT	5α-dihydrotestosterone
DXA	Dual Energy X-ray Absorptiometry
ER	Estrogen receptor
IFNγ	Interferon γ
IL	Interleukin
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
MMP	Matrix metalloproteinase
Orx	Orchiectomy
Ovx	Ovariectomy
PCOS	Polycystic ovary syndrome
SMC	Smooth muscle cell
TNFα	Tumor necrosis factor α
VCAM-1	Vascular cell adhesion molecule-1
WT	Wild-type

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INTRODUCTION

CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) denotes disorders of the heart and blood vessels and includes e.g. coronary heart disease (CHD), cerebrovascular disease, and peripheral arterial disease. CVD is the leading cause of death in the world, causing about 30% of deaths globally, and more than 40% of deaths in Europe.[1] The global burden of CVD is increasing and CVD will remain the leading cause of death worldwide in 2030.[2] The underlying cause of the majority of clinical CVD events is atherosclerosis.[3]

Atherosclerosis

Atherosclerosis is a disease characterized by the formation of thickenings of the innermost layer of the arterial wall. These thickenings or atherosclerotic lesions/plaques develop in large and medium-sized arteries preferentially at sites with turbulent flow and shear stress, typical of branches, bifurcations, and curvatures.[4] Atherosclerotic lesions are characterized by inflammation, lipid accumulation, cell death and fibrosis. Clinical complications of atherosclerosis may arise from flow-limiting stenoses, but most of the fatal clinical events are caused by the rupture of a plaque. Such rupture exposes the pro-thrombotic material in the plaque to the blood and causes thrombotic occlusion of the artery at the rupture site.[5] Risk factors associated with atherosclerosis include age, gender, dyslipidemia, hypertension, diabetes mellitus, and smoking.[6] However, it is not completely understood how these risk factors affect the complicated mechanisms that underlie the formation of atherosclerotic lesions.

Fatty streaks

Before the concept of atherosclerosis as an inflammatory disease was proposed by Russell Ross, the formation of atherosclerotic lesions was regarded as a consequence of hypercholesterolemia with lipid accumulation and smooth muscle cell proliferation in the vascular wall as characteristic features.[7] However, during the last decade the concept of atherosclerosis as an inflammatory disease has become increasingly recognized.[8]

The initiation of atherosclerotic lesion formation is thought to involve activation of the endothelium in the arteries. This activation increases the permeability of the endothelium, which leads to infiltration and subsequently retention of low-density lipoproteins (LDL) in the intima,[9] initiating an inflammatory response.[10]

In the inflamed intima modification of LDL, through oxidation[11] or enzymatic cleavage, releases lipids that can induce endothelial cells to express leukocyte adhesion molecules.[12] These adhesion molecules

recruit circulating platelets which further activate the endothelial cells.[13] Activated endothelial cells express several types of leukocyte adhesion molecules, causing leukocytes to roll along the vascular surface and adhere at the activated site. One of these adhesion molecules is vascular cell adhesion molecule-1 (VCAM-1), which is up-regulated in response to hypercholesterolemia.[14]

Leukocytes such as monocytes and lymphocytes, bind to VCAM-1 on the activated endothelium. Once attached, these leukocytes migrate into the intima in response to chemokines produced in the inflamed intima.[8] Other cytokines or growth factors produced by the inflamed intima, transform the infiltrating monocytes into macrophages. These macrophages then take up oxidized LDL via their scavenger receptors, and are transformed into foam cells. The inflammatory response in the intima also induces migration and proliferation smooth muscle cells (SMC).

The foam cells are able to present T-lymphocyte-specific antigens on their surface that activate T cells. Such activated T cells produce mainly pro-inflammatory cytokines, interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α). IFN-γ is a macrophage stimulating cytokine that activates macrophages to produce TNF-α and interleukin-1 (IL-1). These cytokines together induce production of many inflammatory and cytotoxic molecules in macrophages and vascular cells. This further increases inflammation and promotes recruitment of additional monocytes and T cells. Together the macrophages and T cells form fatty streaks, which are precursors to the more advanced lesions.[8] Fatty streaks can form early in life, and have been observed already in fetal aortas.[15] However, such fatty streaks do not cause clinical symptoms and may progress to more advanced lesions or regress.[8]

Advanced lesions

As fatty streaks grow and progress to a more advanced lesions, more inflammatory cells and lipids infiltrate the vessel wall. Advanced lesions have a more complex structure than fatty streaks (figure 1); in the centre of the plaque, foam cells and extracellular lipid droplets form a core region which is covered by a fibrous cap of SMCs and a collagen-rich matrix.[5] In the shoulder region, the plaque grows and macrophages and T cells accumulate. Other inflammatory cell types in the lesion include dendritic cells, mast cells, B cells, and natural killer T cells. The lesion can become even more complex, when foam cells in the core region go into necrosis and cholesterol is deposited as cholesterol clefts.

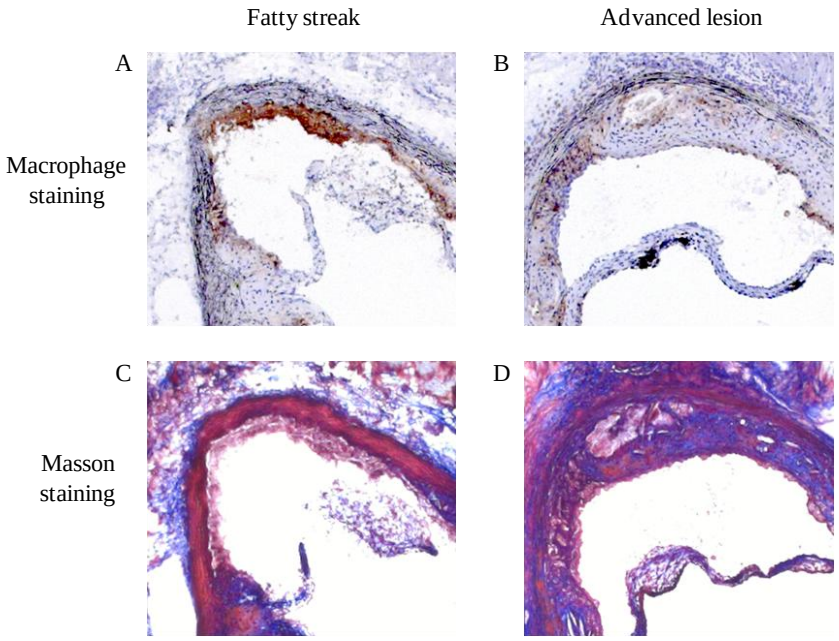

Figure 1. Cryosections of the aortic root from mice showing a fatty streak and an advanced lesion stained for macrophages (A, B) and stained with Masson (C,D). The fatty streak mostly consists of macrophages stained brown-red (A), while there are almost no macrophages present in the advanced lesion (B). Masson staining shows no collagen (blue color) within the fatty streak (C), while the advanced lesion (D) has a collagen-rich matrix (blue), a necrotic core (gray) which is covered by a fibrous cap containing smooth muscle cells (red).

Even though the plaque grows into the intima, the lumen diameter remains constant due to remodeling of the artery wall, which leads to a compensatory enlargement of the vessel lumen.[16] However, some lesions continue to grow into the lumen and become fibrous, with a small lipid pool, abundant SMCs and few macrophages. These stenotic lesions may narrow the lumen and restrict blood flow, particularly under situations of increased cardiac demand. Such lesions lead to ischemia, causing symptoms such as angina pectoris.[17]

Plaque rupture

The fibrous cap that covers the necrotic core prevents contact between the pro-thrombotic material in the plaque and the blood. However, if inflammation persists, extensive remodeling of the plaque and destabilization of the fibrous cap occurs, which eventually leads to rupture or erosion. In these vulnerable plaques, activated immune cells including macrophages, T cells and mast cells produce molecules that can destabilize lesions. Pro-inflammatory cytokines such as IFN-γ and TNF-α inhibit collagen formation, while matrix metalloproteinases (MMPs) and other proteases degrade the collagenous cap. These changes weaken the fibrous cap and make it prone to rupture. When the plaque ruptures, pro-thrombotic agents within the plaque are exposed to the blood. This launches a coagulation cascade that leads to thrombus formation. If unresolved, the thrombus can occlude the vessel and restrict blood flow or it can follow the blood stream to a smaller vessel. In both situations,

the thrombus can cause acute symptoms. In the coronary arteries, thrombi can lead to myocardial infarction, whereas in the arteries that supply the brain, it can cause ischemic stroke.[5]

Mouse models of atherosclerosis

Much of the understanding of the pathogenesis of atherosclerosis derives from animal models. One such model is the laboratory mouse. However, atherosclerosis does not develop spontaneously in mice. Some strains of inbred mice, especially C57BL/6 mice, can develop atherosclerotic lesions when fed a diet that promotes hyperlipidemia; this diet is commonly referred to as the Paigen diet and contains saturated fat, cholesterol and cholate. The inclusion of cholate in the diet is controversial because it induces an inflammatory response.[18, 19] Even after prolonged feeding of Paigen diet, the lesions in C57BL/6 are small, restricted to the aortic root and do not generally evolve beyond lesions containing foam cells.

The possibility to genetically modify the mouse genome has provided tools for the generation of animal models in which complex atherosclerotic lesions develops. These mouse models have in common that they either overexpress or lack genes involved in the regulation of lipoprotein metabolism.[20] Two of these mouse models have become widely used, namely low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice and mice that lack apolipoprotein E ($ApoE^{-/-}$).

$LDLR^{-/-}$ mice consuming chow diet develop a modest hypercholesterolemia and no atherosclerosis, but respond to fat feeding by developing hypercholesterolemia and atherosclerotic plaques. These lesions develop throughout the aorta, with large lesions in the aortic root and coronary arteries.[21] The lesions formed in $LDLR^{-/-}$ mice mainly consist of foam cells; features of advanced lesions such as necrotic cores are only generated after prolonged feeding of high fat diets. The $LDLR^{-/-}$ mouse has a more human-like lipoprotein profile than wild-type (WT) mice with most plasma cholesterol in LDL.

$ApoE^{-/-}$ mice develop spontaneous hypercholesterolemia and atherosclerosis. Lesions are formed throughout the aorta including the aortic root, the ascending aorta, the aortic arch, the abdominal aorta, and the innominate and coronary arteries.[22] The progression of lesions in this strain can be greatly accelerated by an atherogenic diet and these lesions become complex containing foam cells, necrotic cores, and fibrous caps. In $ApoE^{-/-}$ mice most plasma cholesterol is carried in very low-density lipoprotein, rather than in LDL as in humans.[20]

Abdominal Aortic Aneurysm

Abdominal Aortic Aneurysm

Abdominal aortic aneurysm (AAA) rupture is a significant cause of mortality for adults >60 years and has been estimated to be the tenth commonest cause of mortality in Western Countries, causing

approximately 2% of all deaths.[23] General AAA screening programs have been shown to reduce mortality in men aged >65 years,[24, 25] and are now introduced in many countries[26]. AAAs usually do not cause clinical symptoms until rupture occurs, but rupture may lead to death within minutes due to major bleeding.[27]

In humans, AAA most frequently occurs in the infrarenal part of the aorta (figure 2). An AAA is defined as a maximal aortic diameter of ≥3 cm, although other definitions have been used.[28, 29] Epidemiological studies have identified several risk factors that associate with AAA, including male gender, age, smoking, hypertension, obesity, and family history. Several of these risk factors are also associated with atherosclerosis and patients with AAA frequently exhibit increased atherosclerosis. Whether this association is causal or simply due to shared risk factors is unknown.[24]

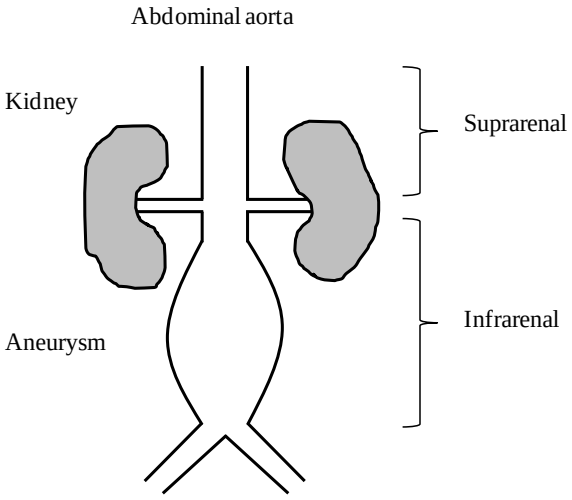

Figure 2. Abdominal aorta with an aneurysm. Aneurysms most frequently occur in the infrarenal part of the aorta in humans and in the suprarenal part of the aorta in mice.

The mechanisms underlying the initiation and formation of AAA are still poorly understood. However, histological features of AAA include chronic adventitial and medial infiltration of inflammatory cells, elastin degradation, and medial attenuation.[30] In AAA, inflammatory cells (neutrophils, T cells, B cells, macrophages, mast cells, NK cells) infiltrate all layers of the vascular wall.[31-34] These cells produce several inflammatory factors including cytokines, chemokines, leukotrienes, reactive oxygen species, and immunoglobulins. In the early stages of AAA formation, mainly Th1 cytokines such as IFN-γ are secreted. Later, in established AAA, the cytokine expression is altered and dominated by Th2 cytokine expression, especially IL-4, IL-5 and IL-10.[35] In this inflammatory environment, endothelial cells, SMCs, fibroblasts or macrophages are all capable of producing enzymes that degrade extracellular matrix proteins such as collagen and elastin. These enzymes include MMPs, serine proteases (e.g. t-PA, u-PA, plasmin and neutrophil elastase), and cysteine proteases (cathepsin D, K, L, and S). When released, these enzymes degrade collagen and

elastin in the aortic vessel wall. The resulting collagen degradation and elastin fragmentation together with smooth muscle cell apoptosis leads to aortic expansion and subsequent aneurysm development.[30]

Animal models of AAA

Mouse models of AAAs have been developed, employing different methods to produce the disease, including genetic manipulation, chemical induction and angiotensin II (AngII) infusion.[36] The genetic manipulations are spontaneous or engineered and include defects in extracellular matrix maturation, increased degradation of elastin and collagen, disturbed cholesterol homeostasis and enhanced production of angiotensin peptides. The chemical approaches include e.g. luminal infusion of elastase and periaortic incubations of calcium chloride.

The models in which AngII is infused into $ApoE^{-/-}$ or $LDLR^{-/-}$ mice demonstrate features of human AAA.[36] Administration of AngII at doses of 500-1000 ng/kg per minute, leads to AAA in the suprarenal part within the 28-day infusion period.[37] The maximal dose (1000 ng/kg per minute) often increases blood pressure.[38, 39] However, a blood pressure increase is not required for formation of AAA, because interventions that eliminate AAA do this independently of the effects on blood pressure.[40]

GENDER DIFFERENCE IN CARDIOVASCULAR DISEASE

Gender difference in coronary heart disease and atherosclerosis

There is a marked gender difference in life span throughout the world. Men in most countries have shorter life expectancy than women. In fact, men live on average 5.6 years shorter than women in countries above a minimal economic development (Gross Domestic Product (GDP) in U.S. dollars, $3000 per capita).[41] This gap in life expectancy is to a large extent driven by a male predisposition to clinical CVD events caused by atherosclerosis. Coronary heart disease deaths in men exceed those in age-matched women by 2.5-to -4.5-fold up until the age of 75. The age-specific CHD death rates curves are parallel for men and women, but with a 5-10 year early head start in men. The reason for the gender difference in CHD is not known, but is likely to involve genetic, hormonal, and/or lifestyle factors or a combination of mechanisms. The male early head start of atherosclerosis and its complications could reflect biological processes early in life (e.g. perinatal androgen surge in boys) or early in the pathogenesis of atherosclerosis.[41-43] Indeed, gender differences are found in endothelial function, lipid loading of human monocyte-derived macrophages, and abdominal fat deposition, all of which are processes related to the atherogenesis.[43]

Gender differences are also found in animal models of atherosclerosis. Males develop earlier and more extensive atherosclerosis independent of lipid levels in diet-induced models both in non-human

primates[44] and rabbits.[45, 46] Although data are divergent, gender differences are also observed in mouse models of atherosclerosis involving ApoE[47] and LDL receptor deficiency.[48, 49]

Gender difference in abdominal aortic aneurysm

In epidemiological studies, male gender is consistently identified as a strong risk factor associated with AAA.[50-52] Men have a 4-fold increased risk of AAA in population-based studies[23, 53] compared with women.

Several of the rodent models of AAA formation show a gender difference comparable to that of humans.[39, 54, 55] In AngII-induced AAA in ApoE$^{-/-}$ and LDLR$^{-/-}$ mice, males are more susceptible to aneurysm development than females; AAA develops in 80% of the males compared with approximately 20- 30% in female mice.[39]

SEX STEROIDS

Sex steroids

Sex steroids are steroid hormones that are produced predominately by the testes in men and by the ovaries in women. Sex steroids include androgens and estrogens, and progesterone is sometimes included as a third class of sex steroids. In addition to the gonadal sex steroids, the human adrenal cortex produces substantial amounts of sex steroid precursors (C19 androgens) which can be locally converted into androgens and estrogens in both sexes. However, in contrast to humans and higher primates, adult rodents (e.g. rats and mice) produce little or no androgen precursors from the adrenals.[56] Androgens and estrogens exert important effects on growth, development, and morphological differentiation, as well as on the development and regulation of sexual and reproductive behavior in both sexes.[57]

Androgens

In males, testosterone is the major potent circulating androgen with more than 95% derived from testicular secretion. Testosterone is synthesized in the Leydig cells of the testes and circulates bound to albumin and sex hormone binding globulin. There are three phases with high testosterone production in a male´s life. The first phase takes place during fetal development, leading to differentiation of the male reproductive organs. The second phase occurs shortly after birth, and the third phase starts at puberty and continues through adulthood. After peaking at approximately 20 years of age, the testosterone levels begin declining with age.[58]

In females, androgens are secreted from the ovaries and androgen precursors from the adrenal cortex. Testosterone is the most important androgen also in females, but testosterone levels in the circulation are less than 1/10 of those of young men.[59]

Activation of the androgen receptor (AR) mediates an important part of the physiological effects of testosterone.[41, 43] The AR is stimulated either directly by testosterone or by its locally formed metabolite 5α-dihydrotestosterone (DHT). Although not present at high levels in the circulation, DHT is the main source of androgenic activity in many tissues as it is the most potent endogenous androgen. Aromatization of testosterone to estradiol provides an alternative pathway for the effects of testosterone. Estradiol exerts its effects in turn via signaling pathways distinct from the AR, most importantly via the estrogen receptor α (ERα). Testosterone may also exert effects independently of the classical sex steroid receptors[41](figure 3).

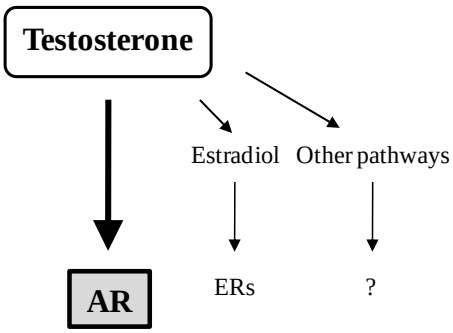

Figure 3. Pathways for the effect of testosterone. AR=androgen receptor, ERs=estrogen receptors.

The androgens affect most organs and tissues in the body. Their actions include development of reproductive organs and secondary sex characteristics, regulation of reproduction, behavior, muscle mass and strength, and distribution of body fat. These effects are physiologically important and well characterized in males. In contrast, the physiological actions of androgens in females have received less attention.[60, 61]

The androgen receptor

The AR is a 110-kDa nuclear protein with 918 amino acid residues. It is located on the X chromosome and ubiquitously expressed in tissues.[41, 43] The AR gene has three major functional domains, as illustrated in Figure 4. The N-terminal domain, which serves a modulatory function, is encoded by exon 1. Exons 2 and 3 encode the DNA-binding domain. The ligand-binding domain is encoded by exons 4-8. The AR belongs to the nuclear receptor super-family together with receptors for other steroid hormones such as thyroid hormone, vitamin D and retinoic acid. These receptors are ligand-activated transcription factors with a highly conserved structure. In the classical model of steroid hormone action the binding of hormone to these receptors induces an allosteric change. This allows

the hormone-receptor complex to pass into the nucleus and bind to high affinity sites on the chromatin and affect transcription.[62]

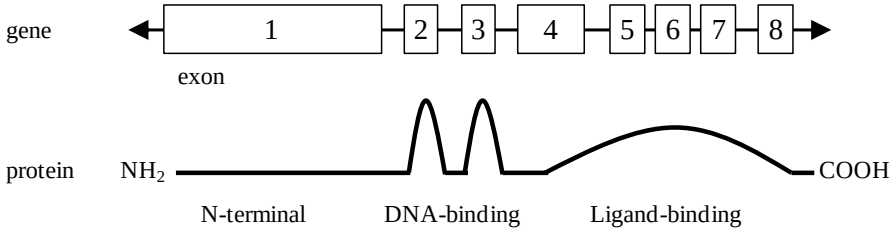

Figure 4. Structural organization of the AR gene and protein

As a homodimer, the androgen-AR complex can induce or suppress androgen-responsive genes by interacting with specific hormone responsive elements known as androgen responsive elements.[63]

In addition to the classical genomic effects, there are indications of rapid, non-genomic effects of sex steroids. The non-genomic effects of androgens involve the activation of second messenger signal transduction cascades, including increases in cytosolic calcium and activation of protein kinase A, protein kinase C and MAPK. These non-genomic rapid effects of androgens are suggested to be mediated by a hitherto uncharacterized membrane receptor.[41, 64, 65]

Androgen receptor deficient mice

Animal models of androgen insensitivity are useful for studies investigating the physiological AR-dependent actions of androgens. In the 1970s, Lyon and Hawkes reported an X-linked gene for testicular feminization (Tfm) in the mouse.[66] The Tfm mice carry a single nucleotide deletion in exon 1 of the AR gene, which leads to premature termination of the AR.[67] Tfm mice are infertile and their testes are small and abdominally located in the inguinal region, as in some patients with complete androgen insensitivity syndrome.

Beside the Tfm mouse, there are several genetically modified mouse models with ubiquitous knockout of the AR (ARKO).[68] Since male ARKO mice are infertile and the AR is located on the X chromosome, generation of ARKO male and female mice uses Cre-loxP technology. This involves a Cre transgenic mouse that expresses Cre recombinase ubiquitously and a mouse strain in which a part of the AR is flanked by loxP sites. This part of the gene, typically an exon, is referred to as floxed. When these mouse strains are crossed, the offspring will express Cre recombinase which recognizes and cuts out the floxed exon, resulting in knockout of the AR.

Table. Description of the constructs and metabolic phenotypes of the different ARKO mouse models

Ref	Floxed exon	Promoter of Cre recombinase	Result of recombination	Detectable AR protein	Obesity reported	Lipids
[69] Japan	Exon 1	CMV	Deletion of exon 1, insertion of early stop codon	No	Yes	Cholesterol ↔ Triglycerides ↔
[70] USA	Exon 2	β-actin	Deletion of exon 2, insertion of early stop codon	No	Yes	Cholesterol ↔ Triglycerides ↑
[71] Belgium	Exon 2	PGK	Deletion of exon 2, insertion of early stop codon	No	No (see Paper I and III)	Not determined
[72] Australia	Exon 3	CMV	In-frame deletion of exon 3	Yes	No	Not determined

The phenotype of the different ARKO males is dominated by female-like external organs, absent male internal genitalia as well as testes that are small and abdominally located. In addition, these mice have increased LH levels and decreased levels of testosterone.[68] Two of the ARKO mice models exhibit late onset of obesity (see Table). There are also reports of osteopenia in the male ARKO model.[73, 74]

The ARKO female mice have been less studied. They are fertile, but with lower follicle numbers and impaired mammary development and produce half the number of pups per litter. At 40 weeks of age, the ARKO female mice are infertile because of complete loss of follicles.[75-78]

Estradiol and metabolism of estradiol

Estradiol is the most important circulating estrogen in premenopausal women, and more than 95% of serum estradiol derives from ovarian secretion.[59] However, several other organs and tissues produce estradiol from sex steroid precursors, including adipose tissue, skin, endometrium, vaginal mucosa, breast, liver, blood vessels, and heart.[79] Estradiol levels vary greatly during the ovarian cycle, until menopause when serum levels of estradiol fall to levels below those found in men.[80] The actions of estrogens are fundamental in female reproductive function and metabolism.[81]

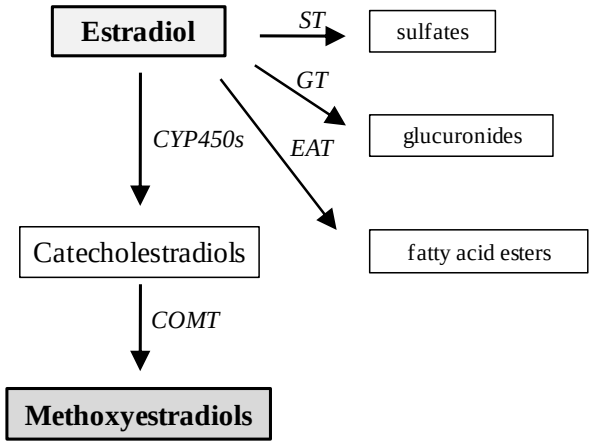

Figure 5. Metabolism of estradiol. GT=glucurunosyltransferase, ST=sulfotransferase, EAT=estrogen acyltransferase, COMT=catechol-*O*-methyltransferase.

Elimination of estradiol is mainly mediated via metabolism into water-soluble compounds that are eliminated by the kidneys (urine) and liver (faeces). It includes glucuronidation, sulfation, esterification, or *O*-methylation of estradiol or its hydroxylated metabolites. The hydroxylation of estradiol is mediated by several CYP450 isoforms. Most of the oxidative metabolism of estradiol takes place in the liver, but some isoforms of CYP450 are expressed locally in the periphery. Through the action of CYP1A1 and CYP1B1, estradiol is metabolized to catecholestradiols, such as 2-hydroxyestradiol and 4-hydroxyestradiol. The catecholestradiols can then be methylated via enzymatic *O*-methylation into 2-methoxyestradiol and 4-methoxyestradiol by the enzyme catechol-*O*-methyltransferase (COMT)[79] (figure 5). Several estradiol metabolites exert biological effects, which may be dependent or independent of the classical ERs.[82] During the past decade, much interest has focused on 2-methoxyestradiol, a potent inhibitor of cell proliferation.[83]

ANDROGENS AND CARDIOVASCULAR DISEASE IN MALES

Androgens, atherosclerosis and coronary heart disease in men

Low serum testosterone levels have been shown to associate with an adverse metabolic risk profile including increased abdominal fat mass, increased levels of total and LDL-cholesterol and triglycerides, reduced levels of high-density lipoprotein (HDL) cholesterol, insulin resistance, and hyperglycemia.[58, 84-87] Indeed, low testosterone levels in men associate with increased atherosclerosis.[85-89] Further, data from prospective longitudinal studies report increased all-cause mortality in men with low testosterone levels, although the results on the association with CVD mortality are conflicting.[90-93] To date, there are no studies demonstrating a significant association between testosterone levels and incident (fatal/non-fatal) CHD events in men, whereas low testosterone levels recently have been linked to increased risk of stroke.[94]

Data from studies of patients undergoing androgen deprivation therapy for treatment of prostate cancer have provided useful insight into the role of androgens in cardiovascular health. Androgen deprivation therapy, including the use of gonadotropin-releasing hormone agonists and/or AR-antagonists, increases body weight, reduces insulin sensitivity, and elevates serum cholesterol and triglyceride levels.[95] Recent studies also suggest that androgen deprivation therapy may increase the risk for cardiovascular disease and cardiovascular mortality.[96-98]

Androgens and atherosclerosis in male animals

Results from animal models support that testosterone confers a beneficial effect on atherogenesis in males. Testosterone treatment reduces atherosclerosis development in orchiectomized (Orx) male rabbits fed a pro-atherogenic diet.[99-101] Similarly, testosterone treatment reduces atherosclerosis in male ApoE$^{-/-}$ mice[102, 103] and LDL-R$^{-/-}$ mice.[104] Possible mechanisms include reduction of total cholesterol and elevation of HDL levels and reduction of pro-inflammatory cytokines.[41, 43, 102, 104, 105]

The putative pathways for the atheroprotective actions of testosterone in male mice have not been well defined. The beneficial effect of testosterone on atherogenesis is mediated via AR-independent pathways, involving aromatization of testosterone to estradiol. Nathan *et al*.[104] found that an aromatase inhibitor blocked the effect of testosterone in LDL-R$^{-/-}$ mice, indirectly indicating that the AR pathway is less important for atheroprotection by testosterone. However, no study has addressed the role of the AR pathway, for the effect of testosterone on atherosclerosis in mice.

Androgens and abdominal aortic aneurysm in males

Despite epidemiological evidence that male gender is a strong risk factor for AAA, there are few studies examining the role of androgens for AAA formation. As Orx protects against AngII-induced and elastase-induced AAA formation in male rodents,[55, 106] it is feasible that androgens promote AAA formation. Contrasting the results from experimental studies, a recent population-based study in men showed that lower levels of free testosterone, but not total, levels were associated with a higher risk of AAA.[53] It is still unknown whether physiological doses of testosterone can accelerate AAA formation, and whether the AR would mediate such an effect. Studies on AAA formation in AR deficient mice have not yet been performed.

ANDROGENS IN FEMALES

The physiological metabolic effects of androgens in females are poorly known[60, 61] and the importance of androgens in women's cardiovascular health remains to be clarified.[61] Studies on the actions of androgens in females have been hampered by difficulties of assessing low serum levels of androgens as well as determining appropriate, physiological doses for replacement.[61, 107] The fact that

ovariectomy (Ovx) entails loss of both androgens and estrogens is frequently overlooked, potentially contributing to an underestimation of the role of androgens.

Hypo- and hyperandrogenism in women

To date, a definition of androgen deficiency in women is lacking, and consequently the symptoms, signs, and clinical outcomes associated with androgen deficiency are not well characterized.[60, 61] There are several conditions that have been shown to associate with low testosterone levels in women. These include ovarian dysfunction (e.g. oophorectomy, chemotherapy, and radiation), adrenal dysfunction (e.g. adrenal insufficiency and adrenalectomy), hypothalamic-pituitary dysfunction, and drug-related effects (e.g. corticosteroids, antiandrogens, oral contraceptives, oral estrogen replacement therapy).[108] Although available data are scarce, they suggest that androgen deficiency in women is characterized by symptoms such as diminished sense of well-being or dysphoric mood, fatigue, sexual dysfunction, decreased muscle strength and bone mass.[60] However, the metabolic consequences of androgen deficiency in women are not well defined.

In women with polycystic ovary syndrome (PCOS, affecting 6-10% of all women), a cluster of cardiovascular risk factors (e.g. insulin resistance, abdominal obesity, hyperlipdemia and hypertension) is associated with hyperandrogenism.[109] Although most evidence suggest that insulin resistance rather than hyperandrogenism plays a key role in the pathogenesis of PCOS,[110] the link between hyperandrogenism and the metabolic syndrome has led to the paradigm that androgens exert adverse cardiovascular and metabolic effects in women.[111, 112] Whether there is an association between high androgen levels in women with PCOS and increased risk of cardiovascular events is a matter of controversy.[113]

Androgens and atherosclerosis in female animals

Previous experimental studies on androgens and atherosclerosis in females are few. Treatment studies show inconsistent results; some studies suggest that androgen administration accelerates atherogenesis in females,[114, 115] while other studies suggest atheroprotective effects.[102, 103, 116]

VASCULAR ACTIONS OF ESTROGENS

The male early head-start in CVD has often been explained by the protective effects of endogenous estrogens in premenopausal women.[41, 117] Indeed, many reports regarding beneficial effects of estradiol on serum lipids, vascular endothelial function, and experimental atherosclerosis support this assumption. Observational studies demonstrate an association between hormone replacement therapy and reduced CVD risk in postmenopausal women.[80, 118] However, two large randomized controlled

suggest that hormone replacement therapy could increase the risk for myocardial infarction and stroke in postmenopausal women,[119, 120] questioning the belief that estrogens are protective.[121, 122] The mechanisms underlying these results remain unknown and are lively debated.

Vascular actions of estradiol

An atheroprotective effect of estradiol is strongly supported by results from studies in animal models of atherosclerosis. Estradiol prevents atherosclerosis in monkeys, rabbits and mouse models of atherosclerosis.[123-125] The effect is mediated through a direct effect of estradiol on the cells of the arterial wall, rather than an effect on the lipoprotein profile.[126]

Several beneficial actions of estradiol on the endothelium could contribute its atheroprotective effect. This includes decreased expression of adhesion molecules (e.g. VCAM-1)[127] and pro-inflammatory chemokines (e.g. MCP-1 and IL-8).[128] Other beneficial actions of estradiol include decreased endothelial permeability[129] and improved endothelial function (e.g. increased levels of NO and prostacyclin, and reduction of reactive oxygen species).[123] Furthermore, estradiol decreases apoptosis of endothelial cells[130] and accelerates endothelial healing,[131-133] possibly involving an increase in circulating endothelial progenitor cells.[134-136] Estradiol also has effects on vascular SMCs and inhibits migration and proliferation.[137] In addition, estradiol decreases LDL oxidation[138] and lipid accumulation in macrophages,[139] which also could contribute to atheroprotection.

Vascular actions of 2-methoxyestradiol

Many of the effects of estradiol on vascular cells are mediated via a direct interaction of estradiol with ERs. There is also evidence that estradiol exerts biological effects via ER-independent pathways.[140, 141] Because estradiol is metabolized to estrogenic and non-estrogenic metabolites and estradiol metabolites may have biological activity, it is feasible that estradiol metabolites may play a role as mediators of the vasculoprotective actions of estradiol.[140]

An estradiol metabolite of putative importance is 2-methoxyestradiol, which is a potent anti-proliferative agent currently being evaluated in phase II clinical trials for cancer.[83, 142] Although the mechanism of action of 2-methoxyestradiol requires additional clarification, it appears to be independent of the classical estrogen receptors.[83, 143] 2-methoxyestradiol inhibits proliferation of vascular smooth muscle cells as well as injury-induced neointima formation *in vivo*.[144] Moreover, 2-methoxyestradiol has vasculoprotective effects in rat models of drug-induced hypertension and pulmonary hypertension[145, 146] and reduces oxidation of low-density lipoprotein *in vitro*.[147] These findings raise the question whether 2-methoxyestradiol might have anti-atherosclerotic effects. However, the hypothesis that 2-methoxyestradiol can modulate atherogenesis[143] currently remain untested *in vivo*.

AIMS OF THE THESIS

- To determine the role of the AR in atheroprotection by testosterone in male mice (Paper I)
- To investigate the physiological, AR-dependent actions of androgens on the development of atherosclerosis in female mice (Paper II)
- To investigate the role of the AR in the development of AAAs in male mice (Paper III)
- To examine whether 2-methoxyestradiol affects the development of atherosclerosis in female mice (Paper IV)

METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in the Material and Methods sections of the individual papers, while a more general discussion of some of the methods is presented below.

ANIMALS

Since normal (WT) mice do not normally develop atherosclerosis, mice on ApoE$^{-/-}$ background were used in all Papers (I-IV) included in this thesis. In Paper I, II and III, we used male or female ARKO mice on an ApoE$^{-/-}$ background (ApoE-M, C57/BL6, Taconic), which were bred in our laboratory animal facility. In Paper IV, we used female ApoE-/- mice, which were purchased from Taconic (Denmark).

DIETS

In Paper I and II, male and female ARKO mice on an ApoE$^{-/-}$ background were fed a soy-free regular chow diet (2016; Harlan Teklad, Oxfordshire, UK) up to 8 weeks of age and then atherogenic diet (21% fat from lard and 0.15% cholesterol, Special Diets Services, Essex, UK) for 8 weeks to accelerate atherosclerosis formation. In Paper III and IV, we fed male ARKO/ApoE$^{-/-}$ mice and female ApoE$^{-/-}$ mice a soy-free regular chow diet (2016; Harlan Teklad, Oxfordshire, UK). A soy-free diet was used to reduce the levels of phytoestrogens in the chow. As phytoestrogens have estrogenic activity they could potentially influence the results in studies on sex steroid action.

Comment: ApoE$^{-/-}$ mice on chow diet have serum total cholesterol levels of about 20 mM. The atherogenic diet increases the serum total cholesterol levels to approximately 30 mM and thereby accelerates atherogenesis. In addition, the lesions become more advanced with necrotic cores, cholesterol clefts, fibrous caps, and medial extensions.

GONADECTOMY

We used gonadectomy; orchiectomy (Orx) of males and ovariectomy (Ovx) of females, in all papers (I-IV). Given the endocrine functions of the testes and ovaries, respectively, Orx decreases the testosterone levels in male mice, while Ovx decreases both estrogen and androgen levels in female animals.

HORMONAL TREATMENT

All hormonal treatment with sex steroids was administered through slow-release pellets (Innovative Research of America, Sarasota, FL) implanted subcutaneous under the skin between the ear and the shoulder of the mice. In Paper I and III, we used a physiological testosterone dose of 25 μg/day. We evaluated this dose in a pilot study; when administered to Orx mice it restored the weights of seminal

vesicles, ventral prostate and salivary glands to the level of sham-operated control mice. In Paper I**V**, we used an estradiol dose of 6 μg/day because several previous studies had shown that it reduced atherosclerotic lesion development in ApoE$^{-/-}$ mice.[117] This dose is supraphysiological in females. The 2-methoxyestradiol dose of 6.66 μg is equimolar to 6 μg estradiol.

EVALUATION OF ATHEROSCLEROSIS

Atherosclerosis in mice is most frequently assessed *ex vivo* in aortas prepared *en face* or in the aortic root. *En face* prepared aortas are cleared from adventitial fat and connective tissue and cut open longitudinally, from the aortic arch down to the abdominal bifurcation, and pinned out flat on a dark material. This technique can provide information on the distribution of lesions within the aorta, but it cannot be used for detailed analyses of lesion characteristics. Cross-sections of the aortic root can provide information on both lesion size and lesion characteristics. For both techniques, it is common to stain for lipids to visualize the atherosclerotic lesions. To stain for lipids, we used Sudan IV stain for *en face* aortas in Papers I, II and IV, and Oil Red O stain for aortic root sections in Papers I and III. After staining, images were captured, and color quantified using the imaging software BioPix.

In aortic root sections lesion characteristics can be evaluated using histological and immunohistochemical techniques. For assessment of lesion composition (collagen content, presence of necrotic core and cholesterol clefts), sections were stained with Masson´s trichrome. For evaluation of macrophage accumulation, aortic root sections were stained with a Mac-2 antibody. Quantitative analyses of collagen and macrophage content as percentage of plaque area were made using Biopix Software. Lesion complexity (presence/absence of necrotic core and cholesterol clefts) was evaluated manually.

Comment: Mac-2 is a surface antigen expressed on mature macrophages.

EVALUATION OF ABDOMINAL AORTIC ANEURYSM

For analysis of AAA formation, the aortas were dissected free from connective and adipose tissue, and the presence or absence of an obvious modest or major outward remodeling of the suprarenal region of the aorta was determined by a blinded observer. An AAA was defined as an outward remodeling of the aorta in ex vivo analysis or death from major bleeding, which was confirmed in a dissection of the animal post mortem.[39] We also determined the maximal outer diameter of the aorta in photos, using an image analysis program (BioPix Software, Göteborg, Sweden).

DUAL ENERGY X-RAY ABSORPTIOMETRY

Dual Energy X-ray Absorptiometry (DXA) is a technique that was originally developed for studying bone parameters, but is also useful technique for metabolic phenotyping studies.[148, 149] It measures bone mass, but also lean mass and fat mass. Body composition was assessed in anesthetized animals using a mouse DXA machine (LunarPIXImus), following the instructions of the manufacturer (Paper II).

INSULIN TOLERANCE TEST

An insulin tolerance test measures the glucose–lowering effect of a given dose of insulin. Injection of insulin normally decreases the blood glucose levels in an animal. In an animal with insulin resistance, injection of insulin has reduced or no effect on the blood glucose levels.

RESULTS AND DISCUSSION

PAPER I

In Paper I, we investigated the importance of the AR-pathway for the atheroprotective effect of testosterone in male mice. Because ARKO mice are testosterone-deficient, we sham operated or Orx the mice before puberty and Orx mice were supplemented with placebo or a physiological testosterone dose. From 8 to 16 wk of age, the mice consumed a high-fat diet. We examined the atherosclerotic lesion formation both in the aortic root and in *en face* preparations.

In the aortic root, we found that sham operated ARKO mice had increased lesion area. Orx increased the lesion area in WT but not in ARKO mice. Testosterone significantly reduced lesion area both in WT (by 50%) and in ARKO mice (by 24%). Importantly, lesion area was larger in testosterone-supplemented ARKO mice compared with testosterone- supplemented WT mice. *En face* analysis showed similar trends as in the aortic root, with some differences between different aortic regions. Thus, in the thoracic aorta, testosterone reduced lesion area in both WT (-53%) and ARKO (-31%) mice, while in the whole aorta there was an effect of testosterone in WT (-54%), but only a trend in ARKO mice (-21%, $P = 0.075$). When we assessed lesion complexity, we found that testosterone reduced the presence of a necrotic core in WT mice, while there was no significant effect in ARKO mice. We did not detect significant differences across the groups in other measures of lesion composition, such as cholesterol clefts, collagen content, neutral lipids, and macrophages.

We clearly demonstrated that the AR-pathway is a major pathway for the effect of testosterone on atherosclerosis in our mouse model. The atheroprotective effect of testosterone in ARKO mice was about half of the effect observed in WT controls, suggesting that a major part of the effect of testosterone is mediated via the AR. These results are supported by previous studies showing that flutamide, an AR antagonist, inhibited most of the protective effect of testosterone on atherosclerosis in rabbits[150] and that the AR agonist DHT reduced atherosclerosis in male ApoE$^{-/-}$mice.[103]

In contrast to the results in Paper I, Nathan *et al*.[104] found that an aromatase inhibitor blocked the effect of testosterone in LDL-R$^{-/-}$ mice, indirectly indicating that the AR pathway is less important for atheroprotection by testosterone. In a recent study, Nettleship and coworkers[151] studied Tfm mice (which carry a naturally occurring AR mutant allele) on a cholate-containing diet. They found that exogenous testosterone reduced fatty streak formation in the Tfm mice suggesting an effect of testosterone that is independent of the AR. Contrasting the results of Nathan *et al*. the atheroprotection by testosterone in Tfm mice was not prevented by treatment with either the aromatase inhibitor anastrazole or the ERα antagonist fulvestrant. Importantly, Nettleship *et al*. did not treat WT controls

with testosterone, and thus could not determine the relative importance of AR-dependent versus AR-independent pathways.

Because testosterone reduced atherosclerotic lesion area in ARKO mice, these data also demonstrate an AR-independent action of testosterone. Aromatization of testosterone to estradiol may mediate an AR-independent effect of testosterone via activation of the ERs. ERα is important in atheroprotection by estradiol, while ERβ reportedly is less important.[117] Therefore, we examined whether the decreased atheroprotection of testosterone in ARKO mice may be explained by decreased ERα or aromatase expression in the ARKO model. However, ERα mRNA expression did not decrease in the femoral artery or liver and we could not detect aromatase expression in either the femoral artery or the liver of ARKO mice. Hence, we found no support for altered ERα or aromatase expression accounting for the reduced effect of testosterone in ARKO mice. However, we cannot exclude the possibility of regulation of local aromatase expression in the ARKO model.

It is not unlikely that the testosterone dose influences the relative importance of the different pathways for the testosterone effect. In Paper I, we used a physiological testosterone dose (slow-release pellets 25μg/day), which increased both serum testosterone levels and the weights of androgen-sensitive organs to the level of intact WT controls. However, this dose appeared slightly supraphysiological in its effects on cholesterol at the study end, although the luteinizing hormone levels at the same time point do not support that the testosterone levels were too high. The higher testosterone dose used by Nathan et al (5.6 times higher than the dose used in our study) could affect the relative importance of the aromatization pathway. Although Nettleship *et al.* claimed a physiological testosterone dose, serum testosterone measurements revealed that the testosterone treatment was intermittently supraphysiological. This treatment regimen, combined with the absence of testosterone-treated WT controls, may contribute to an overestimation of the role of AR-independent pathways.

We investigated possible mechanisms for the increased atherosclerosis and reduced effect of testosterone in ARKO mice, including serum lipids and markers of systemic inflammation. In agreement with previous studies,[102, 104] testosterone treatment lowered serum total cholesterol also in our study. Testosterone reduced serum cholesterol in WT but not ARKO mice, suggesting a role of the AR in the cholesterol-lowering effect of testosterone. However, serum cholesterol did not differ between sham operated WT and ARKO mice despite the difference in atherosclerosis between these groups. This is in line with previous studies that demonstrate no difference in serum cholesterol levels between ARKO and WT mice.[69, 70, 152] Hence, our data on serum cholesterol levels and atherosclerosis are not entirely concordant, suggesting that other mechanisms may be important.

Testosterone has anti-inflammatory effects and these effects could be involved in testosterone´s effect on reducing atherosclerosis.[105] Therefore, we analyzed seven pro-inflammatory cytokines in serum from sham operated ARKO and WT mice. We found increased levels of TNFα in ARKO compared

with WT mice. These results may suggest an effect on the inflammatory response, mediated either via the androgen-AR system or secondary to increased atherogenesis.

Taken together, ARKO mice on apolipoprotein E-deficient background display accelerated atherosclerosis. Testosterone treatment reduced atherosclerosis in both WT and ARKO mice; however, the effect on lesion area and complexity was more pronounced in WT than in ARKO mice. These results are consistent with an AR-dependent as well as an AR-independent component of testosterone atheroprotection in male mice.

PAPER II

In this paper we investigated the physiological, AR-dependent effects of androgens on atherogenesis in female mice.

We assessed atherosclerosis *en face* in 16-week-old $AR^{-/-}ApoE^{-/-}$ females, after 8 weeks on high fat diet.

In the whole aorta, atherosclerotic lesion area was increased in $AR^{-/-}ApoE^{-/-}$ mice**.** Analysis of the different aortic regions showed that lesion area increased in the aortic arch**,** but not significantly in the thoracic aorta.

Studies supporting our finding of beneficial actions of androgens in females include three mouse studies showing atheroprotection by supraphysiological doses of testosterone in ovarian-intact[103, 116] and ovariectomized mice,[102] respectively. Other studies showing adverse or neutral effects of androgen treatment have been conducted in female monkeys or rabbits. In a study by Adams *et al.*[114] testosterone treatment to female monkeys increased atherosclerosis. However, in this study testosterone was given to ovarian-intact animals and completely suppressed ovulation, making it difficult to distinguish between effects of disturbed estrogen action and a direct effect of androgens on atherosclerosis in this study. Obsanjo *et al.*[115] showed that the anabolic steroid nandrolone did not increase atherosclerosis versus placebo (but versus ovarian-intact controls) in ovariectomized monkeys. However, the authors interpreted the study and the paper is referred to[41] as supporting a pro-atherogenic effect of anabolic steroids in females. In female rabbits, Bruck *et al.*[100] found no increase in intimal area by testosterone treatment. Taken together, previous studies on androgen treatment of female animals are scarce and the results are conflicting. Paper II is the first study on atherogenesis in an animal model of female androgen-deficiency.

In $AR^{-/-}ApoE^{-/-}$ females, increased atherosclerosis was associated with an increase in body weight and relative fat mass. After 10 weeks of age, body weight started to increase in $AR^{-/-}ApoE^{-/-}$ relative to $AR^{+/+}ApoE^{-/-}$ mice and was significantly higher in $AR^{-/-}ApoE^{-/-}$ at both 12 weeks and 16 weeks of age.

DEXA analysis showed that absolute and relative fat mass were increased in $AR^{-/-}ApoE^{-/-}$ mice. At the study end, the $AR^{-/-}ApoE^{-/-}$ mice had increased relative weights of all dissected fat depots (gonadal, mesenteric, and inguinal). The metabolic phenotype was further characterized by performing an insulin tolerance test. The glucose-lowering effect of insulin was significantly reduced in the $AR^{-/-}ApoE^{-/-}$ mice, indicating the $AR^{-/-}ApoE^{-/-}$ mice were insulin resistant. Serum total cholesterol and triglycerides levels were elevated at the study end, without any alteration of the lipoprotein profile.

Hence, in addition to the augmented atherosclerosis, we found a yet unreported metabolic phenotype of the $AR^{-/-}$ females characterized by a diet-induced obesity, insulin resistance and dyslipidemia. This is in contrast to previous studies, reporting late-onset obesity in male, but not female, AR-deficient mice.[76, 78, 152, 153] In Paper II, the female $AR^{-/-}$ mice were on an $ApoE^{-/-}$ background and fed a high fat diet containing lard and it is possible that these two factors unmask a phenotype of diet-induced obesity in the females. Our findings demonstrate that the AR-androgen system has important metabolic actions in females under certain conditions. Further studies investigating the mechanisms underlying the obesity in $AR^{-/-}ApoE^{-/-}$ females will be required.

We further addressed the question whether accelerated atherosclerosis in $AR^{-/-}ApoE^{-/-}$ mice was found only in obese mice and/or after feeding high fat diet. *En face* atherosclerosis in 10-week-old chow-fed $AR^{-/-}ApoE^{-/-}$ mice showed increased lesion area in the thoracic aorta and a similar trend for the whole aorta. The young (10-week-old) $AR^{-/-}ApoE^{-/-}$ mice exhibited elevated serum triglyceride levels and a tendency towards augmented cholesterol levels**.** These results showing increased atherosclerosis in non-obese young $AR^{-/-}$ females on chow diet, would argue against obesity as an underlying cause of increased atherosclerosis. However, it cannot be excluded that already 10-week-old mice have an accumulation of fat in the liver, causing dyslipidemia and thereby increased atherogenesis.

Ample data suggest that androgens play important roles in the lipid metabolism in males, but the molecular mechanisms are poorly understood.[154] The results in Paper II on dyslipidemia in $AR^{-/-}$ females are in line with previous findings that testosterone reduces total cholesterol both in female ApoE deficient mice[102] and rabbits[100] and that DHT reduced total cholesterol, but not triglyceride levels in female monkeys.[155] The results in Paper II support that androgens play an important role in the lipid metabolism also in females; however, dissecting the pathways involved will require further research.

To evaluate the impact of the ovarian hormones on atherogenesis in $AR^{-/-}ApoE^{-/-}$ mice, we ovariectomized $AR^{-/-}ApoE^{-/-}$ and $AR^{+/+}ApoE^{-/-}$ mice at 4 weeks age, and assessed atherosclerosis *en face* after 8 weeks on high fat diet. Ovx increased lesion area in $AR^{+/+}ApoE^{-/-}$ mice, but not significantly in $AR^{-/-}ApoE^{-/-}$ mice. Because the ovaries did not protect $AR^{-/-}ApoE^{-/-}$ mice from atherosclerosis, we evaluated the ovarian hormone production. We found no significant differences in

serum estradiol, while serum testosterone levels were significantly increased in $AR^{-/-}ApoE^{-/-}$ females. Neither uterine nor ovarian weights differed between the genotypes.

Our finding that the $AR^{-/-}$ females were not protected from atherosclerosis by their ovaries could indicate either that: 1) factors (e.g. androgens) from the ovaries protect from atherosclerosis in the presence, but not in the absence, of the AR, or 2) that the ovaries in $AR^{-/-}$ females are dysfunctional and do not protect from atherosclerosis. However, the serum estradiol levels were not reduced and uterine weights of $AR^{-/-}$ females did not differ from controls, indicating that peripheral estrogen action is not severely disturbed. Hence, the loss of atheroprotection of the ovaries in $AR^{-/-}$ females may support that androgens from the ovaries protect female mice from atherosclerosis.

To confirm an atheroprotective action in females that is mediated via the AR, we ovariectomized $ApoE^{-/-}$ mice and started treatment with the AR agonist DHT or placebo at 8 weeks of age, followed by high fat diet feeding between 8 and 16 weeks of age. Compared with placebo, DHT reduced atherosclerotic lesion area and serum total cholesterol levels. This finding suggests that beneficial effects of androgens in females are mediated via the AR.

In summary, female $AR^{-/-}$ mice display accelerated atherosclerosis associated with several features of the metabolic syndrome including obesity, insulin resistance and dyslipidemia. These results demonstrate that AR-mediated effects of androgens are crucial for metabolism and cardiovascular health in females (figure 6).

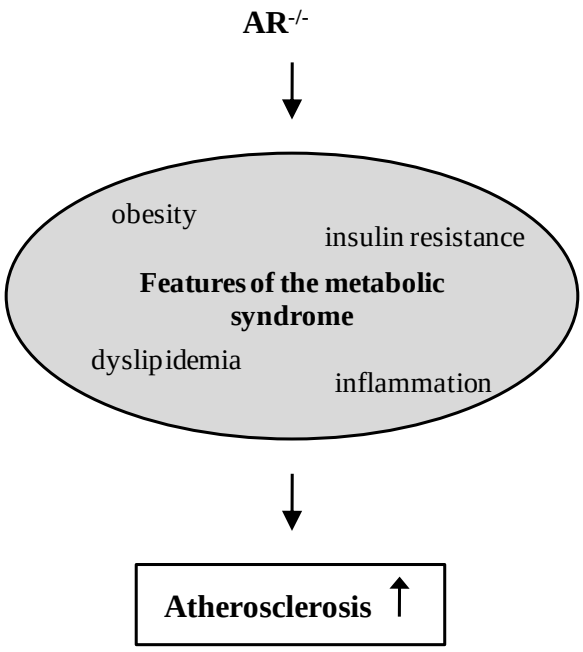

Figure 6. Proposed mechanism(s) for increased atherosclerosis in female $AR^{-/-}$ mice.

PAPER III

In Paper III, we tested the hypothesis that ARKO mice on ApoE deficient background are protected against the development of AAA. We infused ARKO and WT mice with AngII for 4 weeks and examined AAA formation.

We found that formation of AAA was markedly reduced in ARKO compared with WT mice. In accordance, maximal aortic diameter was significantly lower in ARKO vs. WT mice. Our findings are in line with previous studies showing that Orx protects against AAA formation in rodents.[55, 106]

Because the male ARKO mice are testosterone deficient,[156] a testosterone treatment study was performed to distinguish effects of testosterone deficiency from the effects of AR inactivation. AngII-infused Orx ARKO and WT mice were treated with a physiological testosterone dose or placebo. We found that testosterone treatment increased AAA formation in WT, but not ARKO mice. These data demonstrate that activation of the AR by a physiological dose of testosterone increases AAA formation in male mice. Together with data showing that the AR agonist DHT accelerates AAA formation in mice,[157] our data support the notion that the AR may mediate the adverse effect of androgens on AAA formation.

In line with the results in Paper I, atherosclerotic lesion area was increased in the aortic root of both saline- and AngII-treated ARKO mice. Our finding of concomitantly reduced AAA formation and increased atherosclerosis in ARKO mice is surprising because AAA formation shares important mechanistic pathways with atherogenesis (e.g. inflammation, oxidative stress and extracellular matrix degradation). In humans, AAA disease is associated with atherosclerosis as well as an increased risk of CVD events, although the mechanisms for this association are unknown.[24] Importantly, there are differences between the two diseases; risk factors such as hyperlipidemia and hypertension are less strongly associated with AAA formation, whereas diabetes protects against AAA.[23] Although the data on T and AAA formation are scarce, most evidence suggests that testosterone protects against atherosclerotic disease in both experimental and clinical studies.[41, 43] Hence, it is possible, but not yet proven, that androgens have opposite effects on atherogenesis and AAA formation also in humans. Further investigation of the molecular mechanisms that are affected by androgens in atherogenesis and AAA formation will be performed in future studies by our group.

Taken together, Paper III demonstrates that male ARKO mice are protected from the development of AAAs and that testosterone increases AAA formation via an AR-dependent mechanism, possibly contributing to the gender difference in AAA. Further, our data show that atherogenesis and AAA development processes are dissociated and affected in opposite directions in male ARKO mice.

PAPER IV

To investigate whether the estradiol metabolite 2-methoxyestradiol affects atherosclerosis development, female mice were ovariectomized and treated through slow-release pellets with placebo, 17beta-estradiol (6 microg/day), or 2-methoxyestradiol [6.66 microg/day (low-dose) or 66.6 microg/day (high-dose)]. We found that the high-dose 2-methoxyestradiol decreased atherosclerosis evaluated, while the low dose 2-methoxyestradiol showed no effect.

The finding that 2-methoxyestradiol reduced atherosclerotic lesion formation is in accordance with previous studies showing that 2-methoxyestradiol inhibits neo-intima formation *in vivo*[144] and exerts vasculoprotective effects in a rat model of drug-induced hypertension and pulmonary hypertension.[146, 158]

The results in our study imply the possibility that 2-methoxyestradiol may partially mediate the anti-atherosclerotic effect of estradiol in mouse models of atherosclerosis, a notion supported by the fact that estradiol reduces atherosclerosis in ERα$^{-/-}$ mice.[159] Since 2-methoxyestradiol is be unable to activate ERs as an agonist and estradiol is protective in the absence of ERβ,[117] it is possible that the anti-atherosclerotic effects of estradiol in ERα$^{-/-}$ mice are mediated by 2-methoxyestradiol. Interestingly, metabolism of estradiol to methoxyestradiols is critical for the anti-proliferative effects of estradiol on vascular smooth muscle cells.[160] However, the role of this metabolism for the anti-atherogenic effects of estradiol will require further studies, including estradiol treatment in combination with blockade or knockout of the enzymes that convert estradiol to methoxyestradiols.

We investigated two possible mediators, serum lipids and blood pressure, for the effect of 2-methoxyestradiol on atherosclerosis. 2-methoxyestradiol reduced serum total cholesterol levels, which is in line with previous observations in rats.[161] The reduced cholesterol levels may be less important as they occurred in both the low- and high-dose 2-methoxyestradiol groups and atherosclerotic lesion area decreased only in the high-dose group. Since 2-methoxyestradiol did not affect mean arterial blood pressure, it seems unlikely that the reduction of atherosclerosis is mediated through a blood pressure mechanism. However, there are several other possible mechanisms that could contribute to the effect of 2-methoxyestradiol including inhibition of SMC proliferation,[144] reduction of oxidative stress and oxidation of LDL,[79, 147] as well as increased cyclooxygenase-2 expression and prostacyclin generation.[144, 162]

We found that 2-methoxyestradiol shares the effects of estradiol on, for example, serum cholesterol, body weight gain, and uterine weight, although the effects of 2-methoxyestradiol were not as pronounced as those of estradiol. A possible mechanism for these estrogenic effects involves de-methylation of 2-methoxyestradiol to 2-hydroxyestradiol, which can act as an ER agonist. Another possibility is that 2-methoxyestradiol, via cell-specific cofactors, can activate ERs. The role of de-

methylation and/or activation of ERs for the atheroprotective effects of 2-methoxyestradiol in this study remains unclear. Thus, the answer to the question whether estradiol is effective via conversion to 2-methoxyestradiol or whether 2-methoxyestradiol is effective via conversion to estrogenic compounds, or whether both mechanisms are operative, is unknown.

In conclusion, Paper IV demonstrates that 2-methoxyestradiol, an endogenous estradiol metabolite, reduces atherosclerotic lesion formation *in vivo.*

CONCLUSIONS

From the results of the Papers included in this thesis, we conclude the following:

Paper I: ARKO mice on apolipoprotein E-deficient background display accelerated atherosclerosis. Testosterone treatment reduced atherosclerosis in both WT and ARKO mice; however, the effect on lesion area and complexity was more pronounced in WT than in ARKO mice. These results are consistent with an AR-dependent as well as an AR-independent component of testosterone atheroprotection in male mice.

Paper II: Female $AR^{-/-}$ mice display accelerated atherosclerosis associated with several features of the metabolic syndrome including obesity, insulin resistance and dyslipidemia. These results demonstrate that AR-mediated effects of androgens are crucial for metabolism and cardiovascular health in females.

Paper III: Male ARKO mice are protected from the development of AAA and testosterone increases AAA formation via an AR-dependent mechanism, possibly contributing to the gender difference in AAA. Further, our data show that atherogenesis and AAA development processes are dissociated and affected in opposite directions in male ARKO mice.

Paper IV: 2-methoxyestradiol, an endogenous estradiol metabolite, reduces atherosclerotic lesion formation *in vivo*.

CLINICAL IMPLICATIONS

Manipulating the androgen-AR system in men

Testosterone gradually declines as men age.[58] As testosterone has important physiological effects on e.g. muscle, bone, fat mass and brain in men, decreased testosterone levels may contribute to the symptoms and signs of aging, e.g., decreased muscle mass and strength, impaired physical performance and cognitive function, and lack of energy. Recently, much interest has focused on testosterone supplementation in elderly men, as evidenced by a 20-fold increase in testosterone sales in the United States during the 1990s.[58, 92]

There are several short-term studies with testosterone replacement to hypogonadal and/or obese elderly men, reporting beneficial effects on cardiovascular risk factors such as reduced waist circumference, cholesterol and circulating pro-inflammatory cytokines and improved insulin sensitivity. Via yet undefined mechanisms, testosterone increases vasodilatation and improves vascular reactivity which leads to short-term beneficial effects on cardiac ischemia, angina and chronic heart failure.[90] However, no long-term placebo-controlled studies have yet determined whether testosterone supplementation protects androgen-deficient and/or elderly obese men against cardiovascular disease. A recent study reported that testosterone treatment to older men was associated with an increased risk of cardiovascular adverse events.[163] Hence, it is evident that the hypothesis that testosterone replacement leads to cardiovascular benefit needs further investigation and defining the pathways involved in testosterone atheroprotection in males is of great importance. The data in this thesis suggest that testosterone atheroprotection in male mice involves an AR-dependent as well as an AR-independent component. Importantly, these findings warrant confirmation in men.

Death from AAA rupture is a major health problem, especially in men, and recently a general ultrasound screening of 65 year-old men for AAA was introduced in Sweden and other countries (started in 2008 in the Swedish region Västra Götaland). Our data showing that testosterone treatment of male mice increases the development of AAA in an AR-dependent manner suggests that accelerated formation of AAA may be a potential risk associated with extended use of testosterone supplementation in older men. Further, to date there are no treatment options for AAA besides surgery; our finding of the AR dependence in AAA formation highlights the AR as a potential target for interventions aimed at preventing AAA formation in men at high risk, e.g. due to strong heredity.

The androgen-AR system is extensively manipulated during treatment of prostate cancer in men and androgen deprivation therapy may increase the risk of cardiovascular disease and cardiovascular mortality.[96-98] However, the different treatment options for prostate cancer involve both manipulation of systemic androgen levels and blockade of the AR. Understanding the role of the AR in the

development of atherosclerosis is important for ongoing efforts to limit cardiovascular side effects of the different treatment regimens for prostate cancer.[164-166]

Selective androgen receptor modulators (SARMs) are compounds that activate or inhibit the AR in a tissue-specific way with the goal to achieve beneficial effects on bone and muscle mass without adversely affecting e.g. the prostate.[167] The findings in this thesis suggest that actions mediated via the AR may protect against atherogenesis, while they simultaneously increase the risk of AAA (figure 7). This information is essential in the design and evaluation of SARMs with a beneficial cardiovascular profile or SARMs that could be used in prevention or treatment of cardiovascular disease.

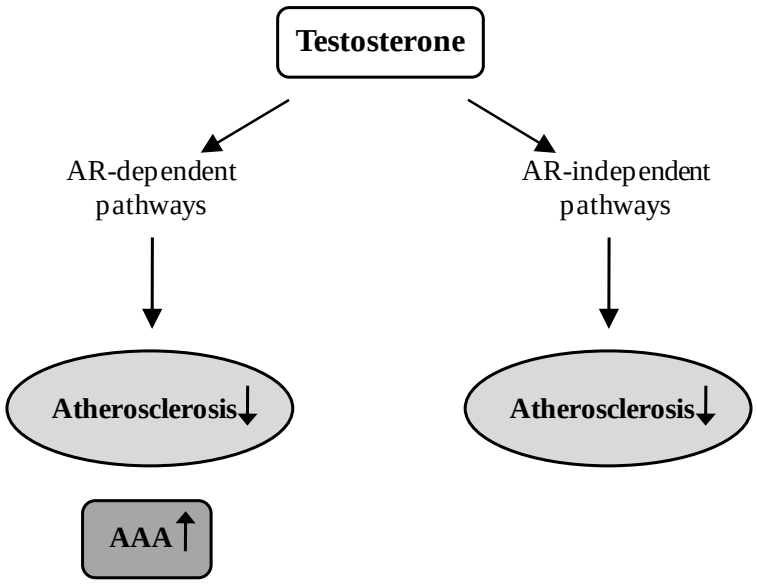

Figure 7. Suggested pathways for the cardiovascular effects of testosterone in males. AR=androgen receptor, AAA=abdominal aortic aneurysm.

Androgens to women?

To date, a clinical definition of female androgen deficiency is lacking[60] and the signs and symptoms associated with a deficient androgen action are therefore poorly understood. The development of female $AR^{-/-}$ mice has provided a valuable tool for determining physiological, AR-mediated actions of androgens in females. In this thesis, female $AR^{-/-}$ mice display accelerated atherosclerosis associated with several features of the metabolic syndrome including obesity, insulin resistance and dyslipidemia. These results demonstrate that AR-mediated effects of androgens are crucial for both metabolism and cardiovascular health in females.

High levels of androgens in women with PCOS are associated with an adverse metabolic risk profile and increased atherosclerosis.[109] This association has favored the idea that androgens exert adverse cardiovascular and metabolic effects in women.[111, 112] However, evidence also supports an association between low androgen levels and atherosclerosis in women.[168-171] Two recent studies reported that both high and low testosterone levels are associated with increased risk of coronary heart disease events in older women.[172, 173] Thus, the clinical studies support an atheroprotective effect of androgens in women.

The clinical recommendation regarding treatment and/or supplementation with testosterone in women is an area of disagreement.[174] However, there is evidence from short-term studies that testosterone treatment to surgically menopausal women improves sexual function.[61, 175, 176] Importantly, many of the published studies on testosterone supplementation in women with sexual dysfunction used supraphysiological doses of testosterone. In addition, there are few studies on the metabolic effects of a physiological androgen therapy to androgen deficient women. However, in a study by Miller *et al*., testosterone treatment improved markers of insulin sensitivity and reduced total cholesterol levels.[177]

The results presented in this thesis highlight the importance of further research to establish an appropriate definition of androgen deficiency in women and to characterize the metabolic phenotype associated with this deficiency. In addition, our results should encourage further investigations of the metabolic effects of androgen supplementation, using physiological doses of androgens, e.g. to women after Ovx.

Implications for hormone replacement therapy

Although most experimental studies that showed anti-atherogenic effects of estrogens employed estradiol, two large clinical trials conducted on hormone replacement therapy in postmenopausal women used conjugated equine estrogens as the estrogen component.[119, 120] These trials unexpectedly demonstrated adverse cardiovascular effects of treatment, raising skepticism about whether the correct estrogen compound was used. Conjugated equine estrogens contain >10 estrogens extracted from horse urine and their exact composition remains undetermined. However, conjugated equine estrogens contain trace amounts of estradiol,[122] thus generating little or no estradiol metabolites.

The results in this thesis on anti-atherogenic activity of an estradiol metabolite lacking estrogen receptor activating capacity suggest that future trials should use estradiol rather than other estrogens; alternatively, such trials might evaluate the use of 2-methoxyestradiol for hormone replacement therapy. An interesting possibility is that 2-methoxyestradiol mediates anti-atherosclerotic effects while the parent hormone estradiol exert certain adverse effects (e.g. inflammatory activation and plaque instability) through the activation of ERs in the setting of established atherosclerosis.[178] Thus,

further definition of 2-methoxyestradiol as a mediator of anti-atherosclerotic actions of estradiol is of great interest.

FUTURE PERSPECTIVES

Although the results reported in this thesis increase our understanding of the role of sex steroids in cardiovascular disease, they also lead to new questions and speculations.

Our results showed that part of the atheroprotective effect of testosterone is mediated via the AR, but the exact mechanism(s) for this AR-dependent effect remains to be determined. Since inflammation is a corner stone in the pathogenesis of atherosclerosis and testosterone has been suggested to exert anti-inflammatory effects, it would be of interest to perform additional studies to characterize the role of the AR in these anti-inflammatory effects in animal models of atherosclerosis. It would also be interesting to examine possible AR-mediated effects of testosterone on for example adhesion, infiltration, and migration of inflammatory cells in the developing atherosclerotic lesions. Studies of possible AR-mediated effects of testosterone on cytokine production by these inflammatory cells would also be of great interest.

The endothelium is considered to play an important role in the initiation of atherosclerosis and endothelial dysfunction is believed to precede the formation of atherosclerotic lesions. Damaged endothelium is repaired by circulating endothelial progenitor cells. Low serum levels of testosterone in men associate with reduced number of circulating endothelial progenitor cells, which suggest a role for androgens in re-endothelialization. Therefore, our group will investigate whether re-endothelialization after endothelial injury is impaired in male ARKO mice and whether this could contribute to the increased atherosclerosis demonstrated in these animals.

To further dissect the mechanisms for the AR-mediated effects of testosterone on a cellular level, it would be interesting to generate mice with cell-specific knockout of the AR, for example in smooth muscle cells, macrophages or endothelial cells, using mice that express Cre recombinase under cell-specific promoters (such as SM22, Lys, or Tie2). These mice would then be used in atherosclerosis studies to investigate the importance of the AR-pathway in specific cell types.

An issue of great importance is to perform further studies to elucidate the mechanisms that underlie the completely opposite effects of testosterone on atherosclerosis and AAA in male mice. One approach could be to run a microarray on aortas from ARKO and WT mice to identify candidate genes involved in these diseases that are differentially regulated. Further, cell-specific AR knockout models could provide valuable insight.

One approach to further investigate the pathways for the atheroprotective effect of testosterone would be to treat ER$\alpha^{-/-}$ mice with a physiological testosterone dose and determine the effect of testosterone

on atherosclerosis. The importance of genomic versus non-genomic effects of testosterone on atherosclerosis could also be studied by treating ER$\alpha^{-/-}$ mice with testosterone together with an AR-antagonist (e.g. flutamide).

Further research to define the role of 2-methoxyestradiol as a mediator of the anti-atherosclerotic actions of estradiol should be performed. Ongoing research in our group involves estradiol treatment to female ovariectomized COMT deficient and WT mice, and we hypothesize that the vascular protective actions of estradiol is attenuated in COMT deficient mice.

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