Immunological, vascular and metabolic actions of androgens

Marta Lantero Rodriguez

Department of Molecular and Clinical Medicine
Institute of Medicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2020

Cover illustration: Graphic summary of androgen actions studied in this thesis.

Immunological, vascular and metabolic actions of androgens

marta.lanterorodriguez@wlab.gu.se

ISBN 978-91-7833-820-7 (PRINT)
ISBN 978-91-7833-821-4 (PDF)
http://hdl.handle.net/2077/63242

Printed in Gothenburg, Sweden 2020
Printed by BrandFactory

A Rik, Elin

y a mis padres.

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”.

-Marie Curie

Immunological, vascular and metabolic actions of androgens

Marta Lantero Rodriguez

Department of Molecular and Clinical Medicine, Institute of Medicine
Sahlgrenska Academy, University of Gothenburg,
Gothenburg, Sweden

ABSTRACT

Men have higher prevalence of cardiovascular disease (CVD) but lower risk of autoimmune disorders than women. The actions of sex steroids may be involved in the sexual dimorphism of these diseases. Although testosterone, the main androgen, seems to protect against autoimmunity, its role in CVD is contradictory and disease-dependent. Androgens, acting mainly via the ubiquitously expressed androgen receptor (AR), regulate multiple physiological processes (e.g. reproduction, immunity, and energy homeostasis) and are potent anabolic hormones. However, the target cells and mechanisms involved in these effects remain poorly defined. The aim of this thesis was to define effects, target cells and mechanisms involved in the actions of androgens on splenic B cell numbers, atherosclerosis, abdominal aortic aneurysms and brown fat activity in male mice.

The main findings were that androgens/AR: 1) control splenic B cell numbers via nervous regulation of splenic stroma and the cytokine BAFF, 2) protect against atherosclerosis in a T cell-dependent manner and that thymic epithelial cells is a likely AR target for atheroprotection, 3) increase angiotensin II-induced aortic neutrophil infiltration and abdominal aortic aneurysms by targeting bone marrow mesenchymal/stromal cells, and 4) reduce brown fat activity and core body temperature in male mice.

In conclusion, our studies support that many immunological actions of androgens are mediated by targeting the stroma of lymphoid organs. Further, these immunological actions contribute to beneficial (atherosclerosis) as well as deleterious (abdominal aortic aneurysms) effects on vascular pathology. We also show that androgens are important regulators of brown adipose tissue thermogenesis in male mice. These findings elucidate androgen actions of potential importance for cardio-metabolic and immunological diseases and may have implications for future development of selective AR modulators.

Keywords: androgens, androgen receptor, immune system, atherosclerosis, abdominal aortic aneurysm, brown fat, mice.

ISBN 978-91-7833-820-7 (PRINT)
ISBN 978-91-7833-821-4 (PDF)

SAMMANFATTNING PÅ SVENSKA

Män har högre risk för hjärt-kärlsjukdom jämfört med kvinnor, men lägre risk för autoimmuna reumatiska sjukdomar. Sannolikt har effekter av könshormoner betydelse för dessa könsskillnader. Mycket talar för att testosteron, som är det viktigaste androgena (“manliga”) könshormonet, skyddar mot autoimmuna sjukdomar, medan dess roll för hjärt-kärlsjukdomar är mer oklar. Androgenerna utövar sina effekter främst via stimulering av androgenreceptorn, d v s “mottagarmolekylen” för androgener. Androgenerna har betydelse för många olika processer i kroppen (såsom fortplanting, immunförsvar och ämnesomsättning) och är viktiga anabola (uppbyggande) hormoner. Det är dock till stora delar okänt vilka som är androgenernas målceller och vilka mekanismer som förklarar dessa effekter. Syftet med denna avhandling var att definiera effekter, målceller och mekanismer bakom androgeners verkan på antal B celler (en särskild typ av antikroppsbildande cell i immunförsvaret) i mjälten, ateroskleros (åderförfettning), aortaaneurysm (bråck på stora kroppspulsådern) och den värmebildande aktiviteten hos brunt fett hos hanmöss.

Huvudfynden i avhandlingen var att androgener 1) reglerar antal B celler i mjälten via en mekanism som inbegriper nervsystemet och den viktiga B cells-stimulerande faktorn BAFF, 2) skyddar mot ateroskleros via en mekanism som inbegriper effekter på thymus (brässen) och T celler (immunförsvarsceller som mognar i thymus) 3) ökar förekomsten av aortaaneurysm hos möss genom att indirekt påverka vita blodkroppar via androgenreceptorer i benceller, och 4) minskar kroppstemperaturen och aktiviteten hos brunt fett hos hanmöss.

Sammanfattningsvis visar studierna i denna avhandling på viktiga mekanismer för androgeners effekter på immunsystemet. Dessa effekter kan i sin tur förklara varför androgener har olika effekter på olika typer av kärlsjukdomar, både bra (som på ateroskleros) och dåliga (som på aortaaneurysm). Avhandlingen visar också att androgener är viktiga för den värmebildande aktiviteten hos brunt fett. Fynden kan få betydelse bl a för framtida behandlingar av hjärt-kärlsjukdomar och sjukdomar i immunsystemet. Fynden kan också bli viktiga för möjligheten att skräddarsy behandlingar mot bl a prostatacancer, där man vill minska androgeneffekter i prostatan utan att få biverkningar från andra organ genom att rikta behandlingen mot androgenreceptorer på specifika målceller.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Wilhelmson A.S, Lantero Rodriguez M, Stubelius A, Fogelstrand P, Johansson I, Buechler M.B, Lianoglou S, Kapoor V.N, Johansson M.E, Fagman J.B, Duhlin A, Tripathi P, Camponeschi T, Porse B.T, Rolink A.G, Nissbrandt H, Turley S.J, Carlsten H, Mårtensson I.L, Karlsson M.C.I and Tivesten Å. **Testosterone is an Endogenous Regulator of BAFF and Splenic B cell Number.** *Nat Commun. 2018 May 25;9(1):2067.*

II. Wilhelmson A.S, Lantero Rodriguez M, Svedlund Eriksson E, Johansson I, Fogelstrand P, Stubelius A, Lindgren S, Fagman J.B, Hansson G.K, Carlsten H, Karlsson M.C.I, Ekwall O and Tivesten Å. **Testosterone Protects against Atherosclerosis in Male Mice by Targeting Thymic Epithelial Cells**. *Arterioscler Thromb Vasc Biol. 2018 Jul; 38(7):1519-1527.*

III. Lantero Rodriguez M, Wilhelmson A.S, Svedlund Eriksson E, Fagman J.B, Alexandersson C, Johansson I, Movérare-Skrtic S, Ohlsson S, Karlsson M.C.I, Langenskiöld M and Tivesten Å. **Depletion of the Androgen Receptor in Osterix-Expressing Bone Cells Protects Against Abdominal Aortic Aneurysms in Male Mice.** *Manuscript*

IV. Lantero Rodriguez M*, Schilperoort M*, Johansson I, Svedlund Eriksson E, Palsdottir V, Kroon J, Ståhlman M, Kooijman S, Ericson M, Borén J, Jansson J.O, Levin M.C, Rensen P.C.N, Tivesten Å. **Testosterone Reduces Brown Fat Activity in Male Mice. ***C*ontributed equally. Manuscript*

Paper II is reprinted with permission from the publisher.

CONTENT

ABBREVIATIONS

AAA	Abdominal aortic aneurysms
ACTH	Adrenocorticotropic hormone
AngII	Angiotensin II
ApoE	Apolipoprotein E
AR	Androgen receptor
ARKO	Androgen receptor knockout
BAFF	B cell activating factor
BAT	Brown adipose tissue
CAR	CXCL12-abundant reticular cells
cBT	Core body temperature
CCL	C-C Motif Chemokine Ligand
CD	Cluster of differentiation
CVD	Cardiovascular disease
CXCL	C-X-C Motif Chemokine Ligand
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DLL4	Delta-like ligand 4
FA	Fatty acids
FDC	Follicular dendritic cell
FO	Follicular
FRC	Fibroblastic reticular cell
FSH	Follicle-stimulating hormone

G-CSF	Granulocyte-colony stimulating factor
Gr-1	Glycosylphosphatidylinositol (GPI)-linked protein or Ly6G/Ly6C
HDL	High-density lipoprotein
IFN-γ	Interferon-gamma
IgM	Immunoglobulin M
IL-7	Interleukin 7
K5	Keratin 5
LDL	Low-density lipoprotein
LH	Luteinizing hormone
LMA	Locomotor activity
Ly6C/Ly6G	Lymphocyte antigen 6 complex, locus C or locus G
Lyz	Lysozyme M
MZ	Marginal zone
NA	Noradrenaline
ORX	Orchiectomy or castration
Osx1	Osterix 1
Pgc1a	Peroxisome proliferator-activated receptor-gamma coactivator 1 alfa
Pgk	Phosphoglycerate Kinase 1
POA	Preoptic area
RA	Rheumatoid arthritis
RQ	Respiratory quotient
SCF	Stem cell factor
Self-pMHC	Self-peptides in major histocompatibility complex
SHBG	Sex hormone binding globulin

SLE	Systemic lupus erythematosus
SNS	Sympathetic nervous system
SSC	Side scatter
Tagln	Transgelin
TCR	T-cell receptor
TEC	Thymic epithelial cell
Ter-119	Glycophorin A-associated protein
Th	T helper
TO	Triolein
Ucp1	Uncoupling protein 1
VCO_2	Carbon dioxide produced
VO_2	Consumed oxygen
WAT	White adipose tissue
WT	Wild type
ZT	Zeitgeber times

1 INTRODUCTION

1.1 ANDROGENS

Androgens are sex steroid hormones required for development of reproductive organs and male fertility. Further, they are involved in immunity and metabolism and are known as anabolic hormones due to their actions on muscle and bone development in both males [1, 2] and females [3]. The focus of this thesis lies on androgen actions in males. Androgens, as other sex steroids, are metabolites of cholesterol synthesized mainly by the gonads (testes in males) and the adrenal cortex (Figure 1). The most important androgen in men is testosterone; 95% of testosterone is produced by the Leydig cells in the testes and 5% is produced by the adrenal cortex from C-19 androgen precursors such as dehydroepiandrosterone (DHEA) and androstenedione [4].

In peripheral target tissues, testosterone can be converted into the more potent androgen dihydrotestosterone (DHT) by the enzyme 5α-reductase and into estradiol by the aromatase enzyme (Figure 1). The testes produce approximately 20% of circulating estradiol in men and 80% derives from DHEA in peripheral tissues. Therefore, androgen actions occur by direct stimulation of the androgen receptor (AR) by testosterone or its metabolite DHT as well as by stimulation of the estrogen receptor after conversion to estradiol.

In the circulation, androgens and other sex steroids are bound to sex hormone binding globulin (SHBG; 50-60%) or albumin (40-50%) and only 1-3% of the sex steroids are in the unbound “free” fraction. Sex steroids have high affinity for SHBG but not for albumin. The free and non-SHBG-bound fractions are defined as the bioavailable fractions. In males, testosterone levels are high during fetal development, shortly after birth and after puberty. In healthy men, circulating total testosterone and estradiol decrease minimally with age, although SHBG markedly increases with age, which results in a pronounced decrease of the bioavailable sex steroids [5-7]. Associations between low testosterone levels and unfavorable body composition have been consistently reported [8].

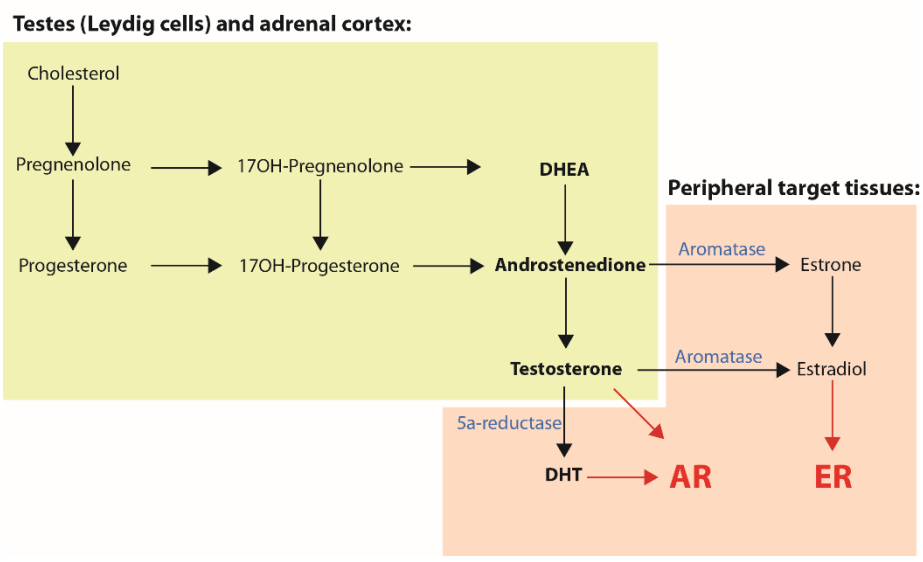

Figure 1. Sex steroid synthesis in males. Early steps in sex steroid synthesis occur mainly in the testes (Leydig cells, green box) and adrenal cortex (green box). Androgen conversion into dihydrotestosterone (DHT) by 5α-reductase (blue) and estradiol by aromatase (blue) occurs mainly in peripheral target tissues, as well as binding of androgens to androgen receptor (AR, red) and estradiol to estrogen receptor (ER, red) (pink box). DHEA, dehydroepiandrosterone.

1.1.1 NEUROENDROCRINE REGULATION OF ANDROGENS

Synthesis and secretion of androgens and other sex hormones from the testes is controlled by the hypothalamic-pituitary-gonadal axis (Figure 2). The hypothalamus secretes gonadotropin-releasing hormone, which in turns activates the pulsatile secretion of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. LH and FSH control spermatogenesis and gonadal steroidogenesis. In turn, sex hormones signal the hypothalamus and pituitary to control physiological hormone levels. Sex steroid hormone production by the adrenal cortex is regulated by the hypothalamic-pituitary-adrenal axis (Figure 2). The hypothalamus secretes corticotropin-releasing hormone, which activates the release of adrenocorticotropic hormone (ACTH) that stimulates the secretion of sex hormones, as well as other hormones (glucocorticoids and mineralocorticoids) from the adrenal cortex.

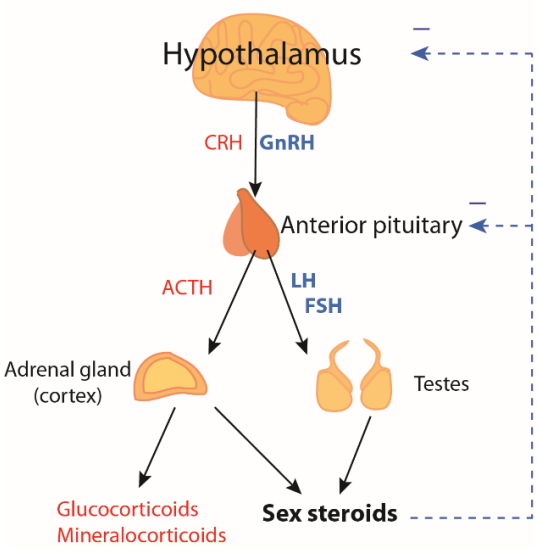

Figure 2. Neuroendocrine regulation of androgens and other sex steroid hormones is controlled via hypothalamus-pituitary-gonadal axis (blue) and hypothalamus-pituitary-adrenal axis (red). GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone, FSH, follicle-stimulating hormone; CRH, corticotropin-releasing hormone and ACTH, adrenocorticotropic hormone.

Actions of androgens (testosterone or DHT) are mainly mediated by binding to the AR, which is ubiquitously expressed in tissues (2). AR is a ligand-inducible transcription factor of the nuclear receptor superfamily present in the cytoplasm of cells. The AR structure consists of an N-terminal domain (encoded in exon 1), a DNA-binding domain (encoded by exon 2 and 3) and a ligand-binding domain (encoded by exons 4-8) linked to the DNA-binding domain by a hinge region [2].

Classical AR signaling occurs when ligands (testosterone or DHT) bind AR at the ligand-binding domain region in the cytoplasm. In turn, AR translocates to the nucleus to bind target genes and regulate their expression. In the nucleus, dimers of AR bind to specific DNA sequences termed androgen response elements. Non-classical AR signaling can be divided into ligand-independent and non-genomic actions. Non-genomic actions occur when the liganded receptor activate second messengers like kinases phosphatases, cytoplasmatic calcium release or nitric oxide synthesis. This can occur within minutes. Ligand-independent activation, which occurs without ligand binding, is mostly triggered via phosphorylation of activation function-1[2].

1.1.2 SPECIES DIFFERENCES IN THE SEX STEROID SYSTEM

Sex steroid metabolism differs between human and rodents. In men, a large proportion of androgens are synthesized in peripheral target tissues from C-19 adrenal androgen precursors, while male rodents synthesize almost all androgens in the testes [9]. Further, aromatase expression is low in peripheral tissues of rodents, which also lack circulating SHBG [10].

1.1.3 ANDROGEN DEFICIENCY IN HUMANS

Androgens have many physiological actions in the body. Androgen deficient conditions, such as Klinefelter syndrome or androgen deprivation therapy for advanced prostate cancer, have been associated with increased risk of other diseases. These include cardiovascular diseases, such as atherosclerosis and myocardial infarction [11], and autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [12]. Androgen deficiency is also associated with many other signs and symptoms such as loss of muscle and bone mass, and hot flashes [13].

1.1.4 SELECTIVE ANDROGEN RECEPTOR MODULATORS

The fact that androgens/AR have many important roles in physiology and pathology of diverse tissues, and that the AR is ubiquitously expressed, complicates the therapeutic use of androgens as well as anti-androgens [14]. However, development of compounds that regulate AR activity in a tissue-specific way, also known as selective AR modulators, is ongoing. A crucial step in the design of selective AR modulators, that promote beneficial/desired (anti-)androgen effects but avoid side-effects, is the identification of target cells for the specific actions of androgens.

1.2 IMMUNE SYSTEM

The immune system consists of an interactive network of lymphoid organs, cells, humoral factors, and cytokines. The main function of the immune system is host defense, and alterations of the immune system function lead to deleterious effects such as infections and tumors (underactivity) or allergic and autoimmune diseases (overactivity) [15]. The immune system can be divided according to the velocity and specificity of the response into innate immunity (rapid "hours" and unspecific response) and adaptive immunity (slow "day-week", antigen-specific). Innate immunity includes neutrophils, monocytes, macrophages, complement, cytokines and acute phase proteins. Physical, chemical, and microbial barriers are also considered a part of the innate immunity. Adaptive immunity consists of antigen-specific reactions mediated by B and T cells. The adaptive immune response has a memory function, and thus subsequent exposures to the same antigen lead to rapid and strong responses.

1.2.1 NEUTROPHILS AND BONE MARROW STROMA

Neutrophils are the first line of defense of the immune system against bacterial and fungal pathogens. However, an exacerbated neutrophil response might damage tissues, and thus neutrophil migration to tissues must be tightly regulated.

Neutrophils are constantly produced in the bone marrow and about 2% of neutrophils can be found in circulation, where they have a short life (7 h in humans and 11 h in mice) [16]. Homeostasis of circulating neutrophils is tightly regulated by the rate of granulopoiesis, egress form the bone marrow and clearance of aged neutrophils (in bone marrow, spleen and liver). These processes are mainly controlled by bone marrow stromal/mesenchymal cells (Figure 3). Bone marrow stroma cells, many of which derive from osterix-expressing progenitors [17], secrete cytokines and chemokines that shape the development and function of the hematopoietic compartment [18]. One such a factor is CXCL12, which has a dual role regulating neutrophil egress from the bone marrow as well as B lymphopoiesis [19]. Neutrophils express the receptor for CXCL12 known as CXCR4, which retains neutrophils in the bone marrow.

In response to inflammation or infection, neutrophils are rapidly released from the bone marrow and migrate into the inflammation site. Inflammation alters normal leukocyte production, promoting granulopoiesis over lymphopoiesis, to achieve neutrophilia. This shift is mediated by reduction of bone marrow CXCL12 levels [20].

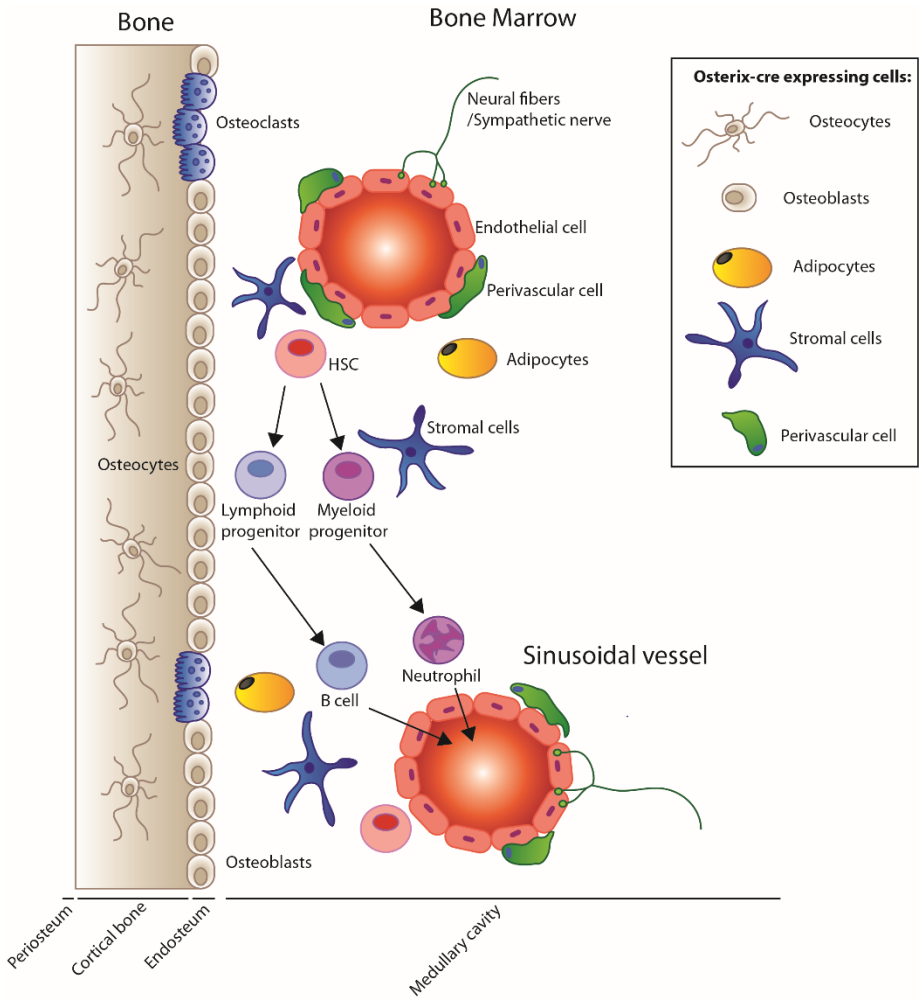

Figure 3. Bone marrow niche. Many bone marrow stromal/mesenchymal cells derive from osterix-expressing progenitors and regulate leukocyte homeostasis. HSC, hematopoietic stem cell.

1.2.2 T CELLS AND THYMIC STROMA

T cells contribute to the adaptive immunity by releasing cytokines and helping other cells, such as B cells and macrophages. T cells are characterized by the surface expression of T-cell receptor (TCR), CD4 or CD8, and CD3. T cells can be divided into T helper (Th) cells ($CD4^+$) and cytotoxic T cells ($CD8^+$). $CD4^+$ cells consist of different subtypes such as Th1 and Th2, which generate immune responses against intracellular (virus, bacteria) and extracellular (helminths) parasites, respectively. Th1 produce interferon-gamma (IFN-γ) which activates macrophages while Th2 activate B cell antibody production by secretion of cytokines.

T cells originate from a bone marrow early T lineage precursor that migrates to the thymus (Figure 4). In the thymus, an adequate microenvironment allows development and selection of T cells that recognize foreign antigens but tolerate self-components [21]. Thymus "education" of T cells is mainly directed by cells from the thymic stroma compartment called thymic epithelial cells (TECs). Historically, the TEC compartment has been anatomically divided into two subsets: cortical and medullary TECs. Cortical TECs orchestrate early checkpoints of the T cell developmental process: (1) early lymphoid progenitor recruitment by secretion of homing factors such as CXCL12, CCL5, and CCL25 (2) T cell lineage commitment by expression of DLL4, and (3) positive selection where $CD4^+CD8^+$ double positive that bind to self-peptides in major histocompatibility complex (self-pMHC) on cortical TECs with good enough affinity get survival signals such as IL-7 and SCF. Medullary TECs organize later steps of T lymphopoiesis: (1) negative selection of self-reactive TCRs, which occurs during the transition from $CD4^+CD8^+$ double positive to single positive thymocytes and (2) agonist selection: low-intermediate affinity between TCR and self-pMHC leads to diversion of the clone into regulatory T cells [21].

Thymus undergoes a dramatic age-dependent involution, which is associated with alterations of the stromal compartment organization and its replacement with adipose tissue. The age-dependent involution also affects maturing thymocytes and immature naïve T cells (recent thymic emigrants) [22]. Thymus involution starts during puberty and correlates with sex hormone levels, both in human and mice [22]. Neonatal thymectomy affects the peripheral T cell pool in humans [23] and is associated with higher risk of autoimmune diseases [24].

1.2.3 B CELLS AND SPLENIC STROMA

B cells contribute to the adaptive immunity by mounting antibody responses, acting as antigen presenting cells for T cells and producing cytokines that affect other cells. In the absence of B cells, the lack of humoral responses increases the susceptibility to serious infections. B cells are classified according to their origin into B1 and B2. B1 cells originate in the fetal liver, populate the peritoneal cavity, and produce natural antibodies (IgM). B2 cells originate from the bone marrow.

Bone marrow B lymphopoiesis leads to formation of immature B cells from common lymphoid progenitors, in a process defined by immunoglobulin rearrangement. Bone marrow stromal or mesenchymal cells are crucial for this stage of maturation (Figure 3). These stromal cells provide the necessary signals for maturation and migration through the bone marrow compartment [25-27]. The resulting immature B cells migrate to the spleen to continue their development (Figure 4).

Immature B cells enter the spleen via the marginal zone and then enter the T cell zone (periarteriolar sheath) where fibroblastic reticular cells (FRCs) are the main stromal cell type. Then they move to the B cell zones (follicle), which contains follicular dendritic cells (FDCs) and FRCs. The spleen is the major site for positive selection of non-self reactive clones. Transitional B cells receive survival signals such as B cell activating factor (BAFF) and develop into mature B cells, follicular and marginal zone B cells. BAFF signaling is also crucial for maintenance of mature B cells and increases in BAFF levels leads to increase in the steady state number of quiescent primary B cells [28] . BAFF is produced by several cells such as macrophages, FDCs and FRCs [7]. Although BAFF production by FDCs is critical for germinal center response, BAFF production by FRCs in the follicles is pivotal for controlling B cell homeostasis and is the primary source of systemic BAFF [29].

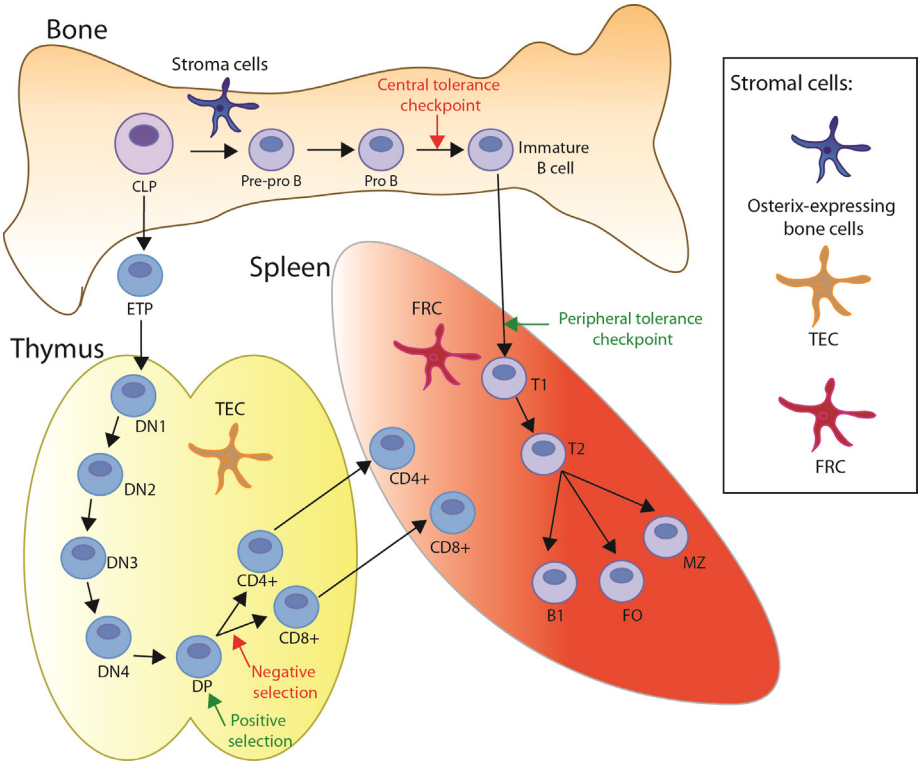

Figure 4. B and T lymphopoiesis and stromal cells involved: osterix-expressing cells, thymic epithelial cells (TECs) and fibroblastic reticular cells (FRCs). CLP, Common lymphoid progenitor; ETP, early T lineage precursor; DN, double negative; DP, double positive; T, transitional; FO, follicular and MZ, marginal zone.

1.2.4 TOLERANCE, AUTOIMMUNITY AND BAFF

Loss of tolerance against self-antigens leads to overexpression of autoantibodies and causes autoimmunity. Autoimmune disorders consist of more than 80 chronic and relapsing diseases. One example of autoimmune disorder is SLE, which is characterized by the presence of autoantibodies (e.g. against double stranded DNA) and autoimmune reactions in several organs in the body [30].

Immune tolerance is defined as the lack of immune response against epitopes/tissues that are normally capable of triggering an immune response, and it is divided into central and peripheral tolerance. Central tolerance

occurs in the thymus (T cells) and in the bone marrow (B cells), and consists on the elimination of autoreactive lymphocyte clones (negative selection). Peripheral tolerance functions as a "backup" strategy of central tolerance. It occurs mainly in secondary lymphoid tissues (spleen and lymph nodes) after B and T cells enter peripheral tissues and lymph nodes. The main goal is to avoid immune responses against the body's own tissues. There are three mechanisms of peripheral tolerance: anergy, deletion and suppression by regulatory T cells [31].

BAFF promotes B-cell survival and differentiation, and is involved in the pathogenesis of autoimmune diseases [7]. BAFF deficiency alters splenic B cell development after the T1 stage and reduces the size of follicular and marginal zone B cell compartments. Consequently, humoral responses are affected. Treatment with BAFF rescues the mature B cell compartment in BAFF-deficient mice [32]. Furthermore, BAFF receptor deletion in humans blocks B cell development after T1 and results in B lymphopenia and impaired humoral responses [33]. A variant in the gene encoding BAFF has been associated with multiple sclerosis and SLE [34]. BAFF transgenic mice have increased number of splenic B cells and develop anti-DNA antibodies and other autoimmune–like signs [35]. BAFF inhibition may be protective in experimental lupus models [36]. Several strategies have been used to block BAFF activity to improve the symptoms of autoimmune diseases and the BAFF inhibitor belimumab has been approved for SLE treatment.

1.2.5 ANDROGEN ACTIONS IN THE IMMUNE SYSTEM

Males are less susceptible to autoimmunity [37] and their response to pathogenic infections and vaccinations is lower than in females [38]. Androgens are generally considered as negative regulators of the immune system and a factor contributing to the sex differences in autoimmunity. Indeed, testosterone has been shown to protect against autoimmune disease in experimental models [39].

Androgen actions on T cells

Androgens are known to affect thymus size. During puberty, the increase in androgen levels contributes to the involution of the thymus in both humans and mice. Androgen deficiency increases thymus size and thymopoiesis while androgen supplementation leads to thymic involution [40-42]. However, the potential effects of this regulation for the pathogenesis of disorders dependent on T cells, such as atherosclerosis, remains to be elucidated.

Androgen actions on B cells

Androgens suppress both bone marrow B lymphopoiesis [43, 44] and splenic B cell number [43]. Low androgen levels, such as those in patients with hypogonadotropic hypogonadism and Klinefelter syndrome, result in high B cell counts, which are reduced by testosterone replacement therapy [45, 46]. We have recently showed that global AR deletion increases bone marrow B cell numbers from the pro-B cell stage and also splenic B cell number [44]. Furthermore, the increased bone marrow B lymphopoiesis, but not the increase splenic B cell number, was mimicked by cell-specific deletion of AR in osterix-expressing bone cells. These data suggest that regulation of splenic B cell numbers is independent of bone marrow B lymphopoiesis [44]. Given the crucial role of BAFF for splenic B cell numbers, a potential mechanism may involve the downregulation of BAFF by androgens. However, whether androgens regulate splenic B cell number in males via downregulation of BAFF is unknown.

Androgen actions on neutrophils

Androgens increase neutrophil accumulation in inflammatory sites [47-49] and are associated with increased inflammation and tissue damage. Furthermore, androgens have important actions on bone marrow leukocyte homeostasis. Cell-specific deletion of AR in osterix-expressing cells (O-ARKO) enhances bone marrow B lymphopoiesis in male mice [44]. However, whether O-ARKO affects neutrophil homeostasis is unclear. Further, potential consequences of such regulation for the pathogenesis of diseases dependent on neutrophils, such as abdominal aortic aneurysms, needs to be further investigated.

1.3 CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) denotes disorders of the blood vessels and the heart. CVD include different diseases such as coronary heart disease, cerebrovascular disease and peripheral arterial disease. CVD is the number one cause of death globally, causing 31% of all deaths [50, 51]. Of CVD deaths, about 85% are due to stroke and myocardial infarction. The main underlying cause of clinical CVD events is atherosclerosis. Several CVD risk factors are behavioral and therefore modifiable, such as tobacco use, unhealthy diet, physical inactivity and harmful use of alcohol. Other risk factors such as age and genetic factors are not modifiable.

1.3.1 ATHEROSCLEROSIS

Atherosclerosis is the major cause of morbidities and mortalities in western countries. It mainly manifests as ischemic heart disease and stroke, and peripheral arterial disease. Common risk factors include male sex, age, hypercholesterolemia, hypertension, smoking, type 2 diabetes and metabolic syndrome [52]. Data from the Canakinumab Antiinflammatory Thrombosis Outcome Study demonstrated that inflammation is a crucial mechanism for atherosclerosis formation [53].

Atherosclerosis is initiated by retention of low-density lipoprotein (LDL) in the intimal layer of arteries. An inflammatory response in the intima modifies trapped LDL via oxidative, lipolytic and proteolytic enzymes and reactive oxygen species that provoke the formation of danger-associated molecular patterns. Danger-associated molecular patterns activate vascular cells and induce immune cell recruitment. Infiltrating monocytes become macrophages in the inflamed intima and start engulfing oxidized LDL, becoming foam cells. Foam cells may activate T cells, and both cell types secrete inflammatory cytokines to recruit more leukocytes. Accumulation of lipid-laden cells forms fatty streaks in the intima, which is an early stage of atherosclerotic plaques. B cells are rare in plaques and mainly found in the adventitial layer of the arterial wall, where tertiary lymphoid organs can be formed.

Atherosclerotic plaques progress by accumulation of lipids and foam cells, and migration of smooth muscle cells from the media to the intima. Smooth muscle cells produce extracellular matrix (collagen and elastin) which thickens the intimal layer. Cell death of macrophages and smooth muscle cells leads to necrotic core formation of the advanced lesions. Subsequent thinning of the fibrous cap might lead to plaque rupture. Human atherosclerotic lesions can be classified as stable or unstable according the characteristics of the fibrous cap. Stable plaques present a thick fibrous cap

while unstable plaques are characterized by a thin fibrous cap which is prone to rupture.

Role of T cells in atherosclerosis

T cells constitute around 10% of all cells in human plaques and are also found in mouse lesions [54]. $CD4^+$ T cells account for about 70% of all T cells in plaques and the rest are identified as $CD8^+$ T cells [55]. Th1 $CD4^+$ cells are the most abundant type in the plaques and an important source of proatherogenic cytokines. Several studies support a proatherogenic role of $CD4^+$ T cells, showing that deficiency of $CD4^+$ T cells in atherosclerosis-prone $apoE^{-/-}$ mice protects against atherosclerosis [56, 57], while the role of $CD8^+$ T cells is less clear [57].

Role of androgens in atherosclerosis

While male gender is a risk factor for atherosclerosis, clinical and experimental studies support an atheroprotective role of androgens. Low testosterone levels in men are associated with enhanced atherosclerosis and greater risk of cardiovascular events [11, 58]. Androgen deprivation therapy in men has been reported to increase cardiovascular risk [59]. Experimental studies have shown that castration-induced atherogenesis is abolished by testosterone supplementation and that global AR deficiency increases atherosclerosis in male mice [60]. Recent studies have tried to identify the target cells for the antiatherogenic actions of testosterone. However, using cell-specific deletion of AR in endothelial and smooth muscle cells there was no differences in atherosclerotic lesion while monocyte/macrophage-specific AR deletion reduced atherosclerosis [61]. Therefore, more research is needed to find the target cell and the mechanism through which androgens/AR protect against atherosclerosis [62].

1.3.2 ABDOMINAL AORTIC ANEURYSMS

Abdominal aortic aneurysms (AAA) denotes the pathological weakening and dilatation of the aortic wall (diameter >3 cm), specifically affecting the infrarenal region of the aorta [63]. AAAs are usually asymptomatic until rupture of the aortic wall and this fatal event is a common cause of death among elderly men [64]. General screening programs using ultrasound in men at 65 years of age have been shown to reduce mortality [65-67]. The prevalence of screening-detected AAA was 1.5% in men aged 65 year in Sweden [68]. Common risks factors and risk markers of AAA include male gender, age, smoking, hypertension and hypercholesterolemia [69]. Hallmarks of human AAAs include inflammation, smooth muscle cell apoptosis, extracellular matrix degradation, mechanical forces and oxidative stress [70].

Role of neutrophils in AAA

Neutrophils are found in the luminal layer of the thrombus in human AAAs [71, 72] and their counts in blood are strongly associated with clinically presented AAA in humans [73]. Experimental studies have demonstrated that neutrophils are important in the early phase of AAA formation in the elastase-induced model [74] and that neutrophil depletion inhibits AAA formation [75]. Furthermore, results from two clinical trials and one preclinical study have shown that treatment with doxycycline depletes aortic wall neutrophils [76, 77] and reduces AAA progression [76-79]. Overall, these studies suggest a negative role of neutrophils in AAA.

Role of androgens in AAA

Male sex is a strong risk factor for AAA development in humans [80-83] and a similar sex difference is found in animal AAA models [84, 85]. Experimental studies have shown that androgens promote AAA formation in males. AR agonists accelerates AAA formation in male mice [86] while androgen deficiency [85, 86] and genetic deletion or pharmacologic blockade of the AR [87, 88] protect against AAA formation [87, 88].

Huang et al. showed that cell-specific AR deletion in macrophages or vascular smooth muscle cells, using lysozyme M (Lyz) Cre and transgelin (Tgln) Cre mice respectively, protected male mice against AAA formation [87]. However, Lyz-Cre and Tagln-Cre target not only macrophages and smooth muscle cells; lysozyme is also expressed in myeloid cells and bone marrow mesenchymal stem cells [89] and Tagln-Cre targets a broad array of bone marrow stromal/mesenchymal cells [90]. Thus, further studies are

needed to better define the target cell for the AAA-provoking actions of testosterone.

1.3.3 AAA AND ATHEROSCLEROSIS ARE DIFFERENT DISEASES

The pathogenesis of AAA is not fully understood. Previous studies have found that atherosclerosis is present in the aneurysmal wall, and thus have linked AAA formation with atherosclerosis [91]. The two diseases share certain risk factors such male sex, increasing age, smoking and hypercholesterolemia. However, the diseases have major differences, for example regarding the role of androgens, location and hallmarks of the lesions, and the clinical presentation of the disease (Table 1). More recent research suggests that atherosclerosis develops in parallel or secondary to aneurysmal dilatations [92].

Table 1. Differences between abdominal aortic aneurysms (AAA) and atherosclerosis.

	Atherosclerosis	**AAA**
Androgens	Protect	Increase
Location	Intima (large/medium arteries)	Media (aorta)
Hallmarks of the lesion	Foam cell formation	Intense oxidative stress
	Matrix formation	Matrix degradation
	Smooth muscle cell proliferation	Smooth muscle cell apoptosis
	Lipid accumulation	
Localization of macrophages	Subintima	Media
Risk factors	Type 2 diabetes	Not type 2 diabetes
Clinical presentation	Myocardial infarction and stroke	AAA rupture

1.4 METABOLISM

Metabolism refers to the sum of all biochemical reactions that occur in an organism to provide energy for vital processes and synthesizing new cellular components. Metabolic reactions that break down molecules (lipid, glucose and proteins) to release energy are defined as catabolic reactions and anabolic reactions utilize energy for synthesizing new molecules.

Energy balance consists of two components, energy intake and energy expenditure. Energy intake depends on the type of foods ingested and digestibility. Energy expenditure reflects the fuels utilized for growth, development, reproduction, body maintenance needs. Substrates that are not consumed might be stored. Energy imbalance over a period of time alters body weight. Thus, positive energy balance, caused by an energy intake larger than energy expenditure, leads to increased body weight, and vice versa [93].

Energy expenditure is divided into: 1) resting energy expenditure (60-75% of energy expenditure) which varies depending on body size and composition. Lean tissues such as brain and heart consume more energy than fat tissue. Men have higher energy expenditure at rest than women due to their larger lean mass [94]. 2) Thermic effect of food (10% of total energy expenditure), which is the energy expenditure associated with digestion of foods. 3) Activity energy expenditure (15-10% of total energy expenditure), which is the energy expenditure during activity such as exercise activity and thermogenesis (non-exercise activity).

1.4.1 CORE BODY TEMPERATURE

Warm-blooded animals can alter their metabolism to maintain their core body temperature (cBT), which is crucial for cellular function and thus organism survival. Significant increases or reductions in cBT have detrimental effects for the cells, e.g. high cBT denatures proteins while lower cBT affects membrane fluidity, ion fluxes and enzyme performance [95].

cBT in humans is maintained within a narrow temperature range of approx. 0.2°C of width defined as the “thermoneutral zone” [96]. cBT higher/lower than the range limits, trigger thermoeffector mechanisms to keep cBT within the range, e.g. high cBT activates vasodilatation and sweating while low cBT activates vasoconstriction, shivering and brown adipose tissue (BAT) thermogenesis. However, cBT is not a static parameter and it varies depending on physiological demands and pathological conditions [97]. Furthermore, cBT has a circadian rhythm (cBT is higher during awake period) and is affected by sex. Female mice have higher cBT than male mice

[98] and women trigger thermoregulatory mechanism at slightly greater cBT (~0.3°C) than men [96].

Central control of cBT works as a thermoregulatory network or "reflex"[99], which consists of three components: a sensory afferent part, integration (preoptic area or POA region in the hypothalamus) and a command efferent part. Sensory afferent neuronal pathways bring information on environmental temperatures (skin thermoreceptors), visceral temperatures and central temperatures (brain and spine) to the thermoregulatory center in the brain, which is located in the POA. The POA signals to the peripheral effectors such as cutaneous vasomotion, shivering thermogenesis and BAT thermogenesis.

1.4.2 BROWN ADIPOSE TISSUE THERMOGENESIS

BAT thermogenesis has an important role in the control of cBT and energy homeostasis. In contrast to the energy storing function of white adipose tissue, BAT dissipates energy by combustion of mainly lipids into heat. BAT depots are crucial for survival of small rodents and neonate humans and are also present in adult humans [100].

Emphasizing the physiological importance of BAT for the body, the thermogenic activity of BAT is controlled by multiple nuclei in the hypothalamus and is mainly driven by sympathetic nervous system (SNS) [101]. Besides sympathetic outflow to BAT, hypothalamic activation results in pituitary activation and release of ACTH and subsequently glucocorticoids (hypothalamus-pituitary-adrenal axis) and thyroid hormone (hypothalamus-pituitary-thyroid axis), which also may regulate BAT activity [101].

BAT activity is enhanced by cold environment but also has a diurnal rhythmicity, showing the highest fatty acid (FA) uptake at the onset of wakening [102]. Sympathetic stimulation of BAT thermogenesis is mediated via noradrenaline (NA) that binds to β3-adrenergic receptor in brown adipocytes leading to transcription of uncoupling protein 1 (*Ucp1*) and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*Pgc1a*), activation of lipolytic enzymes in the lipid droplets to release lipids for mitochondrial β-oxidation and uncoupling of mitochondrial respiration to generate heat [103]. Prolonged activation of brown adipocytes depletes intracellular lipid droplets, which are replenished by uptake of FA from circulating triglycerides, mainly triglyceride-rich lipoproteins. Lipoprotein lipase hydrolyzes FA, which are then taken up by cluster of differentiation 36. Glucose is also taken up from circulation for *de novo* lipogenesis (Figure 5).

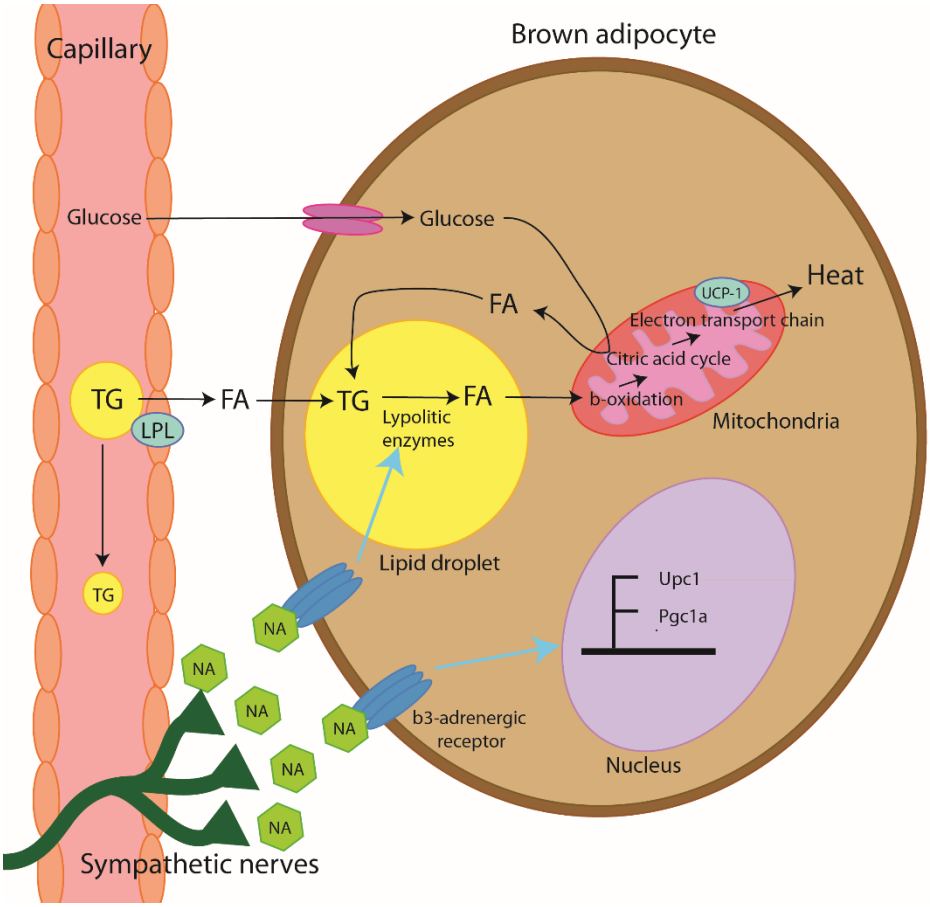

Figure 5. Brown adipose tissue (BAT) activation via sympathetic stimulation leads to fatty acid (FA) uptake from triglyceride (TG)-rich lipoproteins in circulation. NA, noradrenaline; UCP-1, uncoupling protein 1 and LPL, lipoprotein lipase.

Role of androgens on core body temperature and BAT activity

As previously mentioned, core body temperature is affected by sex, where male mice have overall lower cBT than female mice [98]. Castration increases cBT in male mice [98, 104], suggesting that androgens might regulate body temperature. Men activate thermoregulatory mechanisms at significantly lower cBT than women (~0.3°C) [96]. Furthermore, both androgen withdrawal in men receiving androgen deprivation therapy and estrogen withdrawal in women undergoing menopause is associated with the occurrence of hot flashes. Hot flashes in menopausal women seem to be triggered by small elevations in cBT occurring within a narrowed thermoneutral zone [105].

BAT activity is also affected by sex. Men show lower BAT activity, measured by glucose radiotracers, than women [106-108]. BAT from male rats exhibit a lower *Ucp1* expression and mitochondrial respiration than BAT from female rats [109]. Experimental studies have found that androgen deficiency decreases BAT weight [110] and increases BAT *Ucp1* mRNA expression [104], which might be due to an increased BAT activity. Further, *in vitro* experiments have shown that testosterone treatment of brown adipocytes diminishes their lipolysis and downregulates thermogenic genes, such as *Ucp1* and *Pgc1a* [111-113]. BAT expresses AR [114] and *in vivo,* AR agonist treatment increases BAT weight, which may be suggestive of a depressed BAT activity [110]. However, mice with a global AR deficiency show reduced BAT *Ucp1* expression [115, 116]. Whether androgens modulate BAT activity *in vivo* and the potential pathways for such a regulation remains unknown.

2 AIM

The general aim of this thesis was to investigate immunological, vascular and metabolic actions of androgens in male mice.

The specific aims of the four papers included in the thesis were:

I: To define the mechanism by which androgens regulate splenic B cell numbers in males.

II: To test the hypothesis that atherosclerosis induced by androgen deficiency is T cell-dependent and that AR knockout specifically in thymic epithelial cells (epithelial cell-specific AR knockout; E-ARKO) results in increased atherosclerosis in male mice.

III: To test the hypothesis that AR knockout specifically in osterix-expressing bone cells (osterix-expressing bone cell-specific AR knockout; O-ARKO) alters aortic neutrophil recruitment and protects against AAA in male mice.

IV: To clarify the role of androgens and the AR for *in vivo* BAT activity in male mice.

3 METHODOLOGICAL CONSIDERATIONS

This section consists of a general discussion of the methods included in this thesis. A more detail description of the methods can be found in the Material and Methods section of each individual paper.

3.1 ANIMAL MODELS TO STUDY ANDROGEN ACTIONS

In this thesis, two different rodent models were used to study androgen actions; removal of endogenous testosterone (by surgical removal of the testes) and inactivation of the androgen receptor (in genetically modified mice using the Cre/LoxP technology).

3.1.1 CASTRATION AND TESTOSTERONE REPLACEMENT

Castration (orchiectomy; ORX) results in elimination of endogenous androgen production in male mice. ORX renders mice completely testosterone deficient since mice lack adrenal androgen production. This model was used in Papers I-IV.

For supplementation with testosterone (in Papers I-III) we used subcutaneous slow-release pellets (Innovative Research of America, Sarasota, FL, USA) that assure steady testosterone physiological levels [60]. For a short-term experiment (in Paper IV) we used testosterone replacement protocol based on subcutaneous injections of testosterone every other day [117]. This regime is around 2.5-3 mg/kg/day for a 0.025 kg mouse. In a pilot study performed by our group, serum testosterone levels measured 24 h after the first injection was slightly higher than those of intact mice. However, wet weights of seminal vesicles and salivary glands matched those from sham-operated controls after a treatment period of 2 weeks (injections every 3 days).

3.1.2 INACTIVATION OF THE ANDROGEN RECEPTOR

The second model consists on the inactivation of the AR in all cells (G-ARKO mice) or in specific target cells such as B cells (B-ARKO), epithelial cells (E-ARKO mice), osterix-expressing bone cells (O-ARKO mice) or brown adipocytes (BAT-ARKO mice) (Table 2).

Table 2. Different ARKO mice models used in Papers I-IV.

Mice	Paper	Cre promoter	Target cell	Control mice
G-ARKO	I, III	Phosphoglycerate Kinase 1	General	Pgk-Cre$^{+/-}$ [118]
B-ARKO	I	CD79a	Early-pro-B cells	Cd79a-Cre$^{+/-}$ or Mb1-Cre$^{+/-}$ [119]
	I	CD19	Pre-B cells	Cd19-Cre$^{+/-}$(Jackson Laboratories)
E-ARKO	II	Bovine keratin 5	Epithelial cells	K5-Cre$^{+/-}$ [120]
O-ARKO	I, III	Osterix	Osterix-expressing bone cells	Osx1-Cre$^{+/-}$(Jackson Laboratories)
BAT-ARKO	IV	Uncoupling protein 1	Brown/ brite adipocytes	Ucp1-Cre$^{+/-}$ (Jackson Laboratories)

To generate the different ARKO mice, we used the Cre-LoxP technology; we bred female AR$^{+/flox}$ (LoxP sites introduced around exon 2, corresponding to the DNA-binding domain) mice with Cre$^{+/-}$ males. The 25% of the offspring expresses Cre recombinase which recognizes and cuts the "floxed" exon 2 and introduces a stop codon before exon 3, resulting in the knockout of the AR.

To assess the efficacy and tissue/cell specificity of the deletion of AR, we quantified exon 2 ("floxed") and compared it to exon 3 (not "floxed") in genomic DNA. Varying ratios of AR exon 2 to exon 3 genomic DNA may be considered as good knockout efficacy. Higher efficiencies (90%) are expected when analyzing organs from G-ARKO mice or isolated cells from cell specific-ARKO (e.g. B cells from B-ARKO mice) while lower efficiencies (40%) are expected when analyzing whole organs in cell-specific-ARKO mice (e.g. BAT from BAT-ARKO mice). The lower efficiencies might be due to presence of other cell types when analyzing a whole organ (e.g. BAT), contamination issues during cell isolation, or that AR knockout might not be 100% efficient.

3.1.3 DIETARY CONSIDERATIONS

Phytoestrogens, such as isoflavones, are plant-derived compounds that mimic estrogen actions and can potentially alter the results from studies on sex steroid actions. In order to avoid potential estrogenic effects, we used regular chow diets formulated to exclude soybean meal such as 2016 Teklad Global 16% Protein Rodent Diet, Harlan, UK; RM3 (E) Soya free, Special Diet Services, UK or R70, Lantmännen Lantbruk, Sweden.

3.2 ANIMAL MODELS OF ATHEROSCLEROSIS

Animal models provide certain insights into the complex mechanisms of atherosclerosis. Despite their highly debated relevance for human disease, mice are a common animal model used for atherosclerosis studies because they can be genetically manipulated, are relatively easy to handle, cheap, and reproduce certain characteristics of the human disease.

There are major differences between mice and humans regarding lipoprotein metabolism and bile acid composition [121]. Mice transport cholesterol mainly in high-density lipoprotein (HDL), have low plasma cholesterol levels, lack cholesteryl ester transfer protein, have a different bile acid composition which reduces cholesterol uptake in the intestine. Overall, these properties protect mice against atherosclerosis when fed a regular chow diet with low cholesterol content (0.02-0.03%) (Table 3).

Table 3. Species differences in lipoprotein metabolism and atherosclerosis.

	Humans	**Wild-type mice**
Cholesterol	LDL	HDL
CETP	High levels	No
Bile acids composition		α- and β-muricholic acids (↓intestinal cholesterol uptake)
Cholesterol levels	High	Low
Lesion location	Coronary arteries, carotids and peripheral vessels (iliac artery)	No lesions
Atherosclerosis	Atherosclerosis	Atheroprotected

A combination of dietary and genetic manipulations has been able to generate different mouse models for studies of the pathophysiology of atherosclerosis. Diets with higher cholesterol and fat content are able to induce atherosclerosis in atherosclerosis-prone strains such as C56BL/6 mice and accelerate the process in atherosclerosis-prone genetic mouse models. Nowadays, there are several available atherosclerosis-prone genetic mouse models.

3.2.1 APOLIPOPROTEIN E-DEFICENT MOUSE MODEL

In Paper II, we used the apolipoprotein E-deficient ($apoE^{-/-}$) model. Genetic ablation of ApoE leads to spontaneous hyperlipidemia and development of atheromatous plaques that show some resemblance with human plaques. $ApoE^{-/-}$ mice still differ with humans in some parameters: 1) $apoE^{-/-}$ mice carry plasma cholesterol in very-low-density lipoprotein and chylomicron particles, 2) $apoE^{-/-}$ mice preferentially develop lesions in aortic root, carotid artery and aortic branches 3) $apoE^{-/-}$ mice seldom present plaque rupture, thrombosis or calcifications.

3.2.2 EVALUATION OF ATHEROSCLEROSIS

Evaluation of atherosclerosis is normally assessed *ex vivo* in aortas prepared *en face* or in aortic root sections, stained for lipids (Sudan IV and Oil Red O respectively). *En face* analysis shows the distribution of lesions throughout the aorta but it does not provide information about the characteristics of the lesion. To study the characteristics and size of the lesion is necessary to examine cross-sections of aortic root stained with immunohistochemical and histological techniques. In Paper II, we evaluated lesion size in Oil Red O stained cross-sections of aortic root at different levels as well as the presence of T cells (CD3) and leukocytes (CD18) in the plaques.

3.3 ANIMAL MODELS OF AAA

Animal model of disease should mimic cellular and biochemical features of the progression of the human disease. However, this premise is difficult to fulfil in the case of AAAs. Characteristics of human AAAs have mainly been defined from tissues collected at late stages of the disease, when surgical repaired is needed (AAA diameter > 5 cm), while little information about initiation and progression of human AAAs is available. Several mouse models of AAA have been developed by inducing the disease via genetic manipulations (defects of the extracellular matrix, in matrix metalloproteinases, overexpression of renin and angiotensinogen and hyperlipidemic mice) and chemical induction (elastase infusion, calcium chloride and angiotensin II infusion). These models include some of the

characteristics present in the human disease such as inflammation, medial degeneration, thrombus formation and rupture of the aortic wall [122, 123] and may provide insights into the pathophysiology and modulation of the disease.

3.3.1 ANGIOTENSIN II-INDUCED AAA MODEL

The angiotensin II (AngII)-induced AAA model was chosen in Paper III. This model presents several features of human AAAs, including medial degeneration, inflammation, and thrombus formation [122], as well as higher susceptibility of males compared to females [122]. However, the AAAs locate in the infrarenal area of the aorta in humans and suprarenal area in mice exposed to AngII [123]. In this model, Ang II is administrated at doses between 500-1000 ng/kg/min to AAA-prone apoE$^{-/-}$ mice. Of note, wild-type C56BL/6 mice also develop AAA in the suprarenal region of the aorta in response to AngII, but the incidence of AAA is lower [122]. The temporal characteristics for initiation and progression of AngII-induced AAAs have been well described over a 56-day period (Table 4).

Table 4. Temporal sequence of events in AngII-induced AAA model [123].

Time (days)	Changes in the suprarenal region of abdominal aorta
1-4	– Medial accumulation of macrophages – Disruption of elastin fibers – Adventitial accumulation of macrophages (suprarenal aorta, thoracic and sinus)
4-10	– Vascular hematoma – 10% of the mice die (aortic rupture) – Development of thrombi and inflammatory reaction (macrophages)
Beyond 14	– Deposition of extracellular matrix in thrombi regions – More macrophages and also T and B cells – Medial disruption still present and disorder elastin fiber between broken elastin fibers
28	– Changes in endothelium distribution (reendothelialzation of dilated lumen) – Neovascularization of the aneurysmal tissue
Beyond 28	– Atherosclerotic lesions detected (not detected earlier)

AngII, which is a major effector of the renin-angiotensin system, is a peptide hormone that increases blood pressure by vasoconstriction and other mechanisms. AngII infusion at higher doses results in moderate increases in blood pressure. However, it has been shown that elevated blood pressure is not necessary for AngII-induced AAA formation [124]. AngII is also a pro-inflammatory mediator that induces the transcription of pro-inflammatory genes through nuclear factor-κB.

3.4 FATTY ACID UPTAKE BY BROWN FAT

In Paper IV, we evaluated BAT activity by measuring the uptake of FA derived from glycerol tri[^{3}H]oleate ([^{3}H]TO)-labeled lipoprotein-like triglyceride-rich particles. The emulsion particles (80 nm) consist of a hydrophobic core (neutral lipids: triolein, cholesteryl oleate) surrounded by a monolayer of phospholipids (egg yolk phosphatidylcholine, lysophosphatidylcholine and cholesterol). This emulsion mimics chylomicrons *in vivo* and acquire apolipoproteins such as apolipoprotein E when incubated with serum [125].

3.5 INDIRECT CALORIMETRY AND TELEMETRY

Energy expenditure is most commonly assessed by indirect calorimetry, which is a non-invasive technique. Indirect calorimetry estimates energy expenditure by measuring oxygen consumption and carbon dioxide production, in contrast to direct calorimetry that measures heat production (Figure 6). Moreover, it provides important information about the nature of substrate(s) used for metabolism. The respiratory quotients (RQ), calculated by the quota of the amount of carbon dioxide produced (VCO_2) divided by the amount of consumed oxygen (VO_2) by the animal, give information about the actual substrate that the animal metabolizes, i.e. oxidation of lipids and proteins requires more oxygen and produces more energy than glucose oxidation [126]. At the same time, we also monitored cBT and locomotor activity (LMA) by telemetry.

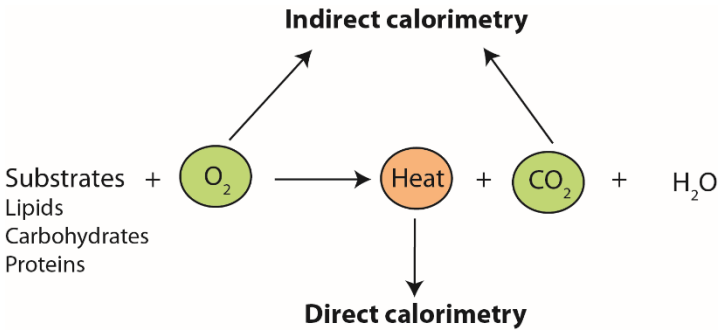

Figure 6. The energy expenditure of an organism can be directly measured (direct calorimetry) or estimated by assessing oxygen (O_2) consumption and carbon dioxide (CO_2) production (indirect calorimetry).

Energy expenditure, VO_2, VCO_2, RQ, cBT and LMA have a circadian rhythm showing higher values during awake period. Therefore, in Paper IV, mice were studied during a 24h study period to take into account the diurnal variations but we excluded the first 3h from the analysis. The reason for exclusion was that during this period, mice needed to adjust to a new environment and that they were caged alone. These new conditions might increase stress levels in mice, which affects the measurements, and in turn the interpretation of the results. Considering the diurnal rhythmicity of BAT activity (whose FA uptake peaks at the onset of wakening) [123] as well as LMA, we specially addressed time different intervals (Table 5).

Table 5. Time intervals specially addressed in Paper IV according to [102]. Time intervals given as Zeitgeber times, ZT0 corresponds to the onset of light period (7:00) and ZT12 corresponds to the onset of the dark phase (19:00). LMA, locomotor activity.

Time interval (ZT)	**BAT activity**	**LMA**
4-6	Low	High
8-12	High	Low
12-18	High	High

Energy expenditure increases with a greater total tissue mass and depends on body composition, e.g. lean tissues (brain, heart and liver) utilize more energy than fat tissue [93]. Therefore, adjustment of VO_2 and VCO_2 to body size or lean mass is done when these parameters differ between groups at the time of indirect calorimetry measurements. However, cBT and LMA are not traditionally adjusted to body size or lean mass. Nowadays, there is still no consensus on whether to adjust or not to body weight and/or lean mass. In Paper IV we showed VO_2 adjusted to lean mass, since mice at the time of indirect calorimetry differ in lean mass (as well as body weight).

3.6 GATING STRATEGIES FOR NEUTROPHILS

In Paper III, two different gating strategies were used to identify bone marrow neutrophils: $CD45^+$ $Gr\text{-}1^+$ (Ly6G/Ly6C) SSC^{hi} and $CD11c^-$ $CD11b^+$ $Ly6G^+$.

CD11b labels myeloid cells (monocytes, neutrophils and eosinophils) in the bone marrow. Ly6G labels immature neutrophils ($Ly6G^{lo}$) and mature neutrophils ($Ly6G^+$) in the bone marrow [127]. Therefore, by using $CD11b^+$ $Ly6G^+$ we identify mature neutrophils in bone marrow.

CD45 is expressed all nucleated hematopoietic cells except for erythroid cells [128]. The anti-Gr-1 binds to both Ly6G and Ly6C. Ly6G is only present in neutrophils while Ly6C labels patrolling monocytes ($Ly6G^{low}$), neutrophils, eosinophils ($Ly6G^{int}$) and inflammatory monocytes ($Ly6G^{hi}$). Side scatter (SSC) gives information about the internal complexity or granularity of cells; neutrophils present more granularity than monocytes and can be easily separated from monocytes. Therefore, by using $CD45^+$ SSC^{hi} $Gr\text{-}1^{hi}$ we exclude monocytes and identify mature neutrophils in bone marrow. However, some eosinophils may also be included (although their numbers (0.4-4.7%) are lower than segmented (8.2-21.5%) and band (13.7-31.6%) neutrophils in the bone marrow [129]. Therefore, both gating strategies are able to identify bone marrow neutrophils.

4 RESULTS AND CONCLUSIONS

This section includes a brief description of the main findings and conclusions of each paper included in the thesis. More details are included in the Results and Conclusion section of each paper.

Paper I: Testosterone is an Endogenous Regulator of BAFF and Splenic B Cell Number.

In this study, we show that testosterone is an endogenous regulator of BAFF. Further, the increased splenic B cells numbers in spleen in males due to testosterone deficiency involves nervous regulation of FRCs and BAFF production.

We conclude that testosterone regulates BAFF and that this regulation may be implicated in testosterone-mediated protection against autoimmunity.

Paper II: Testosterone Protects against Atherosclerosis in Male Mice by Targeting Thymic Epithelial Cells.

In this study, we reported that testosterone-mediated atheroprotection in male mice is T-cell dependent. Further, AR deficiency specifically in thymic epithelial cells increased thymus size and thymus-dependent atherosclerosis.

We conclude that the thymic epithelium is an important target compartment for the atheroprotective actions of testosterone.

Paper III: Depletion of the Androgen Receptor in Osterix-Expressing Bone Cells Protects Against Abdominal Aortic Aneurysms in Male Mice.

In this study, we found that AR deletion specifically in osterix-expressing bone cells reduces neutrophil infiltration to the aorta after AngII exposure and mimicked the protection against AAA observed in castrated and G-ARKO male mice.

We conclude that osterix-expressing bone cells are target cells for the AAA-provoking actions of testosterone.

Paper IV: Testosterone Reduces Brown Fat Activity in Male Mice.

In this study, we demonstrated that androgen deficiency by castration markedly increases BAT activity and cBT *in vivo*. Furthermore, the mechanism for these effects is independent of AR expression in brown adipocytes and might be mediated by an increase sympathetic outflow to BAT, supported by a castration-induced increase in NA levels in BAT.

We conclude that testosterone is a negative regulator of BAT activity and cBT in male mice.

The main findings of the thesis are summarized in the schematic illustration below (Figure 7).

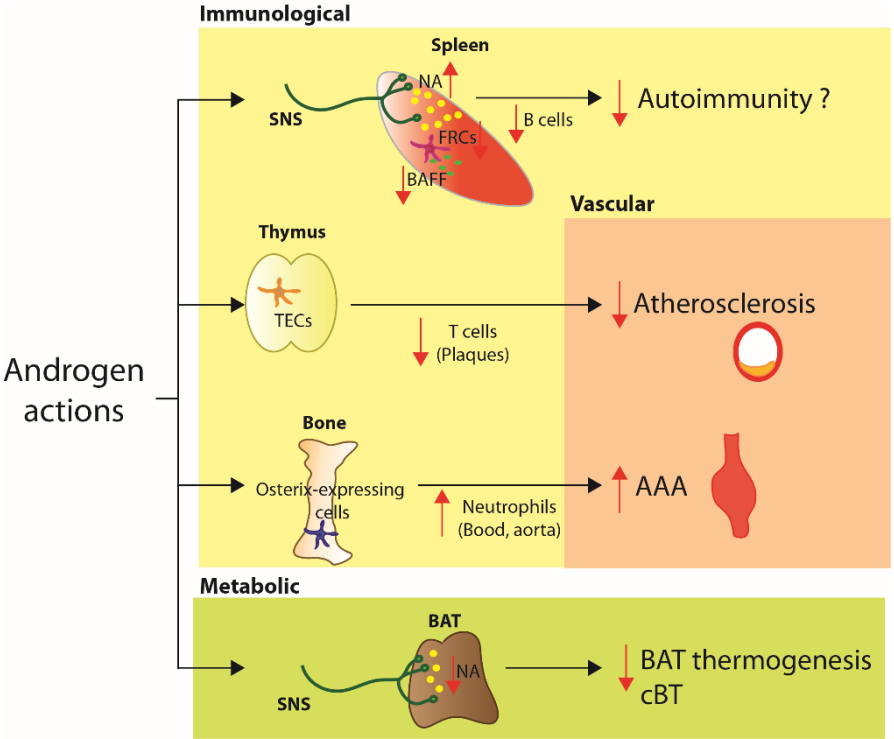

Figure 7. Immunological, vascular and metabolic actions of androgens. SNS, sympathetic nervous system; NA, noradrenaline; FRCs, fibroblastic reticular cells; BAFF, B-cell activating factor; TECs, thymic epithelial cells; AAA, abdominal aortic aneurysm; BAT, brown adipose tissue and cBT, core body temperature.

5 DISCUSSION

5.1 IMMUNOLOGICAL ACTIONS OF ANDROGENS

Androgen actions on the stroma of lymphoid organs

Throughout Papers I-III, we found that androgens regulate immune cells indirectly via the stroma compartment of primary (bone marrow and thymus) and secondary (spleen) lymphoid organs (Figure 7). Stromal cells from primary lymphoid organs are AR targets (TECs in thymus and osterix-expressing cells in bone) while the splenic stroma cells (FRCs) are indirectly regulated via increased signaling from sympathetic nerves to spleen. Notably, immune cells (neutrophils, B and T cell) also express AR and thus androgens can modulate immune cells directly [130]. Studies combining bone marrow transplantation and global AR deletion have shown that AR deletion in thymic stroma, but not in T cells, increases thymus size and cellularity [131]. This finding is in line with our data in Paper II, showing that AR deletion in thymic epithelial cells increases thymus weight.

Direct actions of androgens on B cells have been proposed to regulate splenic B cell numbers. This was reported in a study by Altuwaijri et al, in which B cell-specific AR knockouts (created using CD19-Cre) were compared to wild type mice [132]. In Paper I, we assessed splenic B cell numbers using two different B cell specific-AR knockout models (using CD19 and CD79a Cre promoters) and compared them to their respective Cre expressing littermates, which strengthen our data. We also assessed the efficiency of the AR knockout in splenic B cells, which was 86% and 93%, respectively. Overall, our data do not support that the AR in B cells has a role for the regulation of splenic B cell numbers. Future studies should investigate whether AR in B and T cells might affect cellular functions, which has not been addressed in this thesis.

The use of wild-type, non-littermate controls instead of Cre-expressing littermate controls might complicate the interpretation of the Altuwaijri study. One reason is that Cre recombinase can produce a phenotype of its own. For example, Cre expression can lead to unforeseen outcomes due to "Cre toxicity" [133]. Furthermore, mammalian genomes contain interspersed recombinase recognition sites or cryptic *loxP* sites and recognition of these sites by Cre might lead to DNA and cell damage [134]. Therefore, it is often necessary to include Cre transgenic mice as controls [135] and littermate controls should always be used. Further, the *flox* construct should be tested

for effects on main endpoints, which was done in the papers included in this thesis.

Current evidence on the role of AR in neutrophils is based on data from global AR deletion [136]. Mice with a global AR deletion have an impaired granulopoiesis from the promyelocyte stage, are neutropenic and present impaired host defense [136]. However, mice with AR deletion in myeloid cells (Lyz-Cre) have normal granulopoiesis [130]. In Paper III, we found that granulopoiesis and bone marrow neutrophil numbers were unaltered in castrated and O-ARKO mice in unchallenged conditions. Collectively, findings to date suggest that regulation of granulopoiesis in ARKO mice is not mediated via AR in myeloid cells or osterix-expressing bone cells. Direct actions of androgens have been described in *in vitro* studies. *In vitro* treatment of neutrophils from healthy humans with testosterone directly regulates neutrophil function, by inhibiting superoxide production "antioxidant properties", but increases bactericidal activity [137].

Androgens suppress the adaptive immune system but promote neutrophil recruitment

In Papers I-II, we showed that androgens have profound suppressive effects on the adaptive immune system. Androgens reduce splenic B cell number via nervous regulation of FRC numbers and BAFF production, and reduce the peripheral T cell pool via TECs. In Paper III, we found that androgens, via AR in osterix-expressing bone cells, enhanced AngII-induced neutrophil recruitment to the aorta.

Our findings in Paper III are consistent with previous studies showing that androgens increase neutrophil accumulation in inflammatory sites [47-49], which favors inflammation and tissue damage. Of note, AR blockade during heatstroke reduces neutrophil infiltration in the lung and improves the outcomes of male mice [138]. Surprisingly, testosterone treatment to castrated male rats reduced the bactericidal activity of neutrophils [49]. In contrast, i*n vitro* treatment of neutrophils from healthy humans with testosterone increases microbicide activity of neutrophil at physiological doses (10nM) [137]. Thus, direct and indirect actions of androgens on neutrophils seem to exert functionally opposing effects on neutrophils.

During trauma-hemorrhagic shock and thermal injury, male rats have higher risk to develop acute respiratory distress, sepsis and multi-organ failure [49, 139]. Testosterone promotes neutrophil activation (upregulation of CD11b, which regulates adhesion and migration of neutrophils) after trauma-hemorrhagic shock and burn injuries while castration of male rats prevented

this activation [139, 140]. These data may suggest that neutrophil migration is increased by castration. In contrast, our (unpublished) data show that CD11b expression in blood neutrophils is upregulated in castrated mice during thioglycollate-challenge. This upregulation was however not mimicked in the O-ARKO mice, suggesting alternative mechanisms in the O-ARKO model.

Do androgens increase neutrophil egress from the bone marrow?

In Paper III, we found that O-ARKO mice challenged with AngII have significantly reduced neutrophil numbers in blood and aorta. Although not directly addressed in Paper III, this may indicate that AR in osterix-expressing bone cells modulates neutrophil egress from the bone marrow in response to an inflammatory trigger.

To better understand the neutrophils fluxes of O-ARKO mice, we estimated the total number of neutrophils in bone marrow and blood after AngII challenge (Figure 8). 24h after AngII, the bone marrow of O-ARKO mice tended to have more (+1.36x10^6) neutrophils than Osx1-$Cre^{+/-}$ control mice (Figure 8). Interestingly, the same mice showed a reduction of 1.03x10^6 neutrophils in the blood pool (Figure 8). Thus, the "surplus" of neutrophils in bone marrow is similar to the "deficit" in the bone marrow, which might support the theory of neutrophil retention in bone marrow during AngII-challenge in O-ARKO mice.

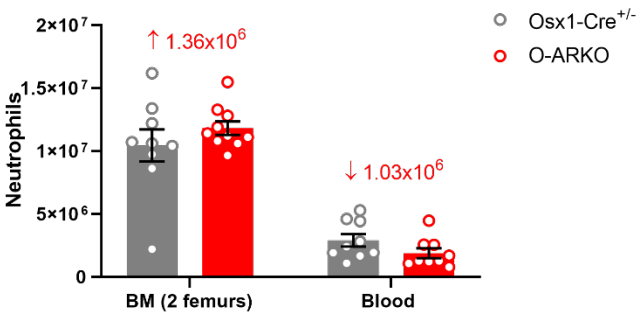

Figure 8. Bone marrow and blood neutrophil pools in O-ARKO and Osx1-Cre+/- mice treated with angiotensin II for 24h. Neutrophils in the blood pool were calculated by multiplying the neutrophil number per µl blood by total blood volume of the mouse (58.5 ml/kg).

In the bone marrow, the production of leukocytes is tightly regulated. In steady state, lymphopoiesis is promoted over granulopoiesis [20]. However, inflammation shifts this balance, promoting the mobilization of neutrophils to the inflammation site and accelerating the production of new granulocytes [20]. These processes are regulated via G-CSF and CXC chemokines. G-CSF disrupts the CXCL12-CXCR4 axis (retention axis), by reducing the absolute number of osteoblasts (osterix-expressing cells) and thus CXCL12 levels. Simultaneously, the expression of CXCL1 and CXCL2 increases in the bone marrow during inflammation. Binding of CXCL1-2 to their receptor CXCR2 on neutrophils facilitate their egress from bone marrow [16].

In Paper III, we found that CXCL12 mRNA levels in $CD45^-$ cells as well as the number of $CD45^-$ cells were increased by castration. Since CXCL12 promotes bone marrow B lymphopoiesis, upregulation of CXCL12 may potentially be responsible for: (i) enhanced bone marrow B cell numbers in O-ARKO [44] and castrated mice in steady state conditions and (ii) neutrophil retention in bone marrow during AngII-challenge and (iii) reduced blood and aortic neutrophil numbers secondary to (ii). Interestingly, treatment with anti-CD20 (Rituximab) has been reported to cause late-onset neutropenia. Following rituximab treatment, B lymphopoiesis is important for the recovery of B cells and this process is associated with a decrease in neutrophils in blood. Such a dual regulation might reflect bone marrow levels of the CXCL12, which promotes B lymphopoiesis and retains neutrophils in the bone marrow [19]. Future studies are needed to define the link between AR in osterix-expressing bone cells and the regulation of neutrophil mobilization to inflammatory sites, including the role of CXCL12.

5.2 VASCULAR ACTIONS OF ANDROGENS

In Papers II-III, we found that certain vascular actions of androgens are mediated via the immune system (Figure 7).

Androgens are atheroprotective but AAA-provoking

In Papers II-III, we show that testosterone have opposite actions depending on disease type, being atheroprotective but AAA-provoking. In Paper II, we demonstrated that the atheroprotective actions of androgens are mediated via AR in thymic epithelial cells, which regulates thymopoiesis and reduces T cell numbers in the plaques. However, in Paper III, we found the AAA-provoking actions of androgens are mediated via AR in osterix-expressing bone cells and affect neutrophil aortic accumulation. The findings of this thesis support that the opposing effects of androgens on atherosclerosis and AAA might reflect the diverse actions of androgens in the immune system.

Androgen levels and AAA in men

Relatively lower testosterone levels are associated with increased risk of CVD events and atherosclerosis [11, 58]. These associations are in agreement with our findings in Paper II and other experimental studies of atherosclerosis [60]. So far, only one study has addressed the association between circulating androgen levels and AAA, showing an association between low free testosterone levels and presence of AAA in elderly men [141]. Thus, these data are not in agreement with our data in Paper III or previous experimental evidence indicating that androgens increase AAA development in animal models [85-88, 142, 143].

The apparent disagreement between the clinical association study and the experimental AAA data may be partly attributed to the effects of inflammation on testosterone levels [144, 145]. Several studies have demonstrated that inflammation, both acute and chronic, causes male hypogonadism [144]. Even a low dose endotoxin challenge produces a transient decline in testosterone serum in lean men, without affecting LH or FSH [144]. Further, *in vitro* treatment of Leydig cells with cytokines reduces testosterone production [145]. Considering the important inflammatory component of AAA, the associations between low serum testosterone and AAA in elderly men may just reflect the presence of chronic inflammation at a later stage of the disease. Future studies may investigate androgens levels at earlier ages of AAA development and/or apply genetic tools for increased understanding of the role of androgens in AAA development.

Role of androgen-mediated regulation of neutrophils in atherosclerosis

Neutrophils are also an important contributor to atherosclerosis [146]. High neutrophil counts are associated with risk of CVD events [73]. In humans, neutrophils are present in the plaques of rupture-prone areas and in intraplaque hemorrhage. Accumulation of neutrophils and their proteases likely drives plaque instability [146]. In experimental models of atherosclerosis, lesion size positively correlates with neutrophil numbers in blood. Neutropenic mice present smaller atherosclerotic lesions at early stages of plaque formation [147]. Neutrophils are found in both early and advanced lesions in murine models, especially in the plaque shoulder and in highly inflammatory areas of the plaque [147].

As demonstrated in Paper III, O-ARKO mice showed reduced neutrophil numbers in blood and aorta during challenge with AngII. Therefore, we hypothesized that O-ARKO might also protect male mice against atherosclerosis. However, O-ARKO apoE$^{-/-}$ male mice develop a similar area of atherosclerotic lesions as control mice (unpublished data).

Although we could not see an effect of O-ARKO on atherosclerotic lesion area, further studies may address whether O-ARKO might have effects on plaque stability. Testosterone replacement therapy used in the treatment of androgen deficiency in young men is not associated with increased CVD risk [148]. However, when used in the treatment of elderly and/or obese men, it increases the risk of CVD events early after the initiation of treatment [148]. The rapid divergence in frequency of CVD events between testosterone and placebo groups indicates acute actions of testosterone that may result in plaque instability. It is tempting to speculate that such acute effects of testosterone treatment might be partly mediated via increasing neutrophils accumulation in blood and/or the atherosclerotic lesion, in turn affecting atherosclerotic plaque stability [59].

Role of androgen-mediated regulation of T and B cells in AAA

T cells constitute the majority of the inflammatory cells in both human and experimental AAA tissue and CD4$^+$ T cells are the most abundant subtype here [149]. Presence of CD4$^+$ T cells has been reported to be essential for AAA formation. CD4-deficient mice and mice lacking IFN-γ (an important cytokine of T cells) are resistant to aneurysm formation [150]. Pro-inflammatory CD4$^+$ T cells contribute to chronic inflammation in the injured aortic wall, which enhances the progression of AAA [149].

As shown in Paper II, androgens reduce thymus size, while E-ARKO increases thymus size, T cell infiltration in the atherosclerotic plaque as well

as atherosclerotic lesion area. Regulation of T cells in E-ARKO might theoretically also result in formation/progression of AAA in male mice. Therefore, it would be interesting to test the hypothesis that E-ARKO promotes AAA progression in male mice.

B cells and immunoglobulins are present in AAA tissue in both mice and humans [151]. Current evidence on the role of B cells in experimental AAA is contradictory, and both protective [152] and pathogenic [153] roles have been described. B cell depletion using anti-CD20, which depletes B cells from the pro-B cell state but not plasma cells, seems to protect against AngII-induced AAA via increased number of regulatory T cells in aorta [153]. B-cell-deficient (μMT) mice present similar AAA frequency as wild-type mice [152] while adoptive transfer of splenic B2 cells to μMT mice protected against AAA formation [152]. We cannot exclude that the protection against AAA in O-ARKO (Paper III) is mediated by modulating the B cell compartment. However, the fact that O-ARKO mice show unaltered levels of B cells in blood makes this possibility seem less likely.

A plausible explanation for the contradictory role of B cells in AAA formation might be illustrated by the effects of B cell depletion on bone marrow homeostasis. The type of B depletion may lead to an increase CXCL12 as a compensatory mechanism to recover B cells. In fact, anti-CD20 has been shown to promote late-onset-neutropenia and alterations in serum CXCL12 levels in humans [19]. In contrast, B cell depletion in μMT mice results in increased number of neutrophils in the infectious lesions [154]. Thus, it may be hypothesized that anti-CD20 protection against AAA formation might be mediated via retention of neutrophils and upregulation of CXCL12, and in line with the importance of the bone marrow stroma for regulating leukocyte responses during inflammation.

5.3 METABOLIC ACTIONS OF ANDROGENS

Potential pathways for androgen-mediated regulation of BAT activity

In Paper IV, we demonstrate that androgen-mediated regulation of BAT activity is accompanied by reduced NA content in BAT, suggesting the involvement of central thermoregulatory pathways. However, although SNS is the main driver of BAT activity, there are also other thermoregulatory pathways, such as the hypothalamus-pituitary-adrenal and hypothalamus-pituitary-thyroid axis [101] that might potentially be targeted by androgens. Castration increases the levels of ACTH [155] and progesterone [156], which are strong activators of BAT activity [101]. Therefore, the markedly increased FA uptake by BAT observed in castrated mice might be due to a combination of effects rather than just due to increased sympathetic signaling to BAT. Future studies combining castration with an anti-ACTH antibody [157] and/or progesterone antagonists might clarify the contribution of these pathways.

Do androgens regulate sympathetic activity in a tissue-specific manner?

SNS plays a critical role in physiological regulation of e.g. heart rate, metabolism, and body temperature. SNS signaling also has profound effects on splenic homeostasis [158] and is the main activator of BAT thermogenic activity [101]. Thus, regulating SNS activity is a powerful pathway for modulation of various physiological functions.

In Paper I and IV, we find indications of androgens regulating SNS output to spleen and BAT, respectively. Testosterone is known to increase general SNS signaling [159, 160]. However, interpreting the data from Paper I and IV, we hypothesize that androgens modulate sympathetic output in a tissue-specific manner. Our findings support that androgens increase sympathetic signaling to spleen while reducing the sympathetic signaling to BAT (Figure 7). We have not studied at what level (e.g. in brain, spine, or ganglia) this regulation occurs and future studies should address this question.

Androgens affect body composition and energy balance

Negative energy balance (energy intake < energy expenditure) over a period of time leads to body weight loss [94]. In Paper IV, we show that androgen depletion results in body weight loss in male mice, as could be expected from previous studies [161, 162]. We have found that food intake is unchanged 3 weeks after castration (unpublished data), which is in line with a previous study showing unaltered food intake during the first three weeks after castration [161]. However, in the long term, castration has been shown to reduce food intake in rodents [161, 162]. Overall, our data suggest that weight loss by androgen depletion in the period studied here (3 weeks) might be due to increased energy expenditure rather than reduced energy intake.

In Paper IV, lean mass, the major contributor to energy expenditure, was reduced by short term (3 weeks) castration. This result is also in line with previous studies [1, 2]. Simultaneously, fat mass was not significantly affected by androgen depletion at this time point. BAT and muscle-based thermogenesis have been shown to compensate each other [163]. Long term castration is known to increase fat mass in male mice [161, 162] and we also have data confirming this effect (unpublished data). The castration-induced increase fat mass might be considered a "side-effect" of the reduction of the highly metabolically active lean mass, given its major contribution to total energy expenditure [94]. That is, loss of lean mass may lead to an excess of energy which needs to be stored as fat. Given that castrated mice become obese, it is somewhat surprising that BAT activity remains increased long term after castration. Consistently, androgen deprivation therapy in adult men is associated with increase fat mass and body weight gain (Table 6).

Table 6. Effects of androgen depletion on body weight, lean mass, fat mass and brown fat activity (BAT) activity in male mice and men.

	Male mice		**Men**
Parameter	**Short term ORX (3 weeks)**	**Long term ORX (>3 weeks)**	**Androgen depletion**
Body weight	↓	↓	↑
Lean mass	↓↓	↓↓	↓
Fat mass	(↓)?	↑	↑
BAT activity	↑	↑	?

5.4 PHYSIOLOGICAL RELEVANCE OF ANDROGEN ACTIONS

In homeostatic conditions, i.e. in the absence of any energy-demanding threats (such as infection, predators, extreme temperatures or lack of food), energy is spent on body maintenance needs, such as growth, basal physical activity, basal metabolism and reproduction [93].

Androgens are potent anabolic hormones that direct energy utilization towards e.g. maintenance/growth of lean and bone mass. Consistent with these roles, we found (Paper IV) that androgen depletion by castration rapidly affects body composition by decreasing lean mass. Simultaneously, androgens prevent energy "loss" by suppressing other tissues/systems, such as the adaptive immune system (e.g. reduces splenic B cell number, peripheral T cell pool; Papers I-II) and reduces BAT activity/heat production and cBT; Paper IV). These suppressive actions of androgens might be a mechanism to prioritize fuel/energy for anabolism and reproduction. The results of this thesis support that androgens are important regulators of energy utilization (Figure 9).

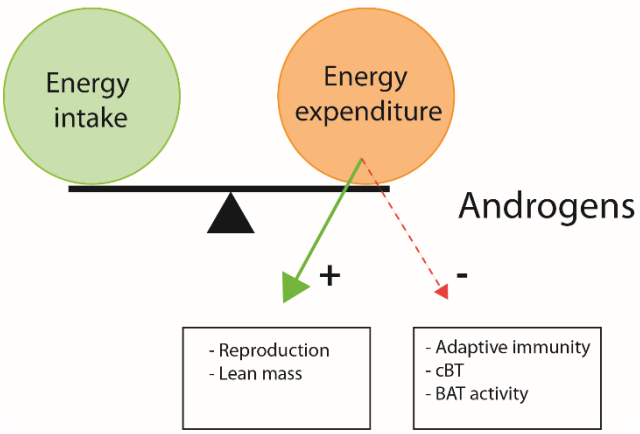

Figure 9. Androgen are important regulators of energy balance, prioritize energy for reproduction and anabolic functions while preventing energy loss via adaptive immunity and BAT activity. BAT, brown adipose tissue and cBT, core body temperature.

The presence of “threats” creates a situation of high-energy demand for the individual. Some examples of extra energy costs on energy expenditure are: inflammation (25-30%), chronic low-grade inflammation (10%), acute pain (up to 60%), and sleep alterations (up to 30%) [164].

Evolution has occurred in the context of limited supply of nutrients [165]. Mental memory (i.e. remembering where to find food/water) and immunological memory (adaptive immune response to a second time exposure) are mechanisms to minimize energy expenditure and protect energy stores [164]. Central nervous and immune systems are the main sites for the regulation of energy utilization. In the presence of threats, the brain and immune system will activate different mechanisms to release energy from the energy stores (adipose tissue, muscle and liver) to cover their needs in order to defend the individual from those threats. However, energy prioritization to the brain and immune system leads to cutbacks on other physiological functions such as reproduction and growth.

Consequently, it seems physiologically relevant to decrease androgen production in the presence of threats such as stress or inflammation [144, 145] - “first survival and then reproduction”- to redistribute energy utilization, prioritizing energy to adaptive immunity while cutting down on anabolism (Table 7).

Interestingly, in response to an inflammatory trigger such as AngII, androgens suppress adaptive immunity, while increasing neutrophil recruitment to inflammation sites (in Paper III). It is plausible that the reduction in androgen levels during inflammation might serve the purpose to shift the focus of the immune activation towards reduction of neutrophil accumulation and promotion adaptive immune responses, which takes place during later stages of an immune reaction [166, 167].

Table 7. Androgen and androgen depletion effect on immune system, body composition, core body temperature (cBT) and reproduction. BAT, brown adipose tissue.

	Androgens	**Androgen depletion**
Immune system	**Adaptive immunity** ↓ Splenic B cells ↓ T cells **Innate immunity** ↑ Neutrophil migration **SAVES ENERGY**	**Adaptive immunity** ↑ Splenic B cells ↑T cells **Innate immunity** ↓ Neutrophil migration **USES ENERGY**
Body composition	↑ Lean mass (muscle) ↓ Fat mass **USES ENERGY**	↓ Lean mass (muscle) ↑ Fat mass **SAVES ENERGY**
cBT	↓cBT (↓BAT thermogenesis) **SAVES ENERGY**	↑cBT (↑BAT thermogenesis) **USES ENERGY**
Reproduction	↑ Reproduction **USES ENERGY**	↓Reproduction **SAVES ENERGY**

6 CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The results of this thesis provide a deeper insight into the diverse actions of androgens in male mice and may have clinical implications for future treatment options for autoimmune diseases and cardio-metabolic diseases. Our data also raise new questions for future investigations. Identification of the target cells and mechanisms involved in the actions of androgens will be crucial for the development of selective AR modulators (see section 1.1.4).

Inhibition of BAFF has been shown to be protective in experimental lupus models [36]. BAFF inhibition is also an approved therapy for SLE, even though their clinical usefulness remains unclear [168]. In Paper I, we found a previously unrecognized regulation of BAFF by androgens, which leads to new questions about the role of BAFF in testosterone-mediated protection against autoimmunity as well as the sex differences in autoimmune disorders.

The finding that adrenergic transmitters regulate FRCs and that testosterone increases splenic NA, suggest that androgens modulate activity of FRCs via the SNS [159, 160]. However, more studies are needed to determine at what level (e.g. brain, medulla, or spine) androgens regulate SNS-mediated control of splenic BAFF production. Such insight may contribute to identify the AR target cell for splenic BAFF regulation. Once the specific AR target cells is found, combination of the cell-specific AR knockout with disease models would give answers to the question whether AR signaling protects against autoimmune disease via BAFF regulation.

Androgen deprivation therapy of men with advanced prostate cancer leads to undesired side effects such as increased risk of CVD, bone and muscle loss as well as hot flashes. Of note, CVD is the leading cause of death among patients with prostate cancer [169]. In Paper II, we conclude that TECs are likely target cells for AR-mediated atheroprotection in male mice. This finding may have major future clinical implications for the development of safer hormonal therapies, e.g. by development of selective AR modulators. Our data suggest that these compounds should avoid targeting or even stimulate the AR in TECs, and thereby might avoid atherosclerosis progression and CVD risk that have been associated with androgen deprivation therapies [59, 169].

Additional experiments aimed to decipher the mechanisms connecting the AR in TEC with atherosclerosis are needed. Of potential interest, castration

of male mice shifts TEC relative numbers, reducing cortical TEC but increasing medullary TEC relative numbers [170]. Further, androgen deficiency increases the fraction of recent thymic emigrants in both humans and mice [171, 172]. Recent thymic emigrants are immature T cells that leave the thymus and migrate to secondary lymphoid organs to continue their maturation into mature naïve T cells. To date, no studies have shown whether the castration-induced shift in TEC relative numbers and/or peripheral T cell pool are replicated in the E-ARKO mice. Ongoing experiments in our lab are addressing these questions.

BAT consumes substantial amounts of lipids and glucose from circulation for heat generation. BAT activity is considered a potential therapeutic target for the treatment of cardio-metabolic diseases, by increasing energy consumption (e.g. in obesity) and/or improving metabolic profile (e.g. hyperlipidemia) [173]. In Paper IV, we found that androgens are potent negative regulators of BAT activity, and this finding raises the question whether androgen deficiency may modulate responses to BAT-directed therapeutic interventions.

Our data suggest that central thermoregulatory pathways might be involved in androgen-mediated regulation of BAT activity, since NA content in BAT is increased by castration. Future studies should address the contribution of the SNS pathway for regulation of BAT activity, as well as the possible contribution of other pathways, as mentioned in section 5.3. Experiments performed in thermo-neutral conditions, in which muscle-shivering and BAT thermogenesis are not necessary to maintain cBT would provide valuable insights. If modulation of BAT-directed SNS activity is an important pathway for the suppressive actions of androgens on BAT, future studies should decipher at what level this regulation occur. Such insights may contribute to the identification of the AR target cell for regulation of BAT activity. More studies are also needed to understand the role of androgens for thermoregulation as well as BAT activity in humans.

Another important question is whether androgen deprivation therapy affects AAA risk in men. In Paper III, we suggest that osterix-expressing bone cells are the target cells for the AAA-provoking actions of testosterone in male mice. Despite the identification of the likely AR target cells, our study opens up for many new questions. More research is needed to understand the connection between AR in osterix-expressing bone cells and AAA. In particular, future studies should address whether the AAA-provoking actions of androgens are mediated by increased neutrophil infiltration to the aorta. Neutrophil depletion may be a useful approach for such studies.

The effects of androgens/AR on bone marrow stromal/mesenchymal cells will also need further consideration. In ongoing experiments, we perform FACS analysis of bone shaft and bone marrow using a more refined definition of stromal cells that excludes erythroblasts ($CD45^-$ $Ter119^-$ $CD31^-$ $CD51^+$) [128]. We expect that these studies, performed in castrated and O-ARKO mice, will provide interesting information on the effects of androgens/AR on bone marrow stromal/mesenchymal cell number, the composition of the stromal compartment (by different subpopulations such as CAR cells), and whether expression levels of CXCL12 and other factors are altered.

ACKNOWLEDGEMENTS

I would not have able to write this thesis without the scientific and personal contribution of many colleagues, family and friends. Therefore, I take the opportunity to specially thank:

Åsa Tivesten, my main supervisor – for being such a great supervisor! From the moment I moved to Gothenburg I felt extremely well taken care of, both scientifically and personally. You helped me getting my first apartment and from the first meeting I felt a great connection. I have always enjoyed discussing the projects and new experiments with you. You give me a lot of energy to do new things. You inspire me with your passion, dedication and your care for everyone in the group. You are a great leader and I could not have been luckier! I will miss the good times we had in the lab and at all the congresses.

Inger Johansson, my co-supervisor – I am really grateful to have you as my co-supervisor. Thanks for all the support and help in the lab during the years. You are a great teacher and I have learned a lot from you, FACS queen☺! It has been really fun to work with you, especially those longer experiment days collecting and isolating cells for FACS. Your happiness and positivity give me a lot of energy. Good luck with writing your book!

Annelie Carlsson, for all your help in the lab. Nobody can take better care of the "little ones" than you, Mouse queen☺! You are great at all the lab techniques. Even the most difficult ones, such as making beautiful bone sections, have no secrets for you. Working at EBM would not have been as fun without you! Finally, I would like to thank you for the fast course in Swedish. I will never forget "*Vi märker* or *jag orkar inte*". Wish you all the best in your new job!

Elin Svedlund Eriksson, it has been really special to go through the PhD and maternity together. Thanks for all your useful tips and motivation. I wish you all the best finishing your projects!

Anna Wilhemson, for all your help when I started my PhD. It has been nice working with you.

Marcus Langenskiöld, my co-supervisor - for the valuable feedback in the manuscript and for stepping in last minute. I look forward to some more discussion of the thesis.

Maaike Schilperoort, **Patrick C.N.Rensen** and **Jan Kroon**, it has been a pleasure to collaborate with you over the last years. Thanks for all your help with the BAT project and for the nice skype and SSARs meetings.

Malin Levin, **Marcus Ståhlman**, **Vilborg Palsdottir** and **John-Olov Jansson,** for being very helpful with BAT project.

Malin Hagberg, for all your help with our beloved osteoblasts. I am glad that I met you.

I am grateful to all members of Wallenberg lab for the nice environment. I want to direct special thanks to **Matias**, **Sivve**, **Mikael, Maria** and **Per,** for all your technical help with the immunoflorescence, antibodies and our complaining scanner. **Linda**, for always answering all my difficult questions. **Malin**, for being always so caring and supportive. It was nice to share a writing room with you. **Eva** and **Liliana**, for all your kindness. **Kristina**, for your help with the injections and **Magnus**, for always being so helpful.

Rosellina, **Kavitha**, **Angela**, **Tony, Piero**, **Andrea**, **Lisa**, **Christina**, **Nadia**, **Esther** and **Oveis,** for all the good times and the help when I needed it.

Gunnar Tobin, you were a great supervisor during my Master thesis and helped me with starting my academic career.

Aditi, Ismena and Ying – my crazy fantastic girls, I am really lucky to have you☺. The PhD studies would have not been the same without all your support, motivation and fun dinners, movie nights and craziness!

Luna, Somita, Johnny, Andrea, Charoula, Liza, Sanaz and **Marie Francoise**, for being such good friends. I can always count on you! ☺ I look forward to many more parties and dancing together!

Isa, **Elvira** y **Meri**, por todo vuestro apoyo durante tantos años. ¡No hay distancia que nos separe!

Janet, **Chris**, **Fijanne**, **Matthijs** en **Juna.** Ik ben zo blij dat ik bij de familie hoor. Kan niet wacthen om straks dicht bij te wonen.

Juan. ¡Ven a Holanda hermano! ¿Qué voy a hacer sin tí después de tantos años juntos?

Papá y **Mamá**, gracias por vuestro gran apoyo durante la tesis y toda mi vida. Sin vosotros no estaría donde estoy ahora. Gracias por siempre confiar en mí y motivarme para alcanzar mis sueños. Os quiero ♥.

Rik, jij bent mijn alles en ik houd heel veel van jou ♥. Jij bent er altijd voor me y sin tí no podría haber recorrido todo este camino. Ik heb heel veel zin om ons nieuwe leven in Nederland te beginnen.

Elin, mi estrellita, mi alegría, mi amor. Tú eres lo mejor que me ha pasado en mi vida. Te quiero con toda alma♥.

REFERENCES

1. Serra, C., et al., *The effects of testosterone deprivation and supplementation on proteasomal and autophagy activity in the skeletal muscle of the male mouse: differential effects on high-androgen responder and low-androgen responder muscle groups.* Endocrinology, 2013. **154**(12): p. 4594-4606.
2. Vanderschueren, D., et al., *Sex steroid actions in male bone.* Endocr Rev, 2014. **35**(6): p. 906-60.
3. Walters, K.A., U. Simanainen, and D.A. Gibson, *Androgen action in female reproductive physiology.* Current opinion in endocrinology, diabetes, and obesity, 2016. **23**(3): p. 291-296.
4. Labrie, F., et al., *Comparable amounts of sex steroids are made outside the gonads in men and women: strong lesson for hormone therapy of prostate and breast cancer.* The Journal of steroid biochemistry and molecular biology, 2009. **113**(1-2): p. 52-56.
5. Bhasin, S., et al., *Reference ranges for testosterone in men generated using liquid chromatography tandem mass spectrometry in a community-based sample of healthy nonobese young men in the Framingham Heart Study and applied to three geographically distinct cohorts.* The Journal of clinical endocrinology and metabolism, 2011. **96**(8): p. 2430-2439.
6. Orwoll, E., et al., *Testosterone and Estradiol among Older Men.* The Journal of Clinical Endocrinology & Metabolism, 2006. **91**(4): p. 1336-1344.
7. Zhou, B., et al., *Therapeutic effects of a novel BAFF blocker on arthritis.* Signal Transduction and Targeted Therapy, 2019. **4**(1): p. 19.
8. Gates, M.A., et al., *Sex steroid hormone levels and body composition in men.* The Journal of clinical endocrinology and metabolism, 2013. **98**(6): p. 2442-2450.
9. van Weerden, W.M., et al., *Adrenal glands of mouse and rat do not synthesize androgens.* Life sciences, 1992. **50**(12): p. 857-861.
10. Jänne, M., et al., *Human Sex Hormone-Binding Globulin Gene Expression in Transgenic Mice.* Molecular Endocrinology, 1998. **12**(1): p. 123-136.
11. Kelly, D.M. and T.H. Jones, *Testosterone: a vascular hormone in health and disease.* J Endocrinol, 2013. **217**(3): p. R47-71.
12. Pikwer, M., et al., *Association between testosterone levels and risk of future rheumatoid arthritis in men: a population-based case-control study.* Ann Rheum Dis, 2014. **73**(3): p. 573-9.
13. Jones, J.M., M. Kohli, and C.L. Loprinzi, *Androgen deprivation therapy-associated vasomotor symptoms.* Asian journal of andrology, 2012. **14**(2): p. 193-197.
14. Narayanan, R., C.C. Coss, and J.T. Dalton, *Development of selective androgen receptor modulators (SARMs).* Mol Cell Endocrinol, 2018. **465**: p. 134-142.
15. Parkin, J. and B. Cohen, *An overview of the immune system.* Lancet, 2001. **357**(9270): p. 1777-89.
16. Sadik, C.D., N.D. Kim, and A.D. Luster, *Neutrophils cascading their way to inflammation.* Trends Immunol, 2011. **32**(10): p. 452-60.
17. Liu, Y., et al., *Osterix-Cre Labeled Progenitor Cells Contribute to the Formation and Maintenance of the Bone Marrow Stroma.* PLOS ONE, 2013. **8**(8): p. e71318.
18. Nombela-Arrieta, C. and S. Isringhausen, *The Role of the Bone Marrow Stromal Compartment in the Hematopoietic Response to Microbial Infections.* Frontiers in Immunology, 2017. **7**(689).

19. Dunleavy, K., et al., *B-cell recovery following rituximab-based therapy is associated with perturbations in stromal derived factor-1 and granulocyte homeostasis.* Blood, 2005. **106**(3): p. 795-802.
20. Ueda, Y., M. Kondo, and G. Kelsoe, *Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow.* J Exp Med, 2005. **201**(11): p. 1771-80.
21. Kadouri, N., et al., *Thymic epithelial cell heterogeneity: TEC by TEC.* Nature Reviews Immunology, 2019.
22. Fink, P.J., *The biology of recent thymic emigrants.* Annu Rev Immunol, 2013. **31**: p. 31-50.
23. van den Broek, T., et al., *Neonatal thymectomy reveals differentiation and plasticity within human naive T cells.* J Clin Invest, 2016. **126**(3): p. 1126-36.
24. Gudmundsdottir, J., et al., *Long-term clinical effects of early thymectomy: Associations with autoimmune diseases, cancer, infections, and atopic diseases.* J Allergy Clin Immunol, 2018. **141**(6): p. 2294-2297.e8.
25. Zhu, J., et al., *Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells.* Blood, 2007. **109**(9): p. 3706-3712.
26. Sato, M., et al., *Osteocytes regulate primary lymphoid organs and fat metabolism.* Cell Metab, 2013. **18**(5): p. 749-58.
27. Nagasawa, T., *Microenvironmental niches in the bone marrow required for B-cell development.* Nat Rev Immunol, 2006. **6**(2): p. 107-16.
28. Cancro, M.P., *Signalling crosstalk in B cells: managing worth and need.* Nature Reviews Immunology, 2009. **9**(9): p. 657-661.
29. Cremasco, V., et al., *B cell homeostasis and follicle confines are governed by fibroblastic reticular cells.* Nature Immunology, 2014. **15**(10): p. 973-981.
30. Moulton, V.R., et al., *Pathogenesis of Human Systemic Lupus Erythematosus: A Cellular Perspective.* Trends in molecular medicine, 2017. **23**(7): p. 615-635.
31. Zhang, P. and Q. Lu, *Genetic and epigenetic influences on the loss of tolerance in autoimmunity.* Cellular & Molecular Immunology, 2018. **15**(6): p. 575-585.
32. Swee, L.K., et al., *Rescue of the mature B cell compartment in BAFF-deficient mice by treatment with recombinant Fc-BAFF.* Immunology letters, 2010. **131**(1): p. 40-48.
33. Smulski, C.R. and H. Eibel, *BAFF and BAFF-Receptor in B Cell Selection and Survival.* Frontiers in Immunology, 2018. **9**(2285).
34. Steri, M., et al., *Overexpression of the Cytokine BAFF and Autoimmunity Risk.* New England Journal of Medicine, 2017. **376**(17): p. 1615-1626.
35. Mackay, F., et al., *Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations.* The Journal of experimental medicine, 1999. **190**(11): p. 1697-1710.
36. Li, W., A.A. Titov, and L. Morel, *An update on lupus animal models.* Current opinion in rheumatology, 2017. **29**(5): p. 434-441.
37. Tedeschi, S.K., B. Bermas, and K.H. Costenbader, *Sexual disparities in the incidence and course of SLE and RA.* Clinical Immunology, 2013. **149**(2): p. 211-218.
38. Trigunaite, A., J. Dimo, and T.N. Jørgensen, *Suppressive effects of androgens on the immune system.* Cellular Immunology, 2015. **294**(2): p. 87-94.
39. Roubinian, J.R., R. Papoian, and N. Talal, *Androgenic hormones modulate autoantibody responses and improve survival in murine lupus.* J Clin Invest, 1977. **59**(6): p. 1066-70.

40. Olsen, N.J., et al., *Androgen receptors in thymic epithelium modulate thymus size and thymocyte development.* Endocrinology, 2001. **142**(3): p. 1278-83.
41. Roden, A.C., et al., *Augmentation of T cell levels and responses induced by androgen deprivation.* J Immunol, 2004. **173**(10): p. 6098-108.
42. Heng, T.S., et al., *Effects of castration on thymocyte development in two different models of thymic involution.* J Immunol, 2005. **175**(5): p. 2982-93.
43. Sakiani, S., N.J. Olsen, and W.J. Kovacs, *Gonadal steroids and humoral immunity.* Nat Rev Endocrinol, 2013. **9**(1): p. 56-62.
44. Wilhelmson, A.S., et al., *Androgens regulate bone marrow B lymphopoiesis in male mice by targeting osteoblast-lineage cells.* Endocrinology, 2015. **156**(4): p. 1228-36.
45. Yesilova, Z., et al., *The effects of gonadotropin treatment on the immunological features of male patients with idiopathic hypogonadotropic hypogonadism.* J Clin Endocrinol Metab, 2000. **85**(1): p. 66-70.
46. Kocar, I.H., et al., *The effect of testosterone replacement treatment on immunological features of patients with Klinefelter's syndrome.* Clin Exp Immunol, 2000. **121**(3): p. 448-52.
47. Cavasin, M.A., et al., *Testosterone enhances early cardiac remodeling after myocardial infarction, causing rupture and degrading cardiac function.* Am J Physiol Heart Circ Physiol, 2006. **290**(5): p. H2043-50.
48. Hofer, M.D., et al., *Androgen supplementation in rats increases the inflammatory response and prolongs urethral healing.* Urology, 2015. **85**(3): p. 691-7.
49. Scalerandi, M.V., et al., *Inefficient N2-Like Neutrophils Are Promoted by Androgens During Infection.* Frontiers in immunology, 2018. **9**: p. 1980-1980.
50. WHO, *Cardiovascular diseases (CVDs)-Key facts.* 2017.
51. Libby, P., et al., *Atherosclerosis.* Nature Reviews Disease Primers, 2019. **5**(1): p. 56.
52. Fruchart, J.-C., et al., *New Risk Factors for Atherosclerosis and Patient Risk Assessment.* Circulation, 2004. **109**(23_suppl_1): p. III-15-III-19.
53. Ridker, P.M., et al., *Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease.* New England Journal of Medicine, 2017. **377**(12): p. 1119-1131.
54. Zhou, X. and G.K. Hansson, *Detection of B cells and proinflammatory cytokines in atherosclerotic plaques of hypercholesterolaemic apolipoprotein E knockout mice.* Scand J Immunol, 1999. **50**(1): p. 25-30.
55. Jonasson, L., et al., *Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque.* Arteriosclerosis, 1986. **6**(2): p. 131-8.
56. Zhou, X., et al., *Lesion Development and Response to Immunization Reveal a Complex Role for CD4 in Atherosclerosis.* Circulation Research, 2005. **96**(4): p. 427-434.
57. Elhage, R., et al., *Deleting TCR alpha beta+ or CD4+ T lymphocytes leads to opposite effects on site-specific atherosclerosis in female apolipoprotein E-deficient mice.* Am J Pathol, 2004. **165**(6): p. 2013-8.
58. Ohlsson, C., et al., *High serum testosterone is associated with reduced risk of cardiovascular events in elderly men. The MrOS (Osteoporotic Fractures in Men) study in Sweden.* J Am Coll Cardiol, 2011. **58**(16): p. 1674-81.
59. Tivesten, A., et al., *Cardiovascular risk with androgen deprivation therapy for prostate cancer: potential mechanisms.* Urol Oncol, 2015. **33**(11): p. 464-75.

60. Bourghardt, J., et al., *Androgen receptor-dependent and independent atheroprotection by testosterone in male mice.* Endocrinology, 2010. **151**(11): p. 5428-37.
61. Huang, C.K., et al., *New therapy via targeting androgen receptor in monocytes/macrophages to battle atherosclerosis.* Hypertension, 2014. **63**(6): p. 1345-53.
62. Takov, K., et al., *The role of androgen receptors in atherosclerosis.* Mol Cell Endocrinol, 2017.
63. Erbel, R., et al., *2014 ESC Guidelines on the diagnosis and treatment of aortic diseases: Document covering acute and chronic aortic diseases of the thoracic and abdominal aorta of the adult. The Task Force for the Diagnosis and Treatment of Aortic Diseases of the European Society of Cardiology (ESC).* Eur Heart J, 2014. **35**(41): p. 2873-926.
64. Golledge, J., *Abdominal aortic aneurysm: update on pathogenesis and medical treatments.* Nature Reviews Cardiology, 2019. **16**(4): p. 225-242.
65. Thompson, S.G., et al., *Screening men for abdominal aortic aneurysm: 10 year mortality and cost effectiveness results from the randomised Multicentre Aneurysm Screening Study.* Bmj, 2009. **338**: p. b2307.
66. Bird, A. and A.M. Davis, *Screening for abdominal aortic aneurysm.* JAMA, 2015. **313**(11): p. 1156-1157.
67. Wanhainen, A., et al., *Outcome of the Swedish Nationwide Abdominal Aortic Aneurysm Screening Program.* Circulation, 2016. **134**(16): p. 1141-1148.
68. Wanhainen, A., et al., *Outcome of the Swedish Nationwide Abdominal Aortic Aneurysm Screening Program.* Circulation, 2016. **134**(16): p. 1141-1148.
69. Forsdahl Signe, H., et al., *Risk Factors for Abdominal Aortic Aneurysms.* Circulation, 2009. **119**(16): p. 2202-2208.
70. Kuivaniemi, H., et al., *Understanding the pathogenesis of abdominal aortic aneurysms.* Expert review of cardiovascular therapy, 2015. **13**(9): p. 975-987.
71. Houard, X., et al., *Mediators of neutrophil recruitment in human abdominal aortic aneurysms.* Cardiovasc Res, 2009. **82**(3): p. 532-41.
72. Fontaine, V., et al., *Involvement of the mural thrombus as a site of protease release and activation in human aortic aneurysms.* Am J Pathol, 2002. **161**(5): p. 1701-10.
73. Shah, A.D., et al., *Neutrophil Counts and Initial Presentation of 12 Cardiovascular Diseases: A CALIBER Cohort Study.* Journal of the American College of Cardiology, 2017. **69**(9): p. 1160-1169.
74. Hannawa, K.K., et al., *L-selectin-mediated neutrophil recruitment in experimental rodent aneurysm formation.* Circulation, 2005. **112**(2): p. 241-7.
75. Eliason, J.L., et al., *Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation.* Circulation, 2005. **112**(2): p. 232-40.
76. Lindeman, J.H., et al., *Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells.* Circulation, 2009. **119**(16): p. 2209-16.
77. Abdul-Hussien, H., et al., *Doxycycline therapy for abdominal aneurysm: Improved proteolytic balance through reduced neutrophil content.* J Vasc Surg, 2009. **49**(3): p. 741-9.
78. Dodd, B.R. and R.A. Spence, *Doxycycline inhibition of abdominal aortic aneurysm growth: a systematic review of the literature.* Curr Vasc Pharmacol, 2011. **9**(4): p. 471-8.

79. Prall, A.K., et al., *Doxycycline in patients with abdominal aortic aneurysms and in mice: Comparison of serum levels and effect on aneurysm growth in mice.* Journal of Vascular Surgery, 2002. **35**(5): p. 923-929.
80. Simoni, G., et al., *Screening for abdominal aortic aneurysms and associated risk factors in a general population.* Eur J Vasc Endovasc Surg, 1995. **10**(2): p. 207-10.
81. Cornuz, J., et al., *Risk factors for asymptomatic abdominal aortic aneurysm: systematic review and meta-analysis of population-based screening studies.* Eur J Public Health, 2004. **14**(4): p. 343-9.
82. Singh, K., et al., *Prevalence of and risk factors for abdominal aortic aneurysms in a population-based study : The Tromso Study.* Am J Epidemiol, 2001. **154**(3): p. 236-44.
83. Golledge, J., et al., *Abdominal Aortic Aneurysm.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2006. **26**(12): p. 2605-2613.
84. Sinha, I., et al., *Female gender attenuates cytokine and chemokine expression and leukocyte recruitment in experimental rodent abdominal aortic aneurysms.* Ann N Y Acad Sci, 2006. **1085**: p. 367-79.
85. Henriques, T.A., et al., *Orchidectomy, but not ovariectomy, regulates angiotensin II-induced vascular diseases in apolipoprotein E-deficient mice.* Endocrinology, 2004. **145**(8): p. 3866-72.
86. Henriques, T., et al., *Androgen increases AT1a receptor expression in abdominal aortas to promote angiotensin II-induced AAAs in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2008. **28**(7): p. 1251-6.
87. Huang, C.K., et al., *Androgen Receptor Promotes Abdominal Aortic Aneurysm Development via Modulating Inflammatory Interleukin-1alpha and Transforming Growth Factor-beta1 Expression.* Hypertension, 2015.
88. Davis, J.P., et al., *Pharmacologic blockade and genetic deletion of androgen receptor attenuates aortic aneurysm formation.* J Vasc Surg, 2016.
89. Silva Jr, W.A., et al., *The Profile of Gene Expression of Human Marrow Mesenchymal Stem Cells.* STEM CELLS, 2003. **21**(6): p. 661-669.
90. Zhang, J. and D.C. Link, *Targeting of Mesenchymal Stromal Cells by Cre-Recombinase Transgenes Commonly Used to Target Osteoblast Lineage Cells.* Journal of Bone and Mineral Research, 2016. **31**(11): p. 2001-2007.
91. Tilson, M.D., *Atherosclerosis and aneurysm disease.* J Vasc Surg, 1990. **12**(3): p. 371-2.
92. Johnsen, S.H., et al., *Atherosclerosis in Abdominal Aortic Aneurysms: A Causal Event or a Process Running in Parallel? The Tromsø Study.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2010. **30**(6): p. 1263-1268.
93. Hall, K.D., et al., *Energy balance and its components: implications for body weight regulation.* The American journal of clinical nutrition, 2012. **95**(4): p. 989-994.
94. Fonseca, D.C., et al., *Body weight control and energy expenditure.* Clinical Nutrition Experimental, 2018. **20**: p. 55-59.
95. Morrison, S.F., *Central control of body temperature.* F1000Research, 2016. **5**: p. F1000 Faculty Rev-880.
96. Lopez, M., et al., *Rate and gender dependence of the sweating, vasoconstriction, and shivering thresholds in humans.* Anesthesiology, 1994. **80**(4): p. 780-788.
97. Siemens, J. and G.B. Kamm, *Cellular populations and thermosensing mechanisms of the hypothalamic thermoregulatory center.* Pflugers Archiv : European journal of physiology, 2018. **470**(5): p. 809-822.

98. Sanchez-Alavez, M., S. Alboni, and B. Conti, *Sex- and age-specific differences in core body temperature of C57Bl/6 mice.* Age (Dordrecht, Netherlands), 2011. **33**(1): p. 89-99.
99. Nakamura, K., *Central circuitries for body temperature regulation and fever.* American journal of physiology. Regulatory, integrative and comparative physiology, 2011. **301**(5): p. R1207-R1228.
100. Rogers, N.H., *Brown adipose tissue during puberty and with aging.* Ann Med, 2015. **47**(2): p. 142-9.
101. Kooijman, S., J.K. van den Heuvel, and P.C. Rensen, *Neuronal Control of Brown Fat Activity.* Trends Endocrinol Metab, 2015. **26**(11): p. 657-68.
102. van den Berg, R., et al., *A Diurnal Rhythm in Brown Adipose Tissue Causes Rapid Clearance and Combustion of Plasma Lipids at Wakening.* Cell Reports, 2018. **22**(13): p. 3521-3533.
103. Hoeke, G., et al., *Role of Brown Fat in Lipoprotein Metabolism and Atherosclerosis.* Circulation Research, 2016. **118**(1): p. 173-182.
104. Hashimoto, O., et al., *Castration induced browning in subcutaneous white adipose tissue in male mice.* Biochem Biophys Res Commun, 2016. **478**(4): p. 1746-50.
105. Freedman, R.R., *Menopausal hot flashes: Mechanisms, endocrinology, treatment.* The Journal of Steroid Biochemistry and Molecular Biology, 2014. **142**: p. 115-120.
106. Dinas, P.C., et al., *Association between habitual physical activity and brown adipose tissue activity in individuals undergoing PET-CT scan.* Clin Endocrinol (Oxf), 2015. **82**(1): p. 147-54.
107. Cypess, A.M., et al., *Identification and importance of brown adipose tissue in adult humans.* N Engl J Med, 2009. **360**(15): p. 1509-17.
108. Pfannenberg, C., et al., *Impact of Age on the Relationships of Brown Adipose Tissue With Sex and Adiposity in Humans.* Diabetes, 2010. **59**(7): p. 1789.
109. Rodriguez-Cuenca, S., et al., *Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue.* J Biol Chem, 2002. **277**(45): p. 42958-63.
110. Movérare-Skrtic, S., et al., *Dihydrotestosterone Treatment Results in Obesity and Altered Lipid Metabolism in Orchidectomized Mice.* Obesity, 2006. **14**(4): p. 662-672.
111. Monjo, M., et al., *Direct effects of testosterone, 17 beta-estradiol, and progesterone on adrenergic regulation in cultured brown adipocytes: potential mechanism for gender-dependent thermogenesis.* Endocrinology, 2003. **144**(11): p. 4923-30.
112. Rodriguez-Cuenca, S., et al., *Expression of mitochondrial biogenesis-signaling factors in brown adipocytes is influenced specifically by 17beta-estradiol, testosterone, and progesterone.* Am J Physiol Endocrinol Metab, 2007. **292**(1): p. E340-6.
113. Rodriguez, A.M., et al., *Opposite actions of testosterone and progesterone on UCP1 mRNA expression in cultured brown adipocytes.* Cell Mol Life Sci, 2002. **59**(10): p. 1714-23.
114. andrRodriguez-Cuenca, S., et al., *Sex steroid receptor expression profile in brown adipose tissue. Effects of hormonal status.* Cell Physiol Biochem, 2007. **20**(6): p. 877-86.
115. Fan, W., et al., *Androgen Receptor Null Male Mice Develop Late-Onset Obesity Caused by Decreased Energy Expenditure and Lipolytic Activity but Show Normal Insulin Sensitivity With High Adiponectin Secretion.* Diabetes, 2005. **54**(4): p. 1000-1008.

116. Yanase, T., et al., *Androgens and metabolic syndrome: lessons from androgen receptor knock out (ARKO) mice.* J Steroid Biochem Mol Biol, 2008. **109**(3-5): p. 254-7.
117. Serra, C., et al., *The effects of testosterone deprivation and supplementation on proteasomal and autophagy activity in the skeletal muscle of the male mouse: differential effects on high-androgen responder and low-androgen responder muscle groups.* Endocrinology, 2013. **154**(12): p. 4594-606.
118. De Gendt, K., et al., *A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1327-32.
119. Hobeika, E., et al., *Testing gene function early in the B cell lineage in mb1-cre mice.* Proceedings of the National Academy of Sciences, 2006. **103**(37): p. 13789-13794.
120. Ramirez, A., et al., *Sequences 5' of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacZ gene in the adult and during development.* Differentiation, 1994. **58**(1): p. 53-64.
121. Oppi, S., T.F. Lüscher, and S. Stein, *Mouse Models for Atherosclerosis Research—Which Is My Line?* Frontiers in Cardiovascular Medicine, 2019. **6**(46).
122. Daugherty, A. and L.A. Cassis, *Mouse models of abdominal aortic aneurysms.* Arterioscler Thromb Vasc Biol, 2004. **24**(3): p. 429-34.
123. Saraff, K., et al., *Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 2003. **23**(9): p. 1621-6.
124. Manning Michael, W., A. Cassis Lisa, and A. Daugherty, *Differential Effects of Doxycycline, a Broad-Spectrum Matrix Metalloproteinase Inhibitor, on Angiotensin II–Induced Atherosclerosis and Abdominal Aortic Aneurysms.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2003. **23**(3): p. 483-488.
125. Rensen, P.C., et al., *Selective liver targeting of antivirals by recombinant chylomicrons--a new therapeutic approach to hepatitis B.* Nat Med, 1995. **1**(3): p. 221-5.
126. Even, P.C. and N.A. Nadkarni, *Indirect calorimetry in laboratory mice and rats: principles, practical considerations, interpretation and perspectives.* Am J Physiol Regul Integr Comp Physiol, 2012. **303**(5): p. R459-76.
127. Yvan-Charvet, L. and L.G. Ng, *Granulopoiesis and Neutrophil Homeostasis: A Metabolic, Daily Balancing Act.* Trends in Immunology, 2019. **40**(7): p. 598-612.
128. Boulais, P.E., et al., *The Majority of CD45(-) Ter119(-) CD31(-) Bone Marrow Cell Fraction Is of Hematopoietic Origin and Contains Erythroid and Lymphoid Progenitors.* Immunity, 2018. **49**(4): p. 627-639.e6.
129. Yang, M., et al., *Morphology and quantitative composition of hematopoietic cells in murine bone marrow and spleen of healthy subjects.* Annals of hematology, 2013. **92**(5): p. 587-594.
130. Lai, J.J., et al., *Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice.* Am J Pathol, 2012. **181**(5): p. 1504-12.
131. Lai, K.P., et al., *Targeting thymic epithelia AR enhances T-cell reconstitution and bone marrow transplant grafting efficacy.* Mol Endocrinol, 2013. **27**(1): p. 25-37.
132. Altuwaijri, S., et al., *Susceptibility to autoimmunity and B cell resistance to apoptosis in mice lacking androgen receptor in B cells.* Mol Endocrinol, 2009. **23**(4): p. 444-53.

133. Pomplun, D., et al., *Alterations of pancreatic beta-cell mass and islet number due to Ins2-controlled expression of Cre recombinase: RIP-Cre revisited; part 2.* Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme, 2007. **39**(5): p. 336-340.
134. Huh, W.J., I.U. Mysorekar, and J.C. Mills, *Inducible activation of Cre recombinase in adult mice causes gastric epithelial atrophy, metaplasia, and regenerative changes in the absence of "floxed" alleles.* American journal of physiology. Gastrointestinal and liver physiology, 2010. **299**(2): p. G368-G380.
135. Schile, A. *SLICING AND DICING CRE/LOX: PART 1.* [cited 2020; Available from: https://www.jax.org/news-and-insights/jax-blog/2011/may/slicing-and-dicing-cre-lox-part-1.
136. Chuang, K.H., et al., *Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor.* J Exp Med, 2009. **206**(5): p. 1181-99.
137. Marin, D.P., et al., *Testosterone suppresses oxidative stress in human neutrophils.* Cell Biochem Funct, 2010. **28**(5): p. 394-402.
138. Lin, C.Y., et al., *Flutamide, an androgen receptor antagonist, improves heatstroke outcomes in mice.* Eur J Pharmacol, 2012. **688**(1-3): p. 62-7.
139. Deitch, E.A., et al., *Neutrophil activation is modulated by sex hormones after trauma-hemorrhagic shock and burn injuries.* American Journal of Physiology-Heart and Circulatory Physiology, 2006. **291**(3): p. H1456-H1465.
140. Sheth, S.U., et al., *Testosterone depletion or blockade in male rats protects against trauma hemorrhagic shock-induced distant organ injury by limiting gut injury and subsequent production of biologically active mesenteric lymph.* The Journal of trauma, 2011. **71**(6): p. 1652-1658.
141. Yeap, B.B., et al., *Associations of total testosterone, sex hormone-binding globulin, calculated free testosterone, and luteinizing hormone with prevalence of abdominal aortic aneurysm in older men.* J Clin Endocrinol Metab, 2010. **95**(3): p. 1123-30.
142. Cho, B.S., et al., *Differential regulation of aortic growth in male and female rodents is associated with AAA development.* J Surg Res, 2009. **155**(2): p. 330-8.
143. Zhang, X., et al., *Castration of male mice prevents the progression of established angiotensin II-induced abdominal aortic aneurysms.* J Vasc Surg, 2015. **61**(3): p. 767-76.
144. Tremellen, K., et al., *Endotoxin-initiated inflammation reduces testosterone production in men of reproductive age.* American Journal of Physiology-Endocrinology and Metabolism, 2018. **314**(3): p. E206-E213.
145. Bini, E.I., et al., *The implication of pro-inflammatory cytokines in the impaired production of gonadal androgens by patients with pulmonary tuberculosis.* Tuberculosis (Edinb), 2015. **95**(6): p. 701-706.
146. Döring, Y., et al., *Neutrophils in Atherosclerosis.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2015. **35**(2): p. 288-295.
147. Drechsler, M., et al., *Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis.* Circulation, 2010. **122**(18): p. 1837-45.
148. Gagliano-Jucá, T. and S. Basaria, *Testosterone replacement therapy and cardiovascular risk.* Nature Reviews Cardiology, 2019. **16**(9): p. 555-574.
149. Dale, M.A., M.K. Ruhlman, and B.T. Baxter, *Inflammatory Cell Phenotypes in AAAs.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2015. **35**(8): p. 1746-1755.

150. Xiong, W., et al., *Key roles of CD4+ T cells and IFN-gamma in the development of abdominal aortic aneurysms in a murine model.* Journal of immunology (Baltimore, Md. : 1950), 2004. **172**(4): p. 2607-2612.
151. Porsch, F. and C.J. Binder, *Impact of B-Cell–Targeted Therapies on Cardiovascular Disease.* Arteriosclerosis, Thrombosis, and Vascular Biology, 2019. **39**(9): p. 1705-1714.
152. Meher, A.K., et al., *B2 cells suppress experimental abdominal aortic aneurysms.* Am J Pathol, 2014. **184**(11): p. 3130-41.
153. Schaheen, B., et al., *B-Cell Depletion Promotes Aortic Infiltration of Immunosuppressive Cells and Is Protective of Experimental Aortic Aneurysm.* Arterioscler Thromb Vasc Biol, 2016. **36**(11): p. 2191-2202.
154. Buendia, A.J., et al., *B-cell-deficient mice show an exacerbated inflammatory response in a model of Chlamydophila abortus infection.* Infect Immun, 2002. **70**(12): p. 6911-8.
155. Fisher, A.D., et al., *Effects of suppressing cortisol following castration of bull calves on adrenocorticotropic hormone, in vitro interferon-gamma production, leukocytes, acute-phase proteins, growth, and feed intake.* Journal of animal science, 1997. **75**(7): p. 1899-1908.
156. Nilsson, M.E., et al., *Measurement of a Comprehensive Sex Steroid Profile in Rodent Serum by High-Sensitive Gas Chromatography-Tandem Mass Spectrometry.* Endocrinology, 2015. **156**(7): p. 2492-502.
157. Feldhaus, A.L., et al., *ALD1613, a Novel Long-Acting Monoclonal Antibody to Control ACTH-Driven Pharmacology.* Endocrinology, 2017. **158**(1): p. 1-8.
158. Procaccini, C., et al., *Neuro-Endocrine Networks Controlling Immune System in Health and Disease.* Frontiers in Immunology, 2014. **5**(143).
159. Ermis, N., et al., *Heart rate variability of young men with idiopathic hypogonadotropic hypogonadism.* Autonomic neuroscience : basic & clinical, 2010. **152**(1-2): p. 84-87.
160. Keast, J.R., *Effects of testosterone on pelvic autonomic pathways: progress and pitfalls.* J Auton Nerv Syst, 2000. **79**(2-3): p. 67-73.
161. Aoki, A., et al., *Male Hypogonadism Causes Obesity Associated with Impairment of Hepatic Gluconeogenesis in Mice.* Biol Pharm Bull, 2016. **39**(4): p. 587-92.
162. Harada, N., et al., *Castration influences intestinal microflora and induces abdominal obesity in high-fat diet-fed mice.* Scientific Reports, 2016. **6**: p. 23001.
163. Bal, N.C., et al., *Both brown adipose tissue and skeletal muscle thermogenesis processes are activated during mild to severe cold adaptation in mice.* The Journal of biological chemistry, 2017. **292**(40): p. 16616-16625.
164. Straub, R.H., *The brain and immune system prompt energy shortage in chronic inflammation and ageing.* Nature reviews. Rheumatology, 2017. **13**(12): p. 743-751.
165. Hotamisligil, G.S., *Inflammation, metaflammation and immunometabolic disorders.* Nature, 2017. **542**(7640): p. 177-185.
166. McDade, T.W., A.V. Georgiev, and C.W. Kuzawa, *Trade-offs between acquired and innate immune defenses in humans.* Evolution, medicine, and public health, 2016. **2016**(1): p. 1-16.
167. Straub, R.H., *The brain and immune system prompt energy shortage in chronic inflammation and ageing.* Nat Rev Rheumatol, 2017. **13**(12): p. 743-751.
168. Stohl, W., *Inhibition of B cell activating factor (BAFF) in the management of systemic lupus erythematosus (SLE).* Expert review of clinical immunology, 2017. **13**(6): p. 623-633.

169. Ketchandji, M., et al., *Cause of death in older men after the diagnosis of prostate cancer.* Journal of the American Geriatrics Society, 2009. **57**(1): p. 24-30.
170. Dumont-Lagacé, M., C. St-Pierre, and C. Perreault, *Sex hormones have pervasive effects on thymic epithelial cells.* Scientific reports, 2015. **5**: p. 12895-12895.
171. Sutherland, J.S., et al., *Activation of thymic regeneration in mice and humans following androgen blockade.* J Immunol, 2005. **175**(4): p. 2741-53.
172. Olsen, N.J. and W.J. Kovacs, *Evidence that androgens modulate human thymic T cell output.* J Investig Med, 2011. **59**(1): p. 32-5.
173. Schilperoort, M., et al., *Relevance of lipid metabolism for brown fat visualization and quantification.* Curr Opin Lipidol, 2016. **27**(3): p. 242-8.