Roles of Androgens in Cardiovascular Physiology and Pathophysiology

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Cover illustration: A coronary angiogram showing a proximal total occlusion of the left anterior descending coronary artery.

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Dedicated to Arvin and Eira

“Research is to persistently ask why until it all makes sense.”

- ESE

Roles of Androgens in Cardiovascular Physiology and Pathophysiology

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ABSTRACT

Atherosclerosis is the major underlying cause of cardiovascular disease, such as myocardial infarction (MI) and stroke, and these conditions are the leading causes of death both in Sweden and globally. The immune system is involved in the pathogenesis of atherosclerosis as well as post-MI cardiac injury. Men develop cardiovascular disease approximately ten years earlier than women and the incidence of MI is higher in men throughout life. It has been suggested that the female sex hormones, estrogens, have cardioprotective properties while the roles of androgens, often described as male sex hormones, in cardiovascular physiology and pathophysiology are less understood. The underlying mechanisms and target cells for the cardiovascular effects of androgens are poorly described and to a large extent unknown. The aim of this thesis was to define roles and underlying mechanisms of androgens in cardiac physiology, atherosclerosis and MI and to identify target cells that mediate these actions in male mice.

The main findings of this thesis were that:

1) atherosclerosis induced by testosterone deficiency in male mice is thymus- and T cell dependent and that the thymic epithelial cell is likely the target cell for the anti-atherogenic actions of testosterone.
2) depletion of the androgen receptor (AR) in bone marrow stromal cells in male mice reduced post-MI neutrophil infiltration, mortality and adverse cardiac remodelling. Hence, we have identified the target cells for these androgenic effects. Further, we demonstrate that androgens promote neutrophil egress from the bone marrow by regulating leukocyte retention factors in bone cells.
3) androgen deficiency in male mice induces metabolic remodelling and expression of the fetal gene program in the heart.
4) concentrations of sex steroids in the heart do not merely reflect serum levels in male mice. We show that progesterone levels are much higher in the heart than in skeletal muscle and that the cardiac levels are reduced during the acute phase post MI.

This thesis demonstrates important effects of androgens on both the cardiovascular and immune systems. Our results provide potential explanations to sex differences in cardiovascular disease and how androgens can exert beneficial effects in some conditions (atherosclerosis) while detrimental in others (post-MI myocardial injury). Our data may pave the way for the development of selective AR modulators for safer treatment of prostate cancer. Further, our data raise new questions, including whether the AR in bone cells may be explored as a treatment target in acute MI and if drugs that reduce androgen levels, which are used in large patient groups, may positively affect the outcome of an MI.

Keywords: androgens, androgen receptor, atherosclerosis, myocardial infarction, immune system, SARMs, male mice

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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Åderförkalkning (ateroskleros) är den viktigaste orsaken till hjärt-kärlsjukdomar, såsom hjärtinfarkt och stroke. Hjärtkärlsjukdomar och deras konsekvenser medför ett stort lidande för individen, höga kostnader för samhället och är den vanligaste dödsorsaken både i Sverige och globalt. Män insjuknar i genomsnitt tio år tidigare än kvinnor i hjärt-kärlsjukdom och risken för hjärtinfarkt är högre för män under hela livet. Det har föreslagits att de kvinnliga könshormonerna, östrogener, utövar skyddande effekter på hjärtkärlsystemet medan effekter av androgener, som ofta kallas de manliga könshormonerna, är ett tämligen outforskat område. Könshormoner spelar komplexa roller i flera av kroppens organsystem och deras receptorer (mottagarmolekyler) finns på nästan alla celltyper i kroppen.

Syftet med denna avhandling var att undersöka androgeners effekter på hjärt-kärlsystemet och deras betydelse för sjukdom, samt att definiera underliggande mekanismer och målceller för dessa effekter.

Huvudfynden i avhandlingen var att;

1) ateroskleros orsakad av androgenbrist hos hanmöss är beroende av det immunologiska organet brässen och immunförsvarets T celler samt att celler i brässen sannolikt är målcellerna för androgeners hämmande effekt på aterosklerosutveckling.
2) hanmöss som saknar androgenreceptorn i specifika benceller har minskad infiltration av neutrofiler (vävnadsskadande immunceller) i hjärtat efter en hjärtinfarkt, lägre dödlighet och minskad risk för ogynnsam omformning av hjärtat. Därmed har vi identifierat målcellerna för androgeners skadliga effekter vid hjärtinfarkt.
3) androgenbrist hos hanmöss försämrar hjärtfunktionen under vissa förhållanden, ändrar hjärtats energiupptagskälla från fett (vilket det tar upp under hälsosamma förhållanden) till socker och omprogrammerar hjärtats genuttryck.
4) nivåerna av könshormoner i hjärtmuskel inte enbart speglar nivåerna i blodet och att könshormonnivåerna i hjärtat ändras under den akuta fasen av en hjärtinfarkt.

Sammantaget visar resultaten i avhandlingen på viktiga effekter av androgener både på hjärt-kärl- och immunsystemen. Våra fynd kan bidra till att förklara könsskillnader vid hjärt-kärlsjukdom och varför androgener kan ha positiva effekter på vissa hjärt-kärlsjukdomar (åderförkalkning) men negativa effekter på andra (hjärtmuskelskada vid hjärtinfarkt). Resultaten kan även komma att få viktig betydelse för utvecklingen av säkrare skräddarsydda läkemedel vid prostatacancer, där en stor del av behandlingen syftar till att sänka androgennivåerna. Vår forskning ger även upphov till nya frågeställningar, till exempel om läkemedel som minskar androgennivåer kan ha en positiv inverkan på konsekvenserna av en hjärtinfarkt.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Wilhelmson AS, Lantero Rodriguez M, Svedlund Eriksson E, Johansson I, Fogelstrand P, Stubelius A, Lindgren S, Fagman JB, Hansson GK, Carlsten H, Karlsson MCI, Ekwall O, Tivesten Å.
Testosterone Protects Against Atherosclerosis in Male Mice by Targeting Thymic Epithelial Cells- Brief Report. Arterioscler Thromb Vasc Biol. 2018 Jul;38(7):1519-1527

II. Svedlund Eriksson E, Lantero Rodriguez M, Johansson I, Mårtensson AKF, Wilhelmson AS, Karlsson MCI, Hagberg Thulin M, Redfors B, Borén J, Omerovic E, Levin MC, Chagin AS, Tivesten Å.
Protection from Post-Myocardial Infarction Complications by Androgen Receptor Depletion in Bone. In manuscript.

III. Svedlund Eriksson E, Johansson I, Mårtensson AKF, Lantero Rodriguez M, Schilperoort M, Kroon J, Kooijman S, Omerovic E, Andersson L, Levin MC, Rensen PCN, Tivesten Å.
Castration of Male Mice Induces Metabolic Remodeling of the Heart. J Endocr Soc. 2022 Sep 1;6(11):bvac132.

IV. Svedlund Eriksson E, Johansson I, Poutanen M, Landin A, Norlén AK, Ryberg H, Levin MC, Ohlsson C, Tivesten Å.
Sex Steroids in the Heart of Male Mice. In manuscript.

CONTENTS

ABBREVIATIONS

ADT	androgen deprivation therapy
AR	androgen receptor
ARKO	androgen receptor knockout
BM	bone marrow
cDNA	complementary DNA
CV	cardiovascular
CVD	cardiovascular disease
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
G-CSF	granulocyte colony stimulating factor
GnRH	gonadotropin-releasing hormone
HDL	high density lipoprotein
LDL	low density lipoprotein
LH	luteinizing hormone
MI	myocardial infarction
ORX	orchiectomy; castration
RT-PCR	real-time PCR
SARM	selective androgen receptor modulator
SHBG	sex hormone-binding globulin
STEMI	ST-elevation myocardial infarction
TECs	thymic epithelial cells
Treg	regulatory T cells
TRT	testosterone replacement therapy
VLDL	very low-density lipoprotein

1 INTRODUCTION

1.1 SEX HORMONES

Steroid hormones are a group of hormones, derived from cholesterol, that acts as chemical messengers in the body. On the basis of their receptors, the hormones are categorized into five major groups: androgens, estrogens, progestogens, glucocorticoids and mineralcorticoids. Androgens, estrogens and progesterone are classified as sex hormones. In this thesis the focus lies on androgens. Sex hormones are produced as precursors or active hormones in the testes and ovaries, the adrenal cortex, and the placenta during pregnancy.

1.2 ANDROGENS

Testosterone, the main circulating androgen, is synthetized mainly by Leydig cells in the testes in males, in response to anterior pituitary luteinizing hormone (LH). Synthesis and release of LH is under the control of the hypothalamus trough gonadotropin-releasing hormone (GnRH) and inhibited by testosterone via a negative feedback mechanism (Fig. 1). The blood levels of testosterone are approximately 10 times higher in men compared to women [1].
Adrenal androgens, such as dehydroepiandrosterone (DHEA) and androstenedione, are steroid hormones with weak androgenic activity that provide a circulating pool of precursors for peripheral conversion into the more potent androgens and estrogens (Fig. 2). Because of the lipophilic properties of sex hormones, they are not soluble in plasma. Instead, they are bound to transport proteins that increase their half-life and insure ubiquitous distribution. The majority of circulating androgens are bound to the transport proteins sex hormone-binding globulin (SHBG) or albumin and only 1-3% is free, active and able to diffuse over cell membranes to activate receptors. Testosterone and its ten times more potent metabolite dihydrotestosterone (DHT) mediate their actions via the androgen receptor (AR). The AR is almost ubiquitously expressed in mammalian tissues [2]. By binding to the AR, androgens can act rapidly within seconds to minutes by non-genomic actions that activates cell signalling pathways, or more slowly within hours through genomic actions by regulating gene transcription [3].

Testosterone and DHT are required for the development and maintenance of the male reproductive system and secondary sex characteristics. The hormones also have a diverse range of important biological actions in the musculoskeletal, cardiovascular, immune, neural and haemopoietic systems. This thesis focuses on roles of androgen in the cardiovascular and immune systems.

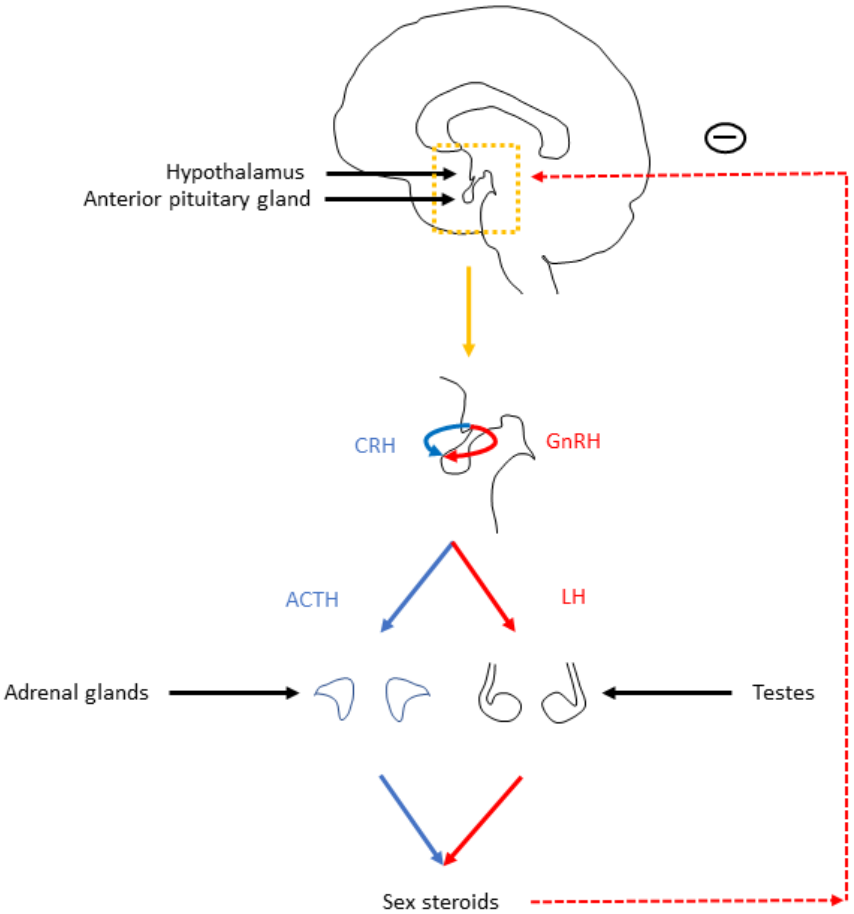


Figure 1. The production of androgens is controlled by the hypothalamic-pituitary-gonadal axis (red) and hypothalamic-pituitary-adrenal axis (blue). GnRH (gonadotropin-releasing-hormone); LH (luteinizing hormone); CRH (corticotropin-releasing-hormone) and ACTH (adrenocorticotropic hormone).

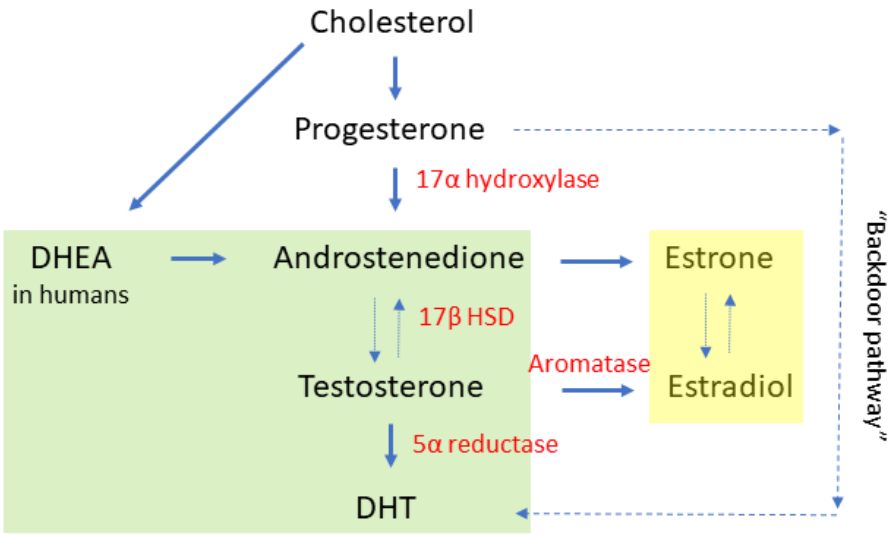


Figure 2. Schematic presentation of sex steroid metabolism. Androgens are indicated in green, estrogens in yellow and metabolizing enzymes are marked in red. Androstenedione can be converted either from DHEA or progesterone to testosterone by the enzyme 17βHSD. Testosterone can be further converted to dihydrotestosterone (DHT) by 5α-reductase or aromatized by aromatase in to estradiol, the most potent estrogen. DHT can also be converted directly from progesterone through a "backdoor pathway" involving several enzymatic processes.

1.2.1 Androgens and the cardiovascular system

The cardiovascular system consists of the heart and the blood vessels and cardiovascular disease (CVD) is a general term for conditions that affect the structure and function of these organs. CVDs are the leading cause of death globally and more than four out of five CVD deaths are due to myocardial infarction and stroke [4].

The question whether androgens play detrimental or beneficial roles in the CV system in health and disease has been debated for decades. On the one hand, men have higher risk of developing CVD compared to age-matched women [5], which may support the hypothesis of harmful effects of androgens in men. On the other hand, numerous clinical studies have reported that low testosterone levels are associated with both CV risk factors and increased mortality [5-8], supporting protective roles of androgens on the CV system. These results are in line with data from both animal and in vitro studies. Testosterone have been shown to protect the myocardium from ischemia/reperfusion injury in rats [9] and to cause vasodilatory effects by inducing relaxation of vascular smooth muscle cells in both human and animal studies [10]. In cultured endothelial cells, testosterone has been shown to increase proliferation and viability [11] and may thereby enhance endothelial repair in vascular

damage. Testosterone has also been shown to stimulate production of proteins involved in the lysis of blood cloths and to stimulate production of anti-inflammatory cytokines [12]. These results are in line with clinical data showing associations between low testosterone levels and reduced levels of anti-thrombotic and anti-inflammatory proteins in humans [12]. In conflict with these results, several studies suggest that increased plasma levels of androgens are associated with increased levels of pro-inflammatory cytokines in humans [13, 14]. Findings also suggests that androgens enhance adhesion of monocytes to endothelial cells [15-18], an initial stage of vascular inflammation that can lead to the development of atherosclerosis.

Supraphysiological doses of testosterone due to misuse of anabolic steroids is well known to cause detrimental cardiac hypertrophy [19, 20]. In animal studies, high doses of testosterone have been shown to cause cardiac fibrosis and apoptosis [21].
Testosterone replacement therapy (TRT) in testosterone deficient men has been shown to exert beneficial effects on lipid profile and may thereby protect from atherosclerosis-related disease [22]. Accordingly, a meta-analysis showed decreased CV risk in men with coronary artery disease and heart failure treated with testosterone [23]. Also, a randomized clinical trial showed improved cardiac function in men with stable angina treated with a low dose of testosterone [24]. Some clinical studies have, on the contrary, reported increased CV events and mortality in men with various comorbidities and TRT [25, 26]. However, these studies have been strongly questioned because of the retrospective design and methodological flaws [27].

Taken together, sub- and supraphysiological levels of androgens seem to be associated with increased CV risk and dysfunction while levels within the physiological range seem to exert protective effects on the CV system. However, conflicting data could possibly be explained by a context-dependent role of androgens. Multiple factors such as genetic and epigenetic variations, comorbidities, hormone interactions and route of testosterone administration likely interact and affects the outcome. To provide clear answers to the roles of androgens in cardiac physiology and pathophysiology the underlying mechanisms must be dissected and studied in detail.

1.2.2 Androgen deprivation therapy

Androgen deprivation therapy (ADT) is the standard treatment for patients with advanced prostate cancer. Pharmacological ADT refers mainly to treatment with GnRH-agonist or GnRH antagonist. GnRH agonists bind to GnRH receptors in the anterior pituitary, causing a continuous release of GnRH. The increase of GnRH causes a transient surge in LH and thereby increased testosterone production from the Leydig cells. Subsequently, the negative feedback mechanism cause downregulation of GnRH receptors and reduction of testosterone to castrate levels. GnRH antagonists binds to GnRH receptors and inhibit the release of LH without an initial testosterone “flare”. Testosterone levels decrease within a few days,

significantly faster than with GnRH agonists. Meta-analyses demonstrate positive associations with MI, stroke and CV death and GnRH agonists [28, 29]. These effects are not consistently reproducible in randomized controlled trials [29]. Animal and human studies suggest that the mechanisms by which ADT increases CV risk include increased atherosclerosis, dyslipidemia, metabolic syndrome, and insulin resistance [29].

1.2.3 Selective androgen receptor modulators

Except for increased CV risk, adverse side effects of ADT also include decreased bone mineral density and muscle mass, decrease libido and sexual function, hot flashes, gynecomastia, anemia and fatigue [30]. Selective androgen receptor modulators (SARMs) are drugs that can exert either agonistic or antagonistic effects on the AR in specific tissues with minimized side effects. These drugs are under development (referens). An important step for the design of SARMs for use in CV disease and/or minimize CV side effects is to identify target cell(s) for the cardiovascular actions of androgens.

1.3 THE IMMUNE SYSTEM

The immune system has two primary lines of defence; innate immunity and adaptive immunity. The former is a rapid, cellular response, initiated within minutes to hours after the aggression of an intruding pathogen. The latter develops, within days to weeks, a more specific response and an immunological memory which can be activated rapidly upon subsequent re-exposure of a stimulus.

1.3.1 Neutrophils and bone marrow stroma

Neutrophils are essential for innate immunity and resistance to microbial pathogens. They are produced and stored mainly in the bone marrow (BM) [31]. Upon an inflammatory stimulus the neutrophils leave the BM and arrive in massive numbers at the site of inflammation within a few hours.

Neutrophils express chemokine receptors that antagonistically regulate retention and egress from the BM [31]. BM stromal cells, most of which derive from cells that express the transcription factor osterix, are a major source of CXCL12. CXCL12 is the ligand to the retention receptor CXCR4 on neutrophils [32-34]. In response to an inflammatory/infectious stimulus, granulocyte-colony stimulating factor (G-CSF) is produced by the inflamed tissue and within the BM. G-CSF reduce the level of CXCL12 in the BM, which attenuates CXCR4 signalling. In parallel, expression of the cytokines CXCL1 and CXCL2 increase in the BM. These cytokines bind to the receptor CXCR2 on neutrophils, which facilitates egress from the BM [31].

Homeostasis of granulopoiesis, egress from the BM and clearance of senescent neutrophils must be tightly regulated since an exaggerated neutrophil response may be detrimental to the host. For example, elevated neutrophil infiltration in the myocardium post MI have been

shown to increase risk of ventricular dilatation [35], which is a well-known state that precede heart failure.

1.3.2 B cells and T cells

There are two types of adaptive immunity; humoral and cell-mediated. B cells are responsible for the humoral immunity via the production of antibodies while cell-mediated immunity mainly depends on T cells. Antibodies either mark virus for destruction or prevent infection by blocking virus-host cell interactions. T cells can either directly eliminate infected cells or indirectly by altering the surrounding microenvironment and by activating macrophages. T cells derive from hematopoietic stems cells in the BM. T cell progenitors migrate from the BM to the thymus where they mature. When antigens are presented to T cells, they differentiate primarily to either cytotoxic T cells (CD8+ cells) or T-helper cells (CD4+ cells). Cytotoxic T cells are mainly involved in the destruction of infected cells and tumour cells. T-helper cells mediate the immune response by directing other cells to execute cytotoxic or phagocytic functions.
Thymic epithelial cells (TECs) account for the majority of stromal cells in the thymus. TECs are essential for the development and maturation of T cells [36].

B cells are classified into two subpopulations B1 and B2. B1 originate from the fetal liver while B2 are produced in the BM. BM lymphopoiesis produce immature B cells which migrate to the spleen for further maturation. B1 are predominately found in the peritoneal and pleural cavities, where they produce “natural antibodies” and secrete immunomodulatory molecules as part of the innate immune system. Natural antibodies are formed without specific immunization and bind to bacteria and self-antigens, such as damaged and apoptotic cells.
BM stromal cells are necessary for the maturation and migration of B2 through the BM compartment [37]. Mature B2 cells circulate between secondary lymphoid organs in search of antigens. When B2 cells are activated by T-helper cells, they can either differentiate into plasma cells or memory cells. Plasma cells are short-lived and provide a rapid response to antigen while memory cells are long-lived survivors of earlier infections. Memory cells can be called upon at a re-infection in which they quickly respond by producing highly specific antibodies directed at the pathogen.

1.3.3 Immunity in atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the vascular wall and the most common underlying cause of MI and stroke. The atherosclerotic process starts by damage to endothelial cells due to hyperlipidemia, hypertension, turbulent blood flow and/or hyperglycemia. This causes retention of low-density lipoprotein (LDL) in the subendothelial space and over-expression of endothelial adhesion molecules that captures monocytes in the vascular wall (Fig. 3). Reactive oxygen species oxidize LDL which attracts more cells from both the innate and adaptive immune system. Macrophages engulf oxidized LDL which turn them into foam cells. Senescent foam cells, accumulated calcium and eventually proliferated

smooth muscle cells build up an atherosclerotic plaque with a necrotic core. When the endothelium above the lesion is damaged, the plaque may rupture and cause thrombotic occlusion of the artery and subsequent ischemic injury. Clinical manifestations of atherosclerosis are highly correlated with plasma levels of cholesterol (total cholesterol, LDL and apolipoproteins) [38].

T lymphocytes are present in the adventitia at early stages of the atherosclerotic process, and later also in the plaques. Interactions between macrophages and T cells lead to a cascade of atherogenic events. T helper 1 cells (Th1) are suggested to have pro-atherogenic properties while regulatory T cells (Treg) act anti-atherogenic in early stages. However, as the disease progresses, a switch to a more pro-atherogenic phenotype seems to occur [39]. If this switch represents a cause or a consequence of atherosclerosis is not yet known.

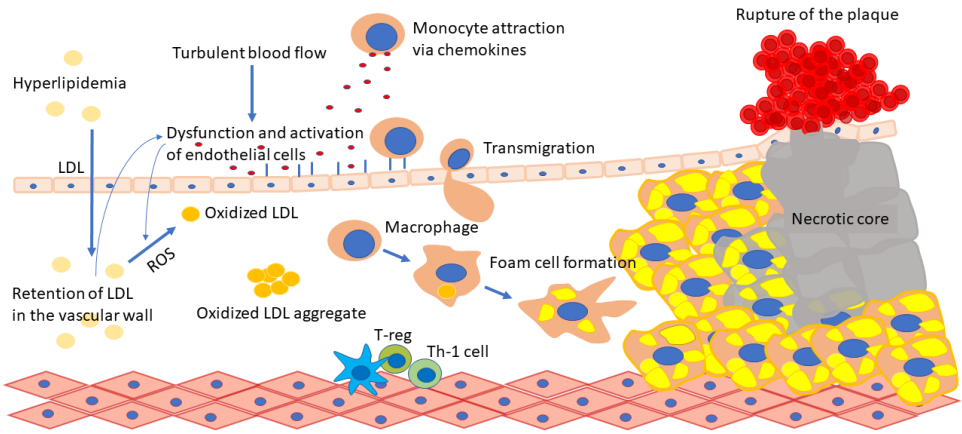


Figure 3. Schematic illustration of the atherosclerotic process. LDL, low density lipoprotein; ROS, reactive oxygen species; T-reg, regulatory T cell; Th-1 cell, T helper 1 cell.

1.3.4 Roles of androgens in the immune system

Several studies suggest immunosuppressive roles of androgens such as stimulation of anti-inflammatory cytokine production, suppression of T cell proliferation and antibody production [40]. The immunosuppressive functions of androgens may contribute to the sexual dimorphism seen in autoimmune- and infectious diseases. Men are generally more susceptible to infections and less prone to autoimmunity compared to women [41, 42].

The AR have been shown to be expressed by all myeloid progenitor cells, indicating a vast potential for androgen modulation on the development and function of the immune system.

Besides direct effects, indirect effects of androgens have been shown by suppression of B lymphocyte development through BM stromal cells [43] and by regulation of the B cell survival factor BAFF [44]. In accordance with this data, androgen deficient disorders are associated with increased B cell numbers which is normalized by TRT [45, 46].
We have previously shown that global AR depletion in mice increase BM B cell number and also splenic B cell numbers [44] In the O-ARKO model, in which the AR is depleted from osterix-expressing bone cells, we saw increased BM B lymphopoiesis but the splenic B cell number was not altered, suggesting that the regulation of splenic B cell numbers is independent of androgen actions in the BM [47].

CXCR4 have been shown to be expressed in B cells during several developmental stages [48]. Previous studies have shown that CXCR4 or CXCL12 deficient mice do not develop B lymphopoiesis [48]. These findings suggest that CXCL12/CXCR4 are important for B cell development and immunity. In inflammatory conditions, reduced levels of CXCL12 promote granulopoiesis over lymphopoiesis [49]. Hence, steady state levels of CXCL12 in the BM might retain neutrophils while promoting B lymphopoiesis.

Furthermore, it is well known that androgens contribute to the decrease of thymus size that occurs during puberty. In androgen deficient disorders, such as Klinefelter syndrome and in hypogonadal men, thymus size and thymopoiesis are increased while these effects are reversed by TRT [50-54]. We have previously shown that the thymic epithelium is a target compartment for androgen/AR-mediated regulation of thymopoiesis [55].

1.3.5 Androgens and neutrophils

Androgens have been shown to increase neutrophil infiltration at inflammatory sites [35, 56] and TRT have been shown to increase neutrophil counts in men [57]. This is in line with decreased neutrophil numbers seen in castrated mice and ARKO mice [40]. In accordance, reduced numbers of neutrophils are seen in patient treated with ADT due to prostate cancer [57].
ARKO mice have been shown to respond less to G-CSF and produce lower levels of pro-inflammatory cytokines [40]. Hence, androgens seem to be of importance for both development and functions of neutrophils.

2 AIM

The general aim of this thesis was to investigate roles and underlying mechanisms of androgens in cardiovascular physiology and pathophysiology.

The specific aims of the papers included in the thesis were;

I: To test the hypothesis that atherosclerosis induced by testosterone deficiency in male mice is T cell dependent and that depletion of the androgen receptor in thymic epithelial cells increase atherosclerotic burden.

II: To test the hypothesis that depletion of the androgen receptor in bone marrow stromal cells reduce neutrophil infiltration in the myocardium and protect male mice from adverse cardiac remodeling post myocardial infarction.

III: To test the hypothesis that testosterone deficiency in male mice induces metabolic remodeling and expression of the fetal gene program in the heart.

IV: To test the hypothesis that the relative levels of sex steroids in the heart differ from other compartments and that sex steroid concentrations in the heart are affected by a myocardial infarction.

3 METHODOLOGICAL CONSIDERATIONS

In this section, the methods in this thesis are discussed in general. Detailed information is presented in the Material and Methods sections of each individual paper.

3.1.1 The mouse as an experimental model for human sex steroid physiology

Mouse models are useful tools for investigating underlying mechanisms and clinical questions that are difficult or impossible to address in humans. In this thesis, two different models were used to study androgen actions; removal of endogenous testosterone by castration and cell-specific inactivation of the AR.

Mouse and human androgen physiology differ in several ways. For example, SHBG is lacking postnatally in mice and androgens are bound only to albumin in plasma. This explains the low and fluctuating total testosterone concentrations seen in serum and pronounced intra-individual variations in mice [58]. Also, mice do not produce DHEA from the adrenal glands [59] and castration renders the mice completely testosterone deficient within a few days.

Further, when laboratory male mice are housed in groups, each individual has a unique dominance rank. Subordinate mice have significantly lower testosterone levels and higher levels of corticosterone than the alpha-cage mate. Thus, the structure of dominance hierarchy may have effects on phenotypic differences within and between cages [60].

3.1.2 Castration and testosterone replacement

The castration model (orchiectomy; ORX) was used in paper I-III. In paper I the mice were ORX and implanted with a subcutaneous slow releasing pellet containing vehicle/placebo (Cat No. SC-111) or a physiological dose of testosterone (25 µg/d; Cat No. SA-151 Innovative Research of America, Sarasota, FL) [61]. In paper II, mice were injected subcutaneously with vehicle (pure 16 corn oil, Cat No. C8267, Sigma) or a physiological dose of testosterone propionate (3 mg/kg/day; 17 Cat No. 86541, Sigma) every three days, for three weeks [62]. Success of ORX or testosterone replacement was verified by weight of the androgen sensitive seminal vesicles at the end of the studies.

3.1.3 Inactivation of the androgen receptor

In paper I and II we used the Cre-LoxP recombination system in order to inactivate the AR specifically in thymic epithelial cells (E-ARKO) and osterix expressing bone cells (O-ARKO), respectively. The AR is located on the X chromosome. ARKO mice were generated by breeding $AR^{+}/^{flox}$ females with $Cre^{+/-}$ males. Cre was under the control of the promotor K5-Cre [63] (created by Dr Ramirez, CIEMAT, Madrid, Spain; transferred from Dr Rognoni, Max Planck Institute, Germany) in the E-ARKO model and Osx-1-Cre (Jackson Laboratory, Bar Harbor, Maine, USA) in the O-ARKO model. E-ARKO were generated on an apoE constitutive knockout background (B6.129P2-Apoetm1UncN11; Taconic) and all mice in all experiments were on a C57BL/6J background. The efficacy and cell specificity of the ARKO were assessed by quantifying exon 2, the floxed exon, compared to exon 3 in genomic DNA (Fig. 4).

Many strains expressing Cre have phenotypes that were not intended when the models were generated. At birth, Osx-cre$^{+/-}$ mice have defects of craniopharyngeal bones and teeth, which result in reduced body weight [64]. These defects are generally normalized by the onset of adulthood. In paper II, Osx1-Cre and O-ARKO mice were fed soaked chow and their teeth were regularly inspected and cut if needed. To ensure accurate interpretation of the observed phenotypes, Osx1-Cre$^{+/-}$ littermates were used as controls in all experiments.

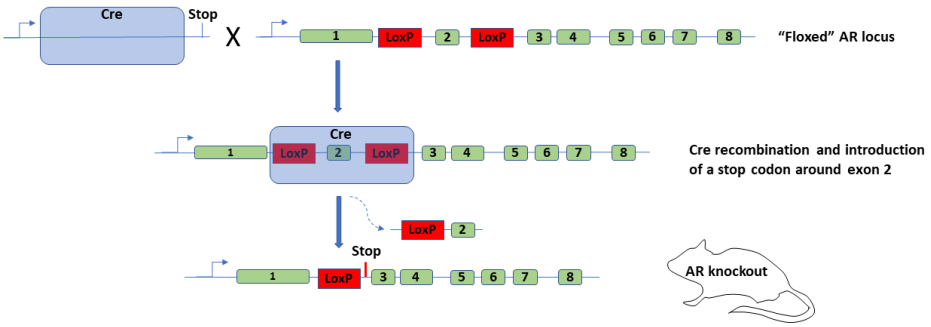


Figure 4. An overview of Cre-LoxP recombination system used for generating cell-specific androgen receptor (AR) knockout mice. The enzyme Cre recombinase recognizes the loxP sites, excises the floxed loci and inactivates the AR by introducing a stop codon before exon 3.

3.1.4 Dietary considerations

Phytoestrogens are plant-derived compounds, structurally and functionally analogous to estrogens. Soy bean and soy products are the richest source of phytoestrogens. In order to avoid potential effects on hormonal responses, all mice were kept on a soy-free regular chow diet (2016 Teklad Global 16% Protein Rodent Diet, Harlan, UK; RM3 € Soya free, Special Diet Services, UK or R70, Lantmännen Lantbruk, Sweden).

3.1.5 Mouse models of atherosclerosis

Mice exhibit major differences in lipid metabolism compared to humans [65]. For example, mice lack cholesteryl ester transfer protein, and subsequently carry most plasma cholesterol in high density lipoprotein (HDL), while humans carry most of it in LDL. Also, the bile composition in mice is different from that in humans, resulting in reduced uptake of cholesterol in the intestine and thereby low plasma cholesterol. Taken together, this may

explain why mice are resistant to developing atherosclerosis. A combination of dietary and genetic manipulations has enabled mice to be useful tools in atherosclerosis research. In paper I we used ApoE$^{-/-}$ mice, the most extensively used mouse model of atherosclerosis. ApoE is a glycoprotein that acts as a ligand for lipoprotein receptor. The protein clears the plasma from chylomicrons and very low-density lipoprotein (VLDL) remnants and is also involved in dietary absorption- and biliary excretion of cholesterol. Deletion of the ApoE gene results in cholesterol levels 4-8 times higher than in wild type mice. On western diet ApoE$^{-/-}$ mice form foam cell-rich lesion containing cholesterol crystals, necrotic cores and calcifications within 12-16 weeks. There are several limitations to mouse models of atherosclerosis, apart from the differences in cholesterol metabolism and lipid profile. First, plaques in humans develop in the coronary and carotid arteries, and progress to larger fibrous atheromas. In mice, lesions are mainly localized in the aortic sinus, proximal aorta, aortic arch and brachiocephalic trunk and progress to less advanced stages [66]. Not even genetically modified mice develop plaque rupture. However, older ApoE$^{-/-}$ mice have been shown to exhibit features of vulnerable human plaques, including formation of a necrotic core, erosion of necrotic mass to the lumen and intraplaque hemorrhage [67].

3.1.6 RNA isolation and real-time qPCR

In paper II and III, gene expressions were determined by real-time PCR (RT-PCR). In this method, RNA is transcribed into complementary DNA (cDNA) from mRNA by reverse transcriptase. The cDNA is then used as a template for the qPCR reaction. Levels of mRNA of the gene of interest can be compared to a reference gene, which gives an estimate of the relative gene expression. The relative gene expressions were calculated using the $2^{-\Delta\Delta ct}$ method.

3.1.7 AR DNA quantification

In paper I-III, the efficacy of the AR knockout models was determined by comparison of the excised exon 2 to in the AR gene to the presence of exon 3. Genomic DNA from different tissue compartments was isolated using the DNeasy Blood and Tissue kit (Qiagen). DNA amplification was analysed using Real-Time PCR System (Applied Biosystems). The $2^{-\Delta\Delta ct}$ method was used to normalize the relative gene expression of Ar exon 2 to Ar exon 3.

3.1.8 Evaluation of atherosclerosis

Atherosclerosis in mice is commonly assessed *ex vivo* in aortas prepared *en face* or in sections of the aortic root. *En face* assessment provide information about plaque burden and distribution throughout the aorta but does not allow examination of plaque composition. Cross-sections of the aortic root provide information on both lesion size and characteristics. In paper I, we used histological- and immunohistochemical techniques to examine cross-sections of the aortic root and the presence of T cell infiltration (CD3) and leukocyte subclasses (CD18) in the vascular wall.

BioPix Software was used for morphometric analysis of the atherosclerotic lesion size. The number of anti-CD3-stained cells was counted manually. All evaluations were performed by a blinded observer.

3.1.9 Mouse model of myocardial infarction

In paper II and IV we used a model of permanent ligation of the left anterior descending artery in order to induce MI. This model is often criticized since it is widely believed that almost all patients with ST-elevation myocardial infarction (STEMI) are revascularized. However, studies have shown that 15-30% of STEMI patients are admitted to the hospital too late for reperfusion therapy [68, 69] and that 50% of STEMI patients do not receive reperfusion treatment when minority populations and patients admitted to smaller regional hospitals are taken into account [70, 71]. The permanent ligation model is therefore directly applicable to these patients. However, neutrophils are recognized as a key component also in ischemia-reperfusion injury which make the results of our studies potentially relevant for this therapeutic procedure too.

3.2.0 Echocardiography

In paper II, MI size and cardiac function was evaluated using a VEVO 2100 echocardiography system (VisualSonics, Ontario, Canada) and a 45-MHz linear transducer (RMV 704). The mice were anesthetized with 1.1% isoflurane, and placed in a supine position on a heating pad with their paws connected to ECG sensors. The probe was rotated 90° from an optimal parasternal long axis projection and cine loops (> 1000 frames/s) was acquired using the ECG-gated kilohertz technique at exactly 1, 3 and 5 mm below the mitral annulus. Stored data were analyzed with Vevo® LAB desktop software version 5.5.0 in a blinded fashion. Cardiac function parameters were calculated according to Simpson's method. Area at risk and MI size was calculated from a 16 segments method, based on the three acquired short axis cine loops.

3.2.1 T cell and neutrophil depletion experiments

In paper I, we performed T cell depletion by using a CD3 antibody (clone 145-2C11 f[ab']2 fragments, Cat No. BE0001-1FAB; BioXCell) or a control antibody (hamster IgG f[ab']2 fragments, Cat No. BE0091-FAB; BioXCell), as described previously [72].
In paper II, we used 1A8 (BE0089, BioXcell), a Ly6G-specific antibody or a rat IgG2a Isotype control (BE0089, BioXcell). A limitation of the model is that newly produced neutrophils have lower ly6G membrane expression and are consequently reduced targets for depletion [73]. This may explain why some neutrophils still could be detected after the depletion in our experiment. Because of the rapid maturation of neutrophils in the BM (2.3 days) and short time of circulation in peripheral blood (0.75 days), we injected the mice every day during the experiments.

3.2.2 Preparation of cells for flow cytometry

To harvest single cells from bone and BM, spleen and myocardium for flow cytometry, tissues were digested and erythrocytes were lysed in an ammonium chloride buffer. The cells were washed and resuspended in FACS buffer and then passed through a 70 µm cell strainer and counted in a NucleoCounter NC-100 (Chemimetec) or Sysmex KX-21 (Sysmex Corporation).

3.2.3 Neutrophil mobilization from bone marrow

In paper III, intraperitoneal injections of G-CSF (filgrastim, Neupogen®, Amgen) were used to mobilize neutrophils from the BM. G-CSF or vehicle were given two times per day, in total five injections. Blood was collected for flow cytometry one hour after the last injection.

3.2.4 Flow cytometry

In paper I and II, flow cytometry was used to analyse phenotypes of different immune cells. Fc-blockage (anti-mouse CD16/CD32, BD bioscience), was first performed to ensure that only detection of antigen specific binding was observed. Fluorochrome-conjugated antibodies was used to detect expression of different cell-surface markers. Cells were analysed in an Accuri C6 or FACS Aria (BD Bioscience) and the relative proportion of the different cell types was determined by FlowJo software (Tree Star, Ashland, OR, USA). Numbers of cell subsets were calculated from the total number of cells in the different organs.

3.2.5 Gating strategies for neutrophils

Figure 1G (blood): Neutrophils were depleted with Ly6G and in the flow cytometry gated as SSC-hi and CD11b+. Since also activated monocytes and eosinophils can have high SSC, a few of these cells may be mistaken for neutrophils. However, the number of eosinophils and activated monocytes in BM are very low compared to neutrophils.

Figure 2 and 3 (heart) and Figure 5C-E (blood and spleen): Cells from the left ventricles were MACS-sorted for $CD45^+$. Neutrophils from left ventricle, blood and spleen were all gated as CD45+, SSC-hi and Gr1-hi (monocytes as CD11b+, Gr1-low, F4/80- and macrophages CD11b+, F4/80+). At the time of this experiment, Ly6G was not established as the most relevant marker for neutrophils.

In figure 5F-G (blood) we identified neutrophils as CD11b+, Ly6G+.

3.2.6 Fatty acid- and glucose uptake in the myocardium

In paper III, we investigated lipid- and glucose uptake in the myocardium by the uptake of [14C]deoxyglucose and fatty acids derived from glycerol tri[3 17H]oleate-labeled 18 triglyceride-rich lipoprotein-like emulsion particles (80 nm) as described previously [74]. The emulsion particles consist of a hydrophobic core of neutral lipids (triolein, cholesteryl oleate) surrounded by a monolayer of phospholipids (egg yolk phosphatidylcholine, cholesterol lysophosphatidylcholin), which makes them imitate chylomicrons *in vivo* [74].

3.2.7 Immunoblotting

In paper III, western blot was used to analyze specific protein expression in the left ventricle. Left ventricles were homogenized and proteins were extracted using Qproteome Mammalian Protein Prep Kit (Qiagen). Immunoblots were visualized with Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore) and a ChemiDoc Touch Imaging System (BioRad). Bands were quantified with Image Lab Software (Bio-Rad) and normalized to the reference gene HPRT1.

3.2.8 Mass spectrometry (GC-MS/MS)

In paper I and IV, serum-, myocardial- and skeletal muscle levels of sex steroid were determined using an in-house gas chromatography-tandem mass spectrometry assay as described previously [59]. The tissues were harvested and kept in -80 °C for later analysis. The tissues were then thawed on ice. 125 mg of myocardium and skeletal muscle was used for preparation and serum samples were adjusted to a volume of 450 µL with deionized water. Sex steroids were extracted, derivatized and quantified as described previously [59]. LLOQ for the assay was 56, 6, 8 and 3.75 pg/mL (in mouse serum) and 75, 7.5, 20 and 8 pg/g (in cardiac and skeletal muscle) for progesterone, androstenedione, and testosterone and DHT, respectively.

4 RESULTS AND CONCLUSIONS

Paper I: Testosterone Protects Against Atherosclerosis in Male Mice by Targeting Thymic Epithelial Cells

In this study, we tested the hypothesis that atherosclerosis caused by testosterone deficiency in male mice is T cell dependent. Further, we hypothesized that androgen receptor depletion in thymic epithelial cells results in increased atherosclerosis.

We found that castration of adult male mice resulted in almost doubled thymus weight after seven days compared to controls. Both thymic medulla and cortex area were increased. Prepubertal castration had similar effect and the effect remained during aging. Castration increased CD4+ T cells in blood and spleen with a similar trend for CD8+ T cells. Testosterone replacement therapy inhibited castration effects on thymus weight and CD4+ and CD8+ T cell numbers.

Castration increased atherosclerosis in male apoE -/- mice. Treatment with a T cell-depleting antibody (anti-CD3) did not affect body weight, weight of the seminal vesicles or thymus, cholesterol levels and had alone no effect on atherosclerotic lesion size. However, T cell depletion prevented castration-induced atherogenesis.

E-ARKO displayed increased thymic weight and the effect was comparable with that found in castrated mice. Further, E-ARKO apoE -/- mice showed more than doubled atherosclerotic lesion size at 16 weeks of age compared to controls. We found no differences in serum cholesterol and triglyceride levels and there were no differences in plaque composition (collagen, neutral lipids, relative macrophages content). We found that the relative numbers of T cells were unchanged in the plaque but increased in the adventitia of E-ARKO mice. Prepubertally thymectomized E-ARKO mice showed no atherosclerosis phenotype at 16 weeks of age.

We conclude that atherosclerosis induced by testosterone deficiency in male mice is thymus and T cell dependent and that the thymic epithelial cell is likely the target cell for antiatherogenic actions of testosterone.

Paper II: Protection from Post-myocardial Infarction Complications by Androgen Receptor Depletion in Bone

In this study we tested the hypotheses that depletion of the AR in BM stromal cells reduces neutrophil infiltration in the myocardium and protects male mice from adverse effects post MI.

We first confirmed that castrated male mice have increased survival due to reduced frequency of cardiac rupture compared to sham-castrated (rupture rate was 7% vs. 44% in castrated and sham-castrated mice, respectively). Castrated mice showed reduced volume of the left ventricle in diastole (-26%) at three weeks post MI.

Castrated mice showed approximately 50% reduction of neutrophil numbers in the left ventricle 48h post MI, while numbers of monocytes and macrophages were unaltered. Injection of the neutrophil depleting antibody 1A8 protected against cardiac dilatation two weeks post MI compared to mice injected with isotype. 1A8 did not add any significant protective effect to left ventricle volume in castrated mice.

Depletion of the AR in BM stromal cells (O-ARKO model) completely mimicked the castration effects on neutrophil infiltration in the heart, cardiac rupture (0% of O-ARKO vs. 42% of control mice) and dilatation of the left ventricle.

Further, we found that G-CSF reduced neutrophil numbers in O-ARKO by approximately 65% while there were no differences in hematopoietic stem and progenitor cells, T cells or B cells in blood. We saw no increased neutrophil activation in blood or difference in neutrophil numbers in the BM at steady state.

Non-hematopoietic BM cells from castrated mice showed increased mRNA levels of the leukocyte retention factors *Cxcl12* and *Angpt1* and O-ARKO mice showed increased levels of Angpt1 mRNA with a similar trend for *CxCl12* mRNA. Further, we demonstrate that the AR agonist DHT reduced Cxcl12 mRNA in osteolinage cells in vitro.

In conclusion, androgens regulate leukocyte retention factors in bone cells and promote neutrophil egress from the BM by targeting BM stromal cells of the osteo-lineage. This facilitated egress increases post-MI neutrophil infiltration, adverse cardiac remodelling and mortality in male mice.

Paper III: Castration of Male Mice Induces Metabolic Remodeling of the Heart

In this study we tested the hypothesis that testosterone deficiency in male mice induces metabolic remodeling and expression of the fetal gene program in the heart.

We observed reduced heart weight adjusted to body weight (-6%) in castrated mice and that this effect was inhibited by testosterone replacement. During pharmacologically induced stress, castrated mice presented reduced heart rate (-7%), stroke volume (-18%), cardiac output (-24%) and cardiac index (-14%) compared to sham-castrated mice, while there were no differences in cardiac function during rest.

Using radiolabelled lipoproteins and glucose, we found that castration shifted energy substrate uptake in the heart from lipids towards glucose and that this effect was inhibited by testosterone replacement. These findings were in accordance with a decrease of Cd36 mRNA (an important fatty acid transporter) in the hearts of castrated mice.

Further, castrated mice showed a large increase in mRNA expression of *βMhc* (+9-fold) and a small reduction in *αMhc* mRNA (-8%) and increased mRNA levels of *Anp* (+54%) and *Bnp* (+24%), all features of the fetal gene programme. These mRNA regulations strongly correlated to the protein expressions of these genes, while testosterone treatment prevented the effects.

We conclude that castration of male mice induces metabolic remodelling and re-expression of the fetal gene program in the heart, in association with a reduced cardiac response to pharmacological stress.

Paper IV: Sex Steroids in the Heart of Male Mice

In this study we tested the hypothesis that sex steroid levels in the heart differs from other compartments in male mice and that sex steroid concentrations in the heart are affected by a myocardial infarction.

We found that progesterone levels were more than 150 times higher in the heart compared to androstenedione and DHT. Testosterone levels were approximately 5-10 times higher than those of androstenedione and DHT. Similar patterns were seen in serum and skeletal muscle.

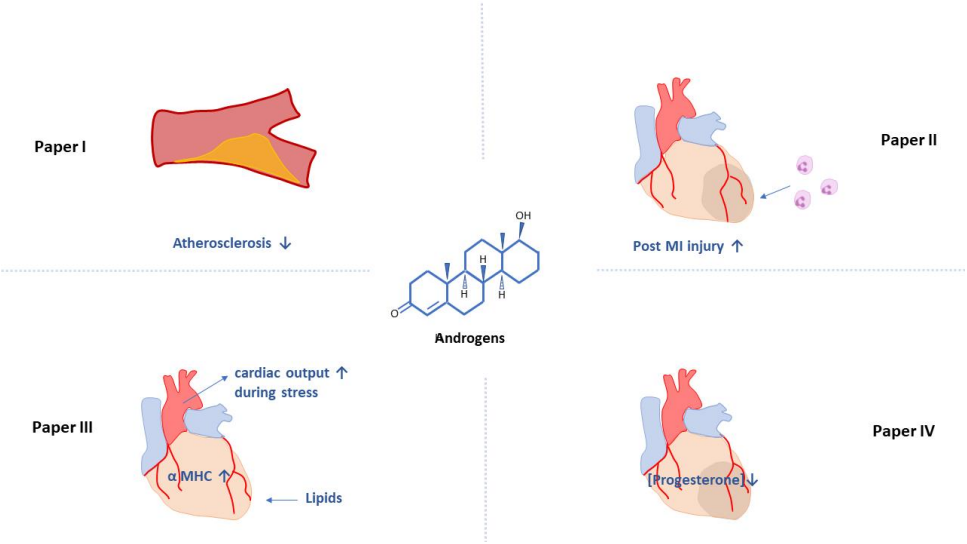
The ratio between heart and serum was above 1.0 for progesterone and DHT, 1.0 for androstenedione and 0.7 for testosterone. In skeletal muscle, DHT showed the highest tissue/serum ratio, while progesterone showed the lowest (median 0.3). The relative concentration of progesterone was approximately five times higher in the heart than in skeletal muscle, with a similar tendency for androstenedione. The tissue/serum ratio of testosterone was slightly higher in the heart while DHT concentrations was relatively lower in the heart compared to skeletal muscle.

As proxies for sex steroid-metabolising enzyme activity, we analysed the ratios between certain sex steroids in the heart and skeletal muscle respectively and compared to the ratios in serum. 5a-reductase activity (DHT/testosterone) was lower in the heart than in skeletal muscle, but higher than in serum. 17β-HSD activity (testosterone/androstenedione) was similar across compartments. 17α-hydroxylase activity (androstenedione/progesterone) was lower in the heart compared to both muscle and serum.

In a pilot study, we analysed sex steroid levels in the heart and serum 48h post myocardial infarction. We found no significant differences in serum or heart levels of sex steroid compared to sham-operated mice. However, the ratio between heart/serum progesterone was lower in the MI group.

We conclude that cardiac concentrations of sex steroids do not merely reflect serum levels in male mice. Progesterone levels were much higher in the heart than in skeletal muscle. In the acute phase post MI, progesterone levels were reduced in the heart.

Summary of results



Illustrative overview of the main findings of the thesis. In paper I, we conclude that the thymic epithelial cell is likely the target cell for the antiatherogenic actions of testosterone. In paper II, we conclude that androgens aggravate post MI injury by increased neutrophil egress from the bone marrow by targeting bone cells. In paper III, we conclude that androgens regulate cardiac metabolism, gene expressions, cardiac function. In paper IV, we conclude that concentrations of certain sex steroids in the heart do not merely reflect serum levels and that local progesterone is reduced in the acute phase post MI.

5 DISCUSSION

In **paper I**, we demonstrate that testosterone deficiency accelerates atherogenesis in male mice. In accordance, the majority of previous animal studies have shown that testosterone exerts anti-atherogenic actions while castration accelerates atherogenesis [75]. These results are in line with the increased risk of CVD-related mortality seen in patients on ADT [76] but in obvious contrast to the higher incidence of MI in men compared to women across all age groups [77]. Although the MI incidence increases in men with age [77] in parallel with a gradual decrease of testosterone levels [78], it remains unclear to which extent the association between coronary artery disease and male sex might be explained by the higher testosterone levels in men.
It is suggested that ADT cause obesity, hyperlipidemia and insulin resistance, which accelerate atherosclerosis development and predispose these patients to increased cardiovascular risk. Previous studies have shown that testosterone deficiency in mice mimics these metabolic effects in humans. Aoki et al showed that castrated mice had increased white adipose mass, serum glucose, triglycerides and non-esterified fatty acids compared to controls [79]. Apart from the above-mentioned metabolic effects, our data suggest that testosterone deficiency may be linked to increased CV risk by immunological mechanisms. Looking into immunomodulating effects of androgens on atherosclerosis development, we found that T cell depletion abolished atherosclerotic burden in castrated mice, suggesting that atherogenesis due to testosterone deficiency is T cell dependent.

In paper I, castration increased CD4+ T cells in blood and spleen with a similar trend for CD8+ T cells and testosterone replacement reversed these effects (shown in spleen). In agreement, treatment with LHRH analogues in humans, resulting in hypogonadotropic hypogonadism, has been shown to increase total peripheral T cells, including CD4+ and CD8+ cells [51, 80]. The increased thymic size in castrated mice, reported by us and others [52, 54, 81], might reflect this increased T cell output.

It has been unclear whether androgens affect thymic size and cellularity by acting directly on thymocytes or indirectly on thymic epithelial and/or stromal cells. We found that E-ARKO mice had increased thymic sizes, comparable with castrated mice, suggesting that androgens play an inhibiting role on thymic growth by targeting epithelial cells. This is in line with previous data that showed increased thymus size in mice with AR depletion in the thymic epithelium while there was no increase of thymus size in mice with androgen resistant thymocytes [52]. The same study also reports that AR expression in thymic epithelium is necessary for androgen-induced thymic involution which is seen in almost all vertebrates from the onset of puberty. The reason why androgens induce thymic involution remains debated. It has been proposed that thymic involution might be beneficial to save energy during the process of ageing and/or to reduce the risk of thymic tumours and autoimmunity due to the rapid turn-over rate of thymic cells [82].

In paper I, we also found that the E-ARKO mice displayed more than doubled atherosclerosis burden. The relative numbers of T cells were unaltered in the atherosclerotic plaques but increased in the adventitia of E-ARKO mice. The adventitia has long been known to accumulate inflammatory cells in atherosclerotic arteries in humans [83, 84]. Although we did not characterize the T cells in detail, the increased burden of atherosclerosis in castrated and E-ARKO male mice might be explained by increased numbers of pro-atherogenic subpopulations of T cells. This would be in line with previously published data that reports androgens to switch T cells towards a decreased pro-inflammatory phenotype [80].

Thymectomized E-ARKO mice did not show any atherosclerotic phenotype, suggesting that the atherosclerotic development caused by depletion of the AR in epithelial cells is thymus dependent. Our findings, together with the data of others [85, 86], suggest that age at the time of thymectomy may be of importance for the subsequent atherosclerotic phenotype in mice. The thymic organogenesis is similar between mice and humans but the immunological output differs considerably [87]. A human infant develop immune cells in utero and the naïve T cell pool is primarily maintained by peripheral proliferation. Mice, on the contrary, depend on sustained thymic output of naïve T cells. Neonatal thymectomies in humans, due to cardiac surgery, has been shown to decrease T cells significantly but does not compromise immune function while neonatal thymectomy in mice results in severe impairment of immune responses [87, 88]. Available studies investigating clinical consequences of early thymectomy include young patients and have short follow-up times. To date, there are no studies investigating if thymectomized patients have increased atherosclerosis burden/CV risk.

In **paper II**, we found that testosterone deficiency induced by castration almost halved the neutrophil infiltration in the myocardium 48 h post MI and reduced subsequent ventricular dilatation and mortality in male mice. Cavasin et al [35] have previously reported reduced neutrophil infiltration in the myocardium post MI in both castrated males and females and that high neutrophil numbers were associated with increased cardiac rupture and left ventricular dilatation. Our data on neutrophil depletion post MI with the antibody 1A8 supports that neutrophils are the causative link between testosterone and ventricular dilatation. This is congruent with data that demonstrate that high neutrophil count is strongly and independently associated with adverse remodelling, heart failure and mortality post MI in humans [89-92]. Data support that there are certain differences in white blood cell count between men and women at admission to hospital due to STEMI (PMID: 32321989), while data at later time points and after reperfusion are currently lacking.

Circulating neutrophil count have been shown to positively correlate to infarct size in patients with acute MI [93]. Several studies have shown that women have higher myocardial salvage and smaller infarct size compared to men [94-96], supporting a pathophysiological difference in infarct evolution between the sexes. Post-mortem data show that women appear to be protected from ischemic induced activation of the apoptotic cascade in the periinfarct region [97]. Somewhat surprising, we found no difference in infarct size three

weeks post MI in castrated or O-ARKO mice. Further, Cavasin et al reported no differences in MI size but lower infarct expansion index in castrated males and females compared to intact males [35]. These discrepant findings might be explained by hypoxia-related adaptive mechanisms that differs between mice and humans. For example, mice lack coronary collaterals which is unique for this species. Instead, mice have been shown to rapidly (within one to two days) form neo-collaterals after coronary occlusion [98], leading to salvage of myocardium. Importantly, our data on MI size at three weeks should be interpreted with caution because of the survival bias due to increased rupture rate in sham-castrated males and O-ARKO controls.

Apart from infarct size, post MI outcomes have been suggested to differ between sexes. Men have been shown to have more pronounced remodeling of the left ventricle post MI [99, 100] and higher risk of ventricular tachyarrhythmia, cardiac arrest and sudden cardiac death compared to women [101]. Further, men have worse short-term prognosis post MI compared to women, regardless of age, when deaths outside hospital and comorbidities are taken into account [102]. Data on long-term mortality are contradictory; women have been reported to have both higher and lower long-term mortality after percutaneous coronary intervention treated STEMI [103-105]. A potentially higher mortality in women could possibly be explained by poorer cardiovascular risk profile in women and the fact that women have higher risk of receiving suboptimal treatment in both the acute and chronic phase of STEMI compared to men [97]. Further, it should be considered that meta-analyses include two-three times more men than women [97]. Observations of poorer outcomes among women could be biased by that only women with more severe MI seek health care.

Several previous studies have reported increased neutrophil infiltration in inflammatory sites in males compared to females and that this correlates with increased severity of tissue damage in males [35, 106-108]. Little is known about the underlying mechanisms of these sex differences. Madalli et al used a model of mesenteric ischemia/reperfusion in rats to investigate neutrophil dynamics [106]. No differences were found in leukocyte motility or ability to interact with blood vessels. Nor did the increased neutrophil response in male appear to be caused by an earlier onset or slower clearance of these cells. Through our studies we demonstrate that the reduced neutrophil infiltration in the myocardium post MI, reduced mortality and decreased ventricular dilatation seen in castrated mice was completely mimicked in the O-ARKO model. We show that androgens cause these adverse effects by increasing neutrophil egress from the BM by targeting BM stromal cells of the osteo-lineage which regulates leukocyte retention factors. Thus, our results provide a potential mechanistical explanation to the sex differences seen in MI complications that may be translational to humans.

In men, neutrophil counts tend to increase with age and numbers are higher compared to women across all age groups [109]. It may be speculated that the higher MI incidence in men is explained by atherosclerotic plaque destabilization promoted by neutrophils. In a previous study, neutrophil-driven smooth muscle cell death destabilized atherosclerotic

plaques in a mouse model [110]. Also, plasma levels of neutrophil extracellular traps have been positively correlated with plaque vulnerability in patients naïve to statins and antithrombotic medication prior to the event [111].

Although neutrophils are considered detrimental to the heart in the setting of MI, accumulating evidence suggest that neutrophils to also exert anti-inflammatory, angiogenic and reparative effects that contributes to cardiac repair [93]. Pharmacological treatments that target neutrophils need to balance their pro-inflammatory and reparative effects, which might possibly explain why several anti-neutrophil treatments have failed [93]. In parallel, inflammatory chemokines have been rising as potential and promising therapeutic targets. A recent trial demonstrated that treatment with the IL-6 blocking antibody tocilizumab increased myocardial salvage in patients with acute STEMI [112]. In addition, CXCL12 therapy reduced infarct size and attenuated angiogenesis and systolic dysfunction in experimental models of MI. Effects of CXCL12 therapy in MI has not yet been studied in humans [113].

Our results from paper II raise the question if anti-androgenic drugs may affect the consequences of an MI. Such drugs are used by large groups of patients for treatment of prostate cancer and benign prostate hyperplasia. Anti-androgenic treatment for prostate hyperplasia with finasteride, which inhibits the enzyme 5 alpha reductase and thereby the conversion of testosterone to DHT, have been shown to attenuated cardiac hypertrophy and left ventricular dysfunction in mice and men with heart failure [114, 115]. These results underline the systemic potency of these drugs. As mentioned previously, ADT is associated with increased risk of CV events. Speculatively, once the myocardial infarction has occurred, perhaps the consequences of the MI are attenuated by the ADT treatment.

Our findings support that testosterone might exert both beneficial and harmful effects on the cardiovascular system through different pathways. The answer to why testosterone seems to aggravate myocardial injury post MI might be explained through the lens of evolution. Evidence show that women respond to infections with increased antibody and autoantibody production while men respond with elevated innate immune activation. Historically, men suffered frequent injuries from hunting accidents and warfare. Testosterone-driven increase in circulating neutrophil numbers might have protected them from bacterial infections after these injuries while the same mechanism happens to cause detrimental effects on MI injury.

In **paper III,** we found that castrated mice had impaired cardiac performance during pharmacological stress, induced by dobutamine, while there was no effect on cardiac function during rest. Dobutamine is a synthetic catecholamine which acts on α1, β1, and β2 adrenergic receptors. Stimulation of these receptors lead to increased cardiac contractility and thereby augment stroke volume. Testosterone have been suggested to affect cardiac contractility through Ca^{2+}-handling mechanisms in ventricular myocytes [116]. In our study, castrated mice showed reduced cardiac output while there was no difference in ejection

fraction compared to sham-castrated mice. This may indicate a diastolic dysfunction rather than impaired cardiac contractility. Testosterone have previously been shown to increase sympathetic nervous signalling [44]. It is plausible that castrated mice have decreased sympathetic tone and/or lower expression of adrenergic receptors in the myocardium which also may explain their decreased heart rate during stress.

ADT of prostate cancer, which suppresses testosterone to castrate levels, is associated with increased risk of heart failure [117]. Heart failure is a chronic and progressive disease that gradually worsen over time. It is possible that the castrated mice would have presented with even more pronounced impairment of the cardiac function during stress, perhaps also during rest, if the ultrasound examination were performed at later time points in our study. Data on repeated echocardiographic assessments in castrated mice over time is currently lacking.

In paper III, we found that castration shifted energy substrate uptake in the heart from lipids towards glucose and that this effect was inhibited by testosterone replacement. These results were in line with a decrease of Cd36 mRNA, an important fatty acid transporter, in the hearts of castrated mice. Further, we found that castration causes a return of the adult heart to the fetal gene program. The fetal gene programme seems to play a causative role in the pathogenesis of heart failure, both in humans and in mouse models [118]. The fetal gene programme may protect the stressed heart from irreversible functional impairment but at a certain point the programme no longer manages to support cardiac structure and function. It is also well known that cardiac function is tightly coupled to cardiac metabolism. It has been proposed that the heart adapts to changes in metabolic substrate access in order to maintain adequate ATP production. When the shift in substrate uptake persists over time ATP production becomes suboptimal and contractile function impairs [119]. A shift towards glucose uptake in the heart might lead to downregulation of enzymes involved in fatty acid oxidation which affects ATP production and, consequently, impairs cardiac contractility. Perhaps, a shift towards glucose may also lead to glucose accumulation and thereby render the heart at risk for glucotoxicity.

Previous studies have shown that low testosterone levels in men with heart failure is associated with poor prognosis and increased mortality [120, 121]. There are several plausible reasons why low testosterone levels are seen in heart failure. For example, testosterone levels could be low due to chronic inflammation, which is an important pathogenetic component of heart failure. Pro-inflammatory cytokines are known to reduce testicular testosterone production through regulation of the hypothalamic-pituitary axis [122]. Also, insulin resistance has been shown to impair endocrine function of Leydig cells [122]. In the clinical setting, it is yet not known whether testosterone deficiency reflects severe chronic disease or if it is a causative risk factor of heart failure. Based on our data, the causality might be bidirectional.

In **paper IV** we hypothesized that the levels of certain sex steroids in the hearts of male mice not merely reflects the levels in serum. Extensive data of sex steroid effects on cardiac function and dysfunction has been reported from both pre-clinical and clinical studies. The united focus has been on circulating sex steroids while effects of local sex steroids in the heart is an almost unexplored field. Previous studies have demonstrated expression of steroidogenic enzymes in cardiac tissues and also shown that androgen-metabolizing enzymes is upregulated in response to cardiac hypertrophy [114, 123]. This suggests that the heart has the ability to produce/metabolize androgens in order to maintain mass and function during challenged conditions.

We found that the concentration of progesterone was higher compared to serum in the unchallenged heart. A growing body of evidence have shown that progesterone lowers blood pressure and has powerful vasodilatory and natriuretic effects [124]. Hence, on a speculative basis, progesterone might accumulate in the heart for cardioprotective purposes. Further, we found concentration of testosterone to be lower in the heart than in serum while cardiac concentrations of DHT was similar to the concentration in serum. The cardiac concentration of testosterone and DHT might therefore correspond to the basal need for maintenance of adequate function.

In paper IV, we also addressed the question whether the cardiac contents of sex steroids is altered during a myocardial infarction. We saw a tendency to increased concentrations of androstenedione and testosterone in the hearts post MI while the left ventricle/serum quotient of progesterone was significantly decreased. The decrease of progesterone in the heart might reflect consumption and conversion into active androgens. This would be in line with previous studies demonstrating that pathological cardiac hypertrophy in both mice and humans is associated with increased levels of DHT [114]. The short time period of 48 hours from MI to tissue harvest may potentially explain some of the negative data in paper IV. Taken together, our results may indicate a previously unknown sex steroid metabolism in the heart that intends to compensate for loss of mass and function when the heart is injured.

6 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The results from this thesis provide new insights to the roles of androgens in cardiovascular physiology and pathophysiology. Our data also suggest new targets for future medical treatments in acute MI and prostate cancer and raise new questions.

In **paper I**, we demonstrate that the thymic epithelial cell is the target cell for the protective effects of testosterone on atherosclerotic development. These data may become important for the development of selective androgen receptor modulators (SARMs; see section 1.2.3) for safer treatment of prostate cancer. Currently available ADT is associated with increased risk of CVD and has have several side effects [125]. Our data suggest that these drugs should avoid targeting the androgen receptor on thymic epithelial cells in order to reduce the risk of atherosclerotic development. The underlying mechanisms of the protective effects on atherosclerotic development through stimulation of the AR on thymic epithelial cells requires further investigation.

In **paper II**, we identified osterix-expressing bone marrow stromal cells as the target cells for the adverse effects of androgens in cardiac injury and outcomes post MI. Our results contribute to a potential explanation of sex differences seen in complications of acute myocardial infarction. The results may become of importance for large patient groups through the development of SARMs, both for safer treatment of prostate cancer but potentially also for medical treatment of acute MI. Further, our data raise the question whether drugs that reduce androgen levels, which are used in large patient groups, might positively affect the outcome of an MI.

In **paper III**, we show that castration of male mice induces metabolic remodelling and re-expression of the fetal gene program in the heart, in association with a reduced cardiac response to pharmacological stress. To our knowledge, this is the first study reporting that castration alters metabolic substrate uptake in the myocardium. Future studies should explore the underlying mechanisms of metabolic remodeling of the heart due to testosterone deficiency and also investigate if these results correspond to human physiology. By characterizing the metabolic profile of the failing heart in detail (transcriptional regulation, post-translational modifications, absolute metabolic rates, mitochondrial biogenesis), targets for future pharmacological treatments with metabolic modulators may be identified. Such treatment could potentially optimize substrate utilization in order to improve cardiac function and ameliorate morbidity and mortality of heart failure.

In **paper IV**, we demonstrate, for the first time to our knowledge, that concentrations of certain sex steroids do not merely reflect serum levels in male mice and that progesterone levels were much higher in the heart than in skeletal muscle. We also show that progesterone levels were reduced in the heart in the acute phase post MI. Increased knowledge of sex steroid physiology and pathophysiology in the heart have the potential to

become of importance to large groups of patients due to development of medical treatment of several cardiovascular conditions. Future studies should investigate if local cardiac androgens are metabolized in the heart in order to maintain adequate cardiac function when the heart is subjected to loading conditions and/or ischemia. The research will also need to include females and investigate if the physiology and pathophysiology can be translated to humans.

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