ANDROGENS AND THEIR INTERACTION WITH BONE AND MUSCLE:
RELEVANCE FOR PEAK BONE MASS ACQUISITION

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<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein 1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARKO</td>
<td>androgen receptor knockout</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B.Pm</td>
<td>bone perimeter</td>
</tr>
<tr>
<td>BFR</td>
<td>bone formation rate</td>
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<tr>
<td>BMC</td>
<td>bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
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<tr>
<td>BMP-2</td>
<td>bone morphogenic protein 2</td>
</tr>
<tr>
<td>BMU</td>
<td>basic multicellular unit</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BV/TV</td>
<td>bone volume / total volume</td>
</tr>
<tr>
<td>cAIS</td>
<td>complete androgen insensitivity syndrome</td>
</tr>
<tr>
<td>Ct.Ar</td>
<td>cortical area</td>
</tr>
<tr>
<td>Ct.th</td>
<td>cortical thickness</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEXA</td>
<td>dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>DHT</td>
<td>5α-dihydrotestosterone</td>
</tr>
<tr>
<td>DPD</td>
<td>deoxypyridinoline</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>Ec.Pm</td>
<td>endocortical perimeter</td>
</tr>
<tr>
<td>EDL</td>
<td>extensor digitorum longus</td>
</tr>
<tr>
<td>ER(α, β)</td>
<td>estrogen receptor (α, β)</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-I</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LA</td>
<td>musculus levator ani</td>
</tr>
<tr>
<td>LRP5</td>
<td>low-density lipoprotein receptor-related protein 5</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAR</td>
<td>mineral apposition rate</td>
</tr>
<tr>
<td>mARKO</td>
<td>myocyte-specific androgen receptor knockout</td>
</tr>
<tr>
<td>mATPase</td>
<td>myosin adenosine triphosphatase</td>
</tr>
<tr>
<td>MCK</td>
<td>muscle creatine kinase</td>
</tr>
<tr>
<td>M-CSF</td>
<td>monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>μCT</td>
<td>microcomputed tomography</td>
</tr>
<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>Min.Pm</td>
<td>mineralizing perimeter</td>
</tr>
<tr>
<td>MRF</td>
<td>myogenic regulatory factor</td>
</tr>
<tr>
<td>mWT</td>
<td>wildtype littermates of mARKO mice</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
</tr>
</tbody>
</table>
Orch: orchidectomy
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PDGF: platelet-derived growth factor
pQCT: peripheral quantitative computed tomography
Ps.Pm: periosteal perimeter
RANK(L): receptor activator of nuclear factor-κB (ligand)
RT-PCR: real-time PCR
Runx2: runt-related transcription factor 2
SAMP6: senescence-accelerated mouse prone 6
SAMR1: senescence-accelerated mouse resistant 1
SARM: selective androgen receptor modulator
SDS: sodium dodecyl sulphate
SE: standard error
Sham: sham-operated
SHBG: sex hormone binding globulin
SSI: strength strain index
T: testosterone
Tb.N: trabecular number
Tb.Th: trabecular thickness
TGFβ: transforming growth factor β
TRAP: tartrate-resistant acid phosphatase
WT: wildtype
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Jill, 12 november 2008
CHAPTER 1: INTRODUCTION

1.1. GENERAL INTRODUCTION

Osteoporosis is a systemic skeletal disease which is characterized by increased bone fragility and susceptibility to fracture. Although known as a women’s disease, osteoporosis affects both genders: 13% of men above the age of 50 will present with an osteoporotic fracture in their lifetime, as compared to 40% of women. Even more so in men than in women, osteoporosis related disability and mortality rates are high. Although osteoporotic fractures are most commonly observed among the elderly, the pathogenesis of osteoporosis starts early in life. Moreover, due to increased life expectancy, the disease is expected to become even more prevalent in the future. For these reasons, obtaining a better understanding of osteoporosis in both genders and its origins in growth is an important challenge for scientists today.

1.2. BONE PHYSIOLOGY

1.2.1. BONE FUNCTION AND STRUCTURE

Bone is a highly specialized connective tissue, conferring strength and rigidity to the skeleton, which is needed for locomotion and protection of vital organs and bone marrow. In addition, it has an important metabolic role as a major source of ions, particularly calcium and phosphate.

The overall macroscopic structure of bone can be divided into two parts: the external part, or cortex, which consist of a thick and dense layer of calcified tissue, and internal spongy form like bone, called trabecular or cancellous bone (Figure 1). In long bones, the diaphysis consists mainly of cortical bone which encloses the medullary cavity where the hematopoietic bone marrow is housed. Towards the metaphysis and epiphysis, the cortex becomes progressively thinner and the internal space is filled with trabecular bone. The spaces enclosed by these thin trabeculae also contain hematopoietic bone marrow and are in continuity with the medullary cavity of the diaphysis. In vertebrae, trabecular bone makes up almost the complete interior. The external (periosteal) surface of the cortex is surrounded by a layer called the periostium, while the internal (endosteal) surface of the cortex and of trabecular bone are lined by the endosteum. Both cortical and trabecular bone are in contact with soft tissue. Because trabecular bone has a high surface to volume ratio, 70%-80% of the interface with soft tissue is at the endosteal bone surface. Therefore, the metabolic function of bone is performed mainly by the trabecular bone, while cortical bone plays a predominantly mechanical and protective role.
Microscopically, bone is composed of cells (Chapter 1.2.2) and an abundant extracellular matrix. The matrix comprises approximately 90% of bone volume and has organic as well as inorganic components. The organic part contains numerous noncollagenous proteins such as proteoglycans, but is predominantly composed of type I collagen fibers, which are usually orientated in a preferential direction and are interconnected by cross-links. The inorganic component of the extracellular bone matrix consists mainly of calcium and phosphate deposits in the form of hydroxyapatite crystals, essential in providing a major portion of the bone’s strength and ion storage.

1.2.2. CELLULAR ORGANIZATION

Bone contains several cell types: osteoblasts, osteocytes and osteoclasts.

Osteoblasts are fully differentiated cells responsible for the production of bone matrix. Located next to matrix which has yet to be mineralized (osteoid), they have a well developed rough endoplasmic reticulum and a prominent Golgi complex which is typical for their high biosynthetic and secretory activity. Osteoblasts originate from mesenchymal stem cells which also give rise to myoblasts, adipocytes, chondrocytes and fibroblasts. Osteoblastogenesis is regulated at the transcriptional level: the development of knockout (KO) mice demonstrated that osteoblastic differentiation requires runt-related transcription factor 2 (Runx2) and Osterix. Mature osteoblasts secrete osteocalcin and alkaline phosphatase, whose concentrations in the serum can be used as an index of bone formation. Actively bone-forming osteoblasts may remain active and ultimately undergo apoptosis. However, they may also become relatively
inactive and form bone lining cells or alternatively, they may be surrounded by matrix and differentiate into osteocytes\textsuperscript{11}.

Osteocytes are located inside lacunae within the bone matrix and are connected to each other and cells of the bone surface by long cytoplasmic processes inside the canalicular network. Osteocytes are believed to sense mechanical deformation and play a central role in the response of bone to mechanical stimuli\textsuperscript{11}.

Osteoclasts are multinucleated cells responsible for bone resorption (Figure 2). They are located within a lacuna (Howship's lacuna) and contain many Golgi complexes, mitochondria and transport vesicles with lysosomal enzymes. The plasma membrane facing the bone matrix is extensively folded (ruffled border) and is surrounded by the sealing zone attaching the osteoclast to the matrix. Osteoclasts are derived from cells of the monocyte/macrophage lineage\textsuperscript{11, 12}. Two cytokines are critical for osteoclastogenesis, namely monocyte-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL)\textsuperscript{13}. M-CSF is secreted by osteoblastic/stromal cells and is important for proliferation of osteoclast precursors. RANKL is a membrane-bound ligand expressed by osteoblastic/stromal cells and binds receptor activator of nuclear factor-κB (RANK) expressed on the surface of osteoclasts. The interaction between RANKL and RANK stimulates the differentiation of osteoclasts and inhibits osteoclast apoptosis\textsuperscript{12}. The bone-resorbing effects of RANKL are physiologically counterbalanced by osteoprotegerin (OPG). OPG is also secreted by the osteoblastic/stromal cell and acts as a decoy receptor for RANKL, preventing it from binding to RANK and activating osteoclasts\textsuperscript{12}. Mature osteoclasts express tartrate-resistant acid phosphatase (TRAP) and digest the organic bone matrix\textsuperscript{14}. The degradation of type I collagen results in the release of collagen cross-links such as deoxypyridinolines (DPD) into urine\textsuperscript{15}. Both TRAP and DPD can be used as parameters of bone resorption.
Bone is a dynamic tissue which undergoes continuous regeneration throughout life. During growth and development, bone size and shape change by a process called modeling. During modeling, bone resorption and formation may occur at different sites, in which case, formation is not always preceded by resorption. Long bones grow in length by a process called endochondral ossification. During this process, mesenchymal stem cells first form a cartilaginous scaffold, which subsequently becomes calcified. After invasion of blood vessels, the cartilage matrix is partially resorbed by osteoclasts, osteoblasts differentiate and bone matrix is formed. Growth of the diameter of bone results from continuous periosteal bone apposition and from resorption and formation of bone at the endosteal bone surface.

During adult life, bone is constantly renewed by a process called bone remodeling, allowing microdamage repair, adaptation to mechanical stress and maintenance of mineral homeostasis. The remodeling of bone requires coordination between bone resorption and formation by local ‘coupling’ of osteoclast and osteoblasts. Replacement of old bone by new bone occurs in a cyclic way within a temporary anatomic structure called the basic multicellular unit (BMU) (Figure 3). In response to a bone-resorbing stimulus, osteoclast precursors migrate to the exposed mineralized bone surface and bone resorption is activated. The osteoclast precursors subsequently fuse to form giant multinucleated osteoclasts, which then begin to remove both
organic matrix and mineral content of bone, producing a resorption pit. The resorption phase is followed by a reversal phase, during which osteoblasts move into the resorption lacuna. Subsequently, during formation, the osteoblasts synthesize the organic matrix (osteoid) which eventually becomes mineralized. After bone formation has ceased, lining cells cover the quiescent bone surface. The mechanism by which bone resorption and formation are coupled is not fully understood. Possible mediators are systemic hormones (e.g. parathyroid hormone or 1,25 dihydroxyvitamin D₃) or locally produced factors, such as insulin-like growth factor(IGF)-I, IGF-II or transforming growth factor(TGF)-β.

Figure 3: Bone remodeling cycle. The cycle begins with the activation of osteoclast precursors which differentiate and mature into multinucleated active osteoclasts. In the resorption phase, osteoclasts digest mineral matrix. This phase is followed by a reversal phase where osteoclasts undergo apoptosis, and preosteoblasts become mature osteoblasts. Osteoblasts synthesize new bone matrix in the formation phase, and when the cavity is filled, osteoblasts undergo apoptosis, become osteocytes or lining cells.

The turnover of bone is determined by the number of BMUs and the relative amount of bone which is resorbed and formed within each BMU. The resorptive phase of the remodeling process has been estimated to last up to 7 weeks in humans, while the bone forming phase lasts approximately 3 months. For this reason, a change in the rate of bone remodeling results in transient loss of bone mass and strength. A BMU imbalance on the other hand, can cause permanent loss of bone from the skeleton.
1.3. BONE GROWTH AND PEAK BONE MASS ACQUISITION

The acquisition of bone mass is not constant throughout life: it increases during childhood, accelerates during the pubertal growth spurt and ends at early adulthood, when peak bone mass is attained. Bone mass levels subsequently start decreasing slowly till old age \(^{22}\). Although this occurs in both genders, there is an accelerated phase of bone loss in women due to estrogen deficiency at the menopause.

Bone mass acquisition during growth and peak bone mass are important determinants of risk for fragility fractures, which occur when bone mass and structure are insufficient to bear the imposed load. During life, the annual rate of bone loss remains relatively constant and little variation occurs in individual areal bone mineral density (BMD) population percentile \(^{5,23,24}\). This ‘tracking’ of bone density implies that an individual with a low BMD at one time point in life maintains a lower BMD throughout the rest of their lifetime \(^{24}\). Computer models predict that a 10% increase in peak bone mass delays the development of osteoporosis by 13 years \(^{25}\). In comparison, a 10% decrease in the rate of non-menopausal bone loss is predicted to merely respite osteoporosis by an approximated 2 years. Therefore, peak bone mass acquisition appears to be one of the major factors involved in the development of osteoporosis.

Heredity seems to play an important role in the acquisition of bone mass: children with a positive maternal or paternal history of osteoporosis appear to be at greater risk of developing the disease themselves \(^{26,27}\). Interestingly, familial resemblance for most bone traits is already present between mothers and their daughters before puberty \(^{28}\). Furthermore, studies show that daughters of women with vertebral fractures have a reduced volumetric BMD but not vertebral size \(^{29}\). Conversely, in daughters of women with hip fractures, the volumetric BMD is unaffected but femoral neck size is increased \(^{29,30}\). Hence, the pathogenesis of bone fragility of the axial and appendicular skeleton seems to be heterogeneous and to have its origins in growth. In addition, a bone deficit in first-degree relatives of men with idiopathic osteoporosis without evidence for age-associated worsening of the bone deficit is consistent with the view that a genetically determined deficient acquisition of bone mass plays a predominant role in the pathogenesis of idiopathic osteoporosis in a majority of affected men \(^{5}\). Through the use of linkage studies, a number of gene polymorphisms (e.g. polymorphisms of the vitamin D receptor and the low-density lipoprotein receptor-related protein 5 [LRP5] \(^{31}\) related to changes in bone mass were identified. However, the exact genetic determinants remain to be discovered.

Despite the strong impact of heritability (genetic factors are estimated to explain 60%-80% of the variance of the adult bone phenotype \(^{32}\), there has been considerable interest in exploring
whether the genetically predetermined bone mineral mass trajectory can be modified by other factors during growth. Normal skeletal development has been shown to depend critically on endocrine factors such as androgens, estrogens and growth hormone (GH)-IGF-I. Other factors affecting peak bone mass are nutrition (e.g. calcium intake) and mechanical factors such as exercise and muscle mass. In men, both androgens and muscle action play an important role in normal bone development, acting by independent as well as cooperative mechanisms.

The effects of androgens and muscle on bone will be discussed in the next chapters.
1.4. ANDROGEN ACTION

1.4.1. ANDROGEN METABOLISM

Androgens are male sex hormones of the steroid family which are secreted by the testes and, to a lesser extent by the adrenals. The major gonadal androgen is testosterone (T) which in the circulation, is mainly bound to albumin and sex hormone binding globulin (SHBG). On average, only 1%-2% of circulating T is free. Together with the albumin-bound fraction, freely circulating T represents the bioavailable fraction which is believed to have rapid access to target tissues. The major circulating adrenal androgens in men are dehydroepiandrosterone and androstenedione.

Like other sex steroids such as estrogen and progesterone, T is synthesized from cholesterol. In peripheral tissues, the 5α-reductase enzyme can irreversibly convert T to 5α-dihydrotestosterone (DHT), a more potent form which, like T, is able to activate the androgen receptor (AR). In a reaction mediated by the aromatase enzyme complex, T can also be converted to 17β-estradiol (E2), which subsequently activates the estrogen receptor (ER). Both 5α-reductase and aromatase have been detected in the gonads, bone and adipose tissue. However, while aromatase has also been detected in skeletal muscle, the expression of 5α-reductase in muscle tissue remains uncertain.

1.4.2. THE MECHANISM OF ACTION OF ANDROGENS

The AR gene is located on the X chromosome (Figure 4). Like receptors for other sex steroids such as the ER, the AR belongs to the nuclear receptor family. The general structure of a steroid receptor consists of several regions: (i) an aminoterminal domain, which has a ligand-independent activation function and is entirely encoded by the first exon, (ii) a DNA-binding domain, responsible for DNA binding as well as for receptor dimerization, and of which the first and second zinc-finger are encoded by exons 2 and 3, (iii) a hinge region, containing a bipartite nuclear localization signal directing the transport of the activated receptor to the nucleus and (iv) a ligand-binding domain, responsible for T or DHT binding, ligand-induced interaction with the aminoterminal domain and transcription of target genes. The latter domain is encoded by part of exon 4 and the complete exons 5 to 8.
According to the classical mechanism of sex steroid action, androgens first diffuse into target cells where they bind with the intracellular AR protein. Binding of androgen causes the AR to undergo a conformational change, dissociate from associated proteins (e.g. heat-shock proteins), translocate to the nucleus, be phosphorylated and dimerize either with other steroid receptor monomers (heterodimerization) or other AR monomers (homodimerization) 43. Finally, in the nucleus, the receptor/ligand complex binds to the androgen response element, a specific DNA sequence present within the promoter region of target genes. In this way, it induces their expression through direct binding to DNA or indirect, via protein-protein interactions with the transcription complex 41, 44.

However, numerous different action mechanisms of androgens have been discovered. In prostate cancer cells for example, the AR has been shown to negatively regulate gene expression through interaction with Ets transcription factors 45. Androgens were also demonstrated to regulate cell viability by influencing the activating protein-1 (AP-1) transcription factor in osteoblasts 46. Interestingly, these hormones can also act nongenomically: they have been shown to have anti-apoptotic effects by activation of the Src/Shc/ERK pathway in murine osteoblasts and osteocytes 44. In prostate cancer cells as well as in cultured myotubes, they are able to activate kinases such as mitogen-activated protein kinases (MAPK), phosphatidyl-inositol 3-kinase and protein kinase C 47-49. However, the biological significance of these different pathways in mediating the actions of sex steroids remains unclear.
1.5. ANDROGEN ACTION ON BONE

1.5.1. THE EFFECT OF ANDROGENS ON BONE GROWTH AND PEAK BONE MASS ACQUISITION

The attainment of peak bone mass differs between men and women for a number of reasons (Figure 5). Firstly, men have a longer period of pre-pubertal growth due to a later onset of puberty. Moreover, they also have a longer pubertal growth spurt 50, during which they have more periosteal bone apposition than women. In turn, women gain more endocortical bone than men 51-54. For these reasons, men have longer and wider long bones, as well as wider vertebrae than women 55,56 and their peak bone mass is 25% greater as compared to the female sex 50. Because both genders show similar increase in trabecular bone thickness during puberty 57 and maintain a fairly constant volumetric BMD during growth 57, adult men have a bigger, but not denser skeleton than women 53.

Figure 5: Effects of puberty on bone development in boys and girls. Longitudinal bone growth (1) occurs by endochondral bone formation at the growth plates. Since boys have a longer prepubertal growth period because of later onset of puberty, they also achieve greater height. Periosteal apposition (2) increases bone width in boys, while endocortical bone apposition (3) is attenuated. In girls, periosteal bone formation is diminished at puberty whereas endocortical bone formation is stimulated. Accordingly, boys acquire a larger bone diameter and an increased medullary area, with the cortical bone mass placed further from the neutral axis of the long bones (adapted from Seeman, 2001 57).

The importance of androgen action in bone growth is demonstrated by the fact that androgen deficiency during growth is associated with failure to acquire normal peak bone mass, in this way, possibly placing men at increased risk of osteoporotic fractures later on in life. Indeed, men with a history of delayed puberty have decreased radial, spinal and femoral areal BMD and a lower peak bone mass 58-61. Spinal and radial areal BMD are also considerably reduced in adolescents suffering from either primary or secondary hypogonadism 62-64. However, since the
areal BMD measurements in these studies are based on projectional methods and are largely influenced by bone size, the lower areal BMD values measured in the patients may result from reduced bone size rather than from changes in bone tissue composition. Interestingly, androgen treatment has been shown to inhibit further bone loss in hypogonadal boys and may result in some gain of volumetric BMD.

Further evidence for the role of androgen action on bone growth comes from studies on men with the complete androgen insensitivity syndrome (cAIS). These patients are genetically male (46 XY), but present with a female phenotype due to a functionally inactive AR. Although the areal and volumetric BMD of spine and hip are decreased in cAIS patients, they show a normal pubertal growth spurt and their epiphyses are fused. Hence, a functional AR seems to be required for normal peak bone mass acquisition, but not for longitudinal growth and epiphyseal fusion.

The use of animal models such as rats and mice has markedly increased our knowledge on the function of androgens during skeletal growth. Surgical castration (also known as orchidectomy or orch) in male rats and mice at the onset of puberty was shown to reduce both cortical and trabecular bone growth. In agreement with the traditional view that androgens stimulate periosteal bone formation, orch in growing rats and mice results in diminished periosteal perimeter, cortical thickness and cortical area. This decrease in cortical bone is primarily caused by attenuated periosteal bone formation and not – or to a lesser extent – by significant changes in the endocortical perimeter. Together with mineralized mass and bone microarchitecture, the external dimensions of bone determine its strength by placing cortical mass further from the neutral bone axis. In accordance, androgen deficiency during growth results in decreased bone strength at maturity. In cancellous bone, orch was shown to cause loss of spinal, femoral and trabecular bone mass associated with high rates of bone turnover. The role of the AR in mediating the actions of androgens on bone is further evidenced by studies of mouse and rat models in which this receptor is deficient. Similar to the effects of orchidectomy, AR inactivation in mice results in reduced cortical and trabecular bone growth. Interestingly, post-orchidectomy administration of androgens was able to reverse the decreased gain of cortical and trabecular bone mass in mice with a functional AR, but not in mice in which the AR was disrupted. Finally, further supporting the role of the AR in bone development, targeted overexpression of AR in the skeleton resulted in enhanced periosteal and trabecular bone formation, but decreased endosteal bone formation without affecting steroid levels in the circulation.
1.5.2. ANDROGEN ACTION ON BONE CELLS

An important indication for possible direct action of androgens on bone was the discovery of AR expression in different types of bone cells. Indeed, AR binding, AR mRNA and/or protein have been observed in human, mouse and rat osteoblasts, osteocytes and osteoclasts.

Several lines of evidence suggest direct action of androgens on osteoblasts. However, androgens have been reported to have both stimulatory and inhibitory effects on the proliferation of these cells. The apparently contradictory results of these studies could be explained by differences in exposure time; more recent investigation showed that although androgens initially stimulate proliferation of osteoblasts, inhibitory effects are observed when treatment of the cells is prolonged. DHT and T have also been shown to stimulate osteoblast differentiation, matrix production and mineralization. Moreover, DHT was able to prevent apoptosis in osteoblastic and osteocytic cell lines. Finally, AR expression levels have been demonstrated to increase throughout osteoblast proliferation with highest AR expression observed in mature osteoblast/osteocyte cultures, suggesting that androgens predominantly affect mature osteoblast cells.

Androgens also have clear effects on formation, activity and apoptosis of osteoclasts. In AR deficient mice, osteoclast development was shown to be up-regulated indirectly by increased RANKL expression in osteoblasts. The effect of androgens on OPG production is not clear, since both suppression and increased production of OPG by osteoblasts have been observed upon administration of androgens. In addition to effects on RANKL/OPG expression, androgens were also demonstrated to regulate osteoblastic/stromal cell production of cytokines implicated in enhanced osteoclastogenesis and bone resorption (e.g. interleukin-6). Besides these indirect action mechanisms of androgens, several studies show that they can influence osteoclast cells directly as well. Indeed, androgens have been reported to suppress RANKL/M-CSF - induced osteoclast formation from primary bone marrow monocytes, to inhibit the activity of mature osteoclasts, and to induce their apoptosis through direct AR-mediated mechanisms.

1.5.3. INDIRECT EFFECTS OF ANDROGENS ON BONE

There are several indications that at least part of the effects of androgens on bone can be explained by indirect mechanisms. T, but not DHT, can be aromatized to E2 and subsequently activate the ER. Two ER subtypes have been described, namely ERα and ERβ, both of which are present in osteoblasts, osteocytes and osteoclasts of different species. The involvement of E2 in male pubertal growth and maturation came to light upon observation of a man suffering from...
estrogen resistance caused by a mutation in the ERα gene. Despite normal T levels, this patient presented with severe osteopenia associated with increased bone turnover. He did not have a pubertal growth spurt and his epiphyses were unfused, suggesting continued linear growth into adulthood. A similar phenotype is seen in men suffering from E2 deficiency due to mutations in the aromatase gene, although these men have normal or elevated T levels. E2 treatment in aromatase-deficient males results in growth plate closure, suppression of bone resorption and elevated bone mass by increased periosteal bone expansion. Similar phenotypes are observed in corresponding KO mice; both male aromatase and ERα KO mice show reduced cortical bone size and longitudinal growth. Male ERβ KO mice, however, do not display a bone phenotype. These studies underscore the fact that E2 action via the ERα but not the ERβ plays an important role in growth of the male skeleton.

Another key player in the process of bone growth is IGF-I, as is demonstrated by the fact that its inhibition, either directly or indirectly via disruption of the GH receptor, reduces both longitudinal and radial cortical bone growth. Several studies suggest a role for the GH/IGF-I axis in mediating the effects of estrogens on the male skeleton. In male ERα KO mice, the observed reduction in skeletal growth may be confounded by changes in IGF-I levels. Indeed, ERα KO mice were shown to have lower serum levels of IGF-I, which in turn, is positively correlated with decreased longitudinal and radial skeletal growth. In humans and mice, androgens indirectly stimulate IGF-I secretion after they are aromatized to estrogens. Therefore, apart from a direct action on bone, clearly androgens can also stimulate growth of bone indirectly after aromatization to estrogens and stimulation of the GH/IGF-I axis.

During puberty in boys, the rise in androgen levels are not only associated with increased bone growth but also with elevated body weight and muscle mass. The elevation of muscle mass may result in enhanced mechanical loading, which is considered to be an important stimulus for skeletal modeling. Increasing the strain on bone, mechanical loads can result in (slight) bone deformation, which, if exceeding the bone's threshold, will result in addition of more bone tissue or in a change in the architecture of bone in order to counteract the increased strain (mechanostat theory). Because the largest load on the skeleton comes from muscles and muscles cause the largest dynamic strains, androgen-induced changes of muscle may have an important impact on growing bone. The effect of androgens on muscle and the effect of muscle on bone will be discussed in the next chapter.
1.6. MECHANICAL ACTION ON BONE

1.6.1. MUSCLE STRUCTURE

Skeletal muscle is a highly organized organ (Figure 6). Connected to the skeleton by tendons, it is vital for human motion and maintaining body posture. Skeletal muscles are covered by a layer of connective tissue called the epimysium, and are made up of fasciculi, which in turn consist of bundled muscle fibers. Both the fasciculi as well as the individual muscle fibers are also covered by specialized layers of connective tissue, respectively termed the peri- and endomysium. Muscle connective tissue is mainly composed of type I and type III collagen and is richly supplied with blood vessels and motor neurons. This connective tissue is important for maintaining muscle integrity, but also contributes to the skeletal muscle's elastic behavior.

Muscle fibers are multinucleated single muscle cells. They are long, cylindrical structures that are bound by a plasma membrane (the sarcolemma) and an overlying basement membrane containing the basal lamina. The basement membrane plays an important role during myogenesis and muscle regeneration, while the sarcolemma forms a physical barrier against the external environment and mediates signals between the exterior and the muscle cell. Upon activation by its motor neuron, the sarcolemma depolarizes. Transverse-tubules (or T-tubules) invaginating the sarcolemma, allow the action-potential signals to penetrate the cell and activate the sarcoplasmic reticulum. When this organelle releases Ca\(^{2+}\), muscle contraction commences, a process primarily mediated by the myofilament proteins, actin and myosin. The rise in intracellular Ca\(^{2+}\) together with the presence of ATP results in the sliding of actin over myosin and, ultimately, in whole-muscle contraction.
Muscle fibers can be classified into several types based on their functional capabilities and enzymatic profiles. Most methods make use of specific myosin profiles, dividing fibers into groups based on myosin heavy chain (MHC) isoform complement (by immunohistochemistry), or myosin adenosine triphosphatase (mATPase) isoform (by histochemistry). There are four major fiber types: slow type I with MHC-Iβ isoform, and three fast types, namely type IIa with MHC-IIa, type IIx with MHC-IIx and type IIb with MHC-IIb isoform. Containing a slow form of mATPase, the slow type I fibers have a high oxidative capacity resulting in slow contraction and relaxation time and low fatigability. The fast fiber types contain a fast form of mATPase and are characterized by higher glycolytic and lower oxidative capacities, fast contraction and relaxation times, and high fatigability (overview see Table 1) \(^{111,114}\). Individual adult skeletal muscles are composed of a mixture of myofiber types, the proportions of which are the main determinants of the overall contractile properties of the muscle \(^{115}\).
# Table 1: General characteristics of type I and type II muscle fibers

<table>
<thead>
<tr>
<th></th>
<th>Type I</th>
<th>Type IIa</th>
<th>Type IIx/b</th>
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</thead>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Myosin ATPase isoform</td>
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<td>Faster</td>
<td>Fastest</td>
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<td>Low content</td>
<td>Higher content</td>
<td>Highest content</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>Glycolytic enzymes</td>
<td>Low concentration</td>
<td>High concentration</td>
<td>Highest concentration</td>
</tr>
<tr>
<td>Oxidative enzymes</td>
<td>High concentration</td>
<td>Low concentration</td>
<td>Lowest concentration</td>
</tr>
<tr>
<td>Mitochondrial content</td>
<td>High</td>
<td>Low</td>
<td>Lowest</td>
</tr>
<tr>
<td><strong>Functional</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak tension</td>
<td>Low</td>
<td>High</td>
<td>Highest</td>
</tr>
<tr>
<td>Contraction speed</td>
<td>Slow</td>
<td>Fast</td>
<td>Fastest</td>
</tr>
<tr>
<td>Relaxation speed</td>
<td>Slow</td>
<td>Fast</td>
<td>Fastest</td>
</tr>
<tr>
<td>Fatigability</td>
<td>Low</td>
<td>High</td>
<td>Highest</td>
</tr>
<tr>
<td><strong>Neural</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recruitment order</td>
<td>Recruited first</td>
<td>Recruited later</td>
<td>Recruited last</td>
</tr>
<tr>
<td>No. of fibers per motor unit</td>
<td>Few</td>
<td>Many</td>
<td>Many</td>
</tr>
</tbody>
</table>

## 1.6.2. MYOGENESIS AND MUSCLE REGENERATION

During the development of vertebrate skeletal muscle, muscle progenitor cells originate from the mesodermal lineage (Figure 7). This process requires the up-regulation of different transcriptional activators of the myogenic regulatory factor family (MRF). First, muscle progenitor cells differentiate into myoblasts which express the MRFs MyoD and Myf5. Next, proliferating myoblasts withdraw from the cell cycle to become terminally differentiated myocytes expressing the MRFs myogenin and MRF4, as well as muscle-specific genes such as MHC and muscle creatine kinase (MCK). These mononucleated myocytes subsequently fuse to form multinucleated myotubes, which eventually mature into contracting muscle fibers.¹¹⁶,¹¹⁷
Figure 7: Schematic view of myogenesis/muscle fiber regeneration. During myogenesis or regeneration, muscle progenitor cells or satellite cells become activated and differentiate into myoblasts expressing the myogenic regulatory factors (MRF) Myf5 and MyoD. These myoblasts differentiate further into myocytes (MRF4 and myogenin expression) expressing muscle creatine kinase (MCK). Next, myocytes fuse to form multinucleated myotubes that can on their turn further mature to myofibers or fuse with already existing myofibers.

During development, a distinct population of myoblasts is formed. These cells fail to differentiate and remain associated to the surface of the developing myofiber as satellite cells. Located between the sarcolemma and the basal lamina, the muscle satellite cells play an important role in postnatal growth and muscle regeneration. When a muscle is stimulated to increase in size or to regenerate after injury, the satellite cells become activated and form muscle precursor cells. In a similar manner as during myogenesis, these muscle precursor cells can differentiate into myotubes and either form new muscle fibers or fuse with existing fibers to increase fiber size and/or myonuclei number 116,118-120. Satellite cells are present in all skeletal muscles, but not in equal distribution. Slow muscle fibers have a higher number of satellite cells than fast fibers 116,119. In addition, satellite cell numbers decrease with age, constituting ±30% of the muscle nuclei in the neonate, ±4% in the adult and ±2% in the senile mouse soleus muscle 119.

Although satellite cells are classically considered the source of myoblasts in postnatal muscle, recent findings have demonstrated that myoblasts can also arise from multipotential stem cells isolated from various tissues such as bone marrow. However, the contribution of non-myogenic cells to the formation of new postnatal skeletal muscle in vivo appears to be minor 120,121.
Androgens are considered to be the main sex steroids regulating body composition. During puberty, boys acquire lean mass at a higher rate and for a longer period of time than girls, resulting in ±35% more muscle mass in males as compared to females. This higher muscle mass mainly results from an increased cross-sectional area of muscle fibers in men. Although all types of muscle fibers are larger in males, the sex difference is especially pronounced in type II fibers, so that the ratio of type II over type I fiber mass is greater in men than in women. In accordance with these differences in fiber size, levels of glycolytic markers were higher in muscles of males as compared to females. These data are in line with the ability of male muscles to generate more strength and power, while female muscles are more resistant to fatigue.

Boys with delayed puberty have lower T levels and lower lean mass as compared to age-matched controls. Reduced lean mass due to reduced protein synthesis is also observed in adult men with acquired hypogonadism or with suppressed serum T due to administration of a gonadotropin-releasing hormone (GnRH) agonist. These men also show increased fat mass due to diminished fat oxidation. Similar observations have been made in elderly men, where age-associated decline of bioavailable T correlates with lower muscle mass, less voluntary muscle strength and higher fat mass. Interestingly, T supplementation is able to increase muscle mass and decrease fat mass in prepubertal boys, as well as hypogonadal young and older men. The effect of androgens on muscle may be dose dependent. Supplementation of physiological amounts of T has been shown to increase synthesis and decrease breakdown of proteins, resulting in hypertrophy of muscle fibers and gain of muscle mass. However, effects on muscle strength are less clear-cut; while several researchers reported that androgens were able to increase voluntary muscle strength, others observed no effect. Possibly, the lack of effect in these latter studies may result from other pathologies and/or enhancement of intrinsic muscle strength (muscle specific tension) or muscle power requires administration of supraphysiological doses of androgens.

In accordance with the data obtained in humans, male mice have higher muscle mass and strength as compared to female mice, but are less fatigue resistant. Castration of male mice or rats results in a reduced muscle mass, which can be restored by T or DHT supplementation. Correspondingly, lean body mass is reduced in mice in which the AR is disrupted. As is the case in humans, the effect of androgens on muscle strength remains unclear in rodents as well. Although orch of mice and rats leads to a reduction in muscle strength
162-164, this is caused by changes in muscle volume but not by differences of intrinsic contractile properties in some 164 but not all studies 163, 165.

1.6.4. MECHANISM OF ANDROGEN ACTION ON MUSCLE

The mechanism by which androgens increase muscle mass is not completely understood (Figure 8). Androgens might act at different steps of the myogenic pathway and may act proximal to both myogenic and adipogenic differentiation pathways, as is suggested by the reciprocal effects of androgens on both muscle and fat mass. Indeed, T and DHT have been shown to promote differentiation of pluripotent mesenchymal C3H10T1/2 cells into the myogenic lineage, while inhibiting adipogenesis 164, 166. As these effects are inhibited by the AR antagonist, bicalutamide, they are mediated by the AR. A possible molecular mechanism by which androgens could regulate lineage commitment, is by activating the Wnt pathway 167. Wnt signaling has been shown to positively regulate myogenesis while inhibiting adipogenesis 168.

However, androgens may also act on other muscle cells. There are several indications that androgens increase muscle mass via an AR-mediated pathway by acting directly on satellite cells. Firstly, both myonuclei and satellite cells of skeletal muscles express AR protein 169-171 and its expression is up-regulated in satellite cells and skeletal muscle after treatment with DHT and T 169, 170. Furthermore, in young men with low serum T due to GnRH agonist administration, T supplementation results in fiber hypertrophy, associated with dose-dependent increases in numbers of satellite cells and myonuclei per fiber 172. Similarly, in rats, T is able to induce satellite cell proliferation and increase myonuclear number by fusion of satellite cells and myofibers 173. Finally, human neck and shoulder muscles contain more AR-positive myonuclei than leg muscles. In accordance, supraphysiological androgen supplementation was shown to increase the number of AR-positive myonuclei more in muscles of the shoulder than of the leg 174, 175.

Figure 8: Mechanism of androgen action on muscle. Androgens can act directly through the androgen receptor (AR) on myonuclei of muscle fibers, satellite cells or multipotential cells. These cells become activated and can form myoblasts which can then further fuse to form myotubes.
### 1.6.5. INDIRECT EFFECTS OF ANDROGENS ON MUSCLE

In addition to direct actions via the AR, androgens can also indirectly affect muscle growth. Accumulating evidence suggests an interaction between androgens and IGF-I in muscle. In men, skeletal muscle IGF-I mRNA levels are decreased due to T deficiency \(^{137}\) and increased by T administration \(^{155}\). Furthermore, T supplementation has also been demonstrated to increase IGF-I protein expression in skeletal muscle of older men \(^{149}\). Similarly, in rats, IGF-I protein levels are elevated after administration of the anabolic steroid and T analogue, nandrolone \(^{176}\). However, the positive effects of T on muscle IGF-I expression are not seen in all studies \(^{67}\). In any case, it is clear that IGF-I and the IGF-I receptor are essential for development of muscle \(^{177}\). As IGF-I administration to cultured muscle cells was shown to stimulate cellular proliferation and subsequent myogenic differentiation \(^{178}\), T-driven intramuscular IGF-I accumulation may possibly promote muscle hypertrophy.

Other potential indirect effects of androgens may be mediated by binding to the glucocorticoid receptor. Indeed, in skeletal muscle, androgens were demonstrated to offset the catabolic effects glucocorticoids have on muscle \(^{179}\). Although ER\(\alpha\) and ER\(\beta\) are expressed in skeletal muscle as well \(^{180,181}\), most studies indicate that estrogens have no significant effects on muscle mass and strength \(^{182}\). However, estrogens may affect fat mass, as is suggested by the findings that disruption of ER\(\alpha\) or aromatase in male mice was shown to increase the mass of fat tissue \(^{183,184}\).

### 1.6.6. MUSCLE ACTION ON BONE GROWTH AND PEAK BONE MASS ACQUISITION

During puberty, growth of muscle and bone are linked. Cross-sectional studies in children and adolescents show that muscle mass and muscle strength are correlated with bone areal BMD and bone strength \(^{52,185-188}\). It is estimated that a 10-20% increment in lean mass is associated with a 5-10% increase in areal BMD and a halving of fracture risk \(^{186}\). Moreover, longitudinal examination of lean body mass and bone mineral content (BMC) during the pubertal growth spurt revealed a correlation between their peak velocities \(^{189}\). Both in boys and girls, peak velocity of muscle gain preceded peak velocity of bone gain, respectively by 0.50 and 0.36 years \(^{189}\). These observations are in accordance with the mechanostat theory which postulates that increased muscle force drives the increase of bone strength during development \(^{188}\). On the other hand, instead of a causal relationship, muscle and bone growth may be independently controlled by other factors such as genetic determinants \(^{186,190}\).

Habitual physical activity has beneficial effects on lean body mass during growth \(^{191}\) and may influence gain of bone mass. Children who are more physically active have been shown to attain more bone mass than their less active peers \(^{192-194}\). In addition, in boys and girls, exercise
intervention studies were demonstrated to increase bone mass during growth\textsuperscript{195-198}. Reportedly, physical activity primarily increases cortical bone size and not volumetric BMD\textsuperscript{187, 199, 200}. Different studies demonstrated that exercise during growth may increase formation of periosteal bone\textsuperscript{187, 197, 200}, endosteal bone\textsuperscript{195, 196} or both\textsuperscript{201}. Compared to bone acquisition on the inner surface, apposition of bone on the periosteal surface is a more effective means of increasing bending and torsial bone strength. For this reason, exercise may be more effective in young bones when periosteal expansion predominates\textsuperscript{202}. The effects of physical activity on trabecular bone are less clear; exercise was shown to increase trabecular bone mass in some\textsuperscript{195, 196, 199, 201} but not all studies\textsuperscript{197, 200}. These differences may result from variations in the type of activity (high-impact or weight-bearing sports versus non-weight-bearing sports)\textsuperscript{199, 203, 204} or in the duration of the exercise intervention\textsuperscript{197, 199}. Whether the beneficial effects of physical activity on the growing skeleton are maintained during adulthood is difficult to prove. However, the results of most\textsuperscript{205-208}, but not all\textsuperscript{209} analyses indicate that the enhancement of bone acquisition during growth caused by exercise interventions, may be long lasting.

Rodent models are often used to elucidate the mechanisms underlying the effects of muscle and loading on bone. As is the case in humans who have sustained spinal cord injury during childhood\textsuperscript{210}, skeletal unloading in rodents was shown to reduce bone mass acquisition at weight-bearing sites\textsuperscript{211-213}. In rats, hind limb unloading during growth decreased calf muscle weight\textsuperscript{211, 212} and resulted in a 40\% reduction of periosteal bone formation in the tibia\textsuperscript{211}. Unloading of the hind limb during growth also decreased trabecular bone mass gain by lowering trabecular number\textsuperscript{212, 213}. Interestingly, studies showed that while reloading by return to normal weight-bearing activity resulted in only a partial restoration of lower bone mass, controlled physical exercise was able to fully reverse the effects of unloading on bone, resulting in complete restoration of trabecular architecture and femoral bone mass in rats\textsuperscript{212, 213}. Similarly, exercise has also been reported to prevent orch-induced bone loss in growing rats\textsuperscript{214}.

The manner in which bone strain is translated into bone formation by osteoblasts (mechanotransduction) remains unclear. Due to their abundance and connectivity in bone tissue, it is generally believed that osteocytes play an important role in this process. These cells can sense fluid movement within the canaliculi caused by bone tissue deformation during loading. The osteocytes subsequently activate lining cells, which in turn differentiate into preosteoblasts\textsuperscript{215, 216}. Several possible messengers mediating mechanotransduction have been proposed. Cultured osteocytes subjected to fluid shear stress were shown to release prostaglandin E\textsubscript{2} and nitric oxide\textsuperscript{217}. When administered to rats, prostaglandin E\textsubscript{2} was able to increase bone formation by stimulating osteoblast recruitment and activity\textsuperscript{218}, while nitric oxide was shown to inhibit the function of osteoclasts\textsuperscript{219}. Another potential mechanism by which
osteocytes may control mechanotransduction, is by adjusting their sclerostin signal. Sclerostin, the product of the SOST gene, antagonizes LRP5, a Wnt co-receptor required for bone formation. Ulnar loading reduces sclerostin levels, resulting in enhanced Wnt signaling and, possibly, in bone formation. Therefore, sclerostin may be an important target for pharmacological modulation of bone mass formation.
CHAPTER 2: AIMS OF THE STUDY

Androgens may affect the male skeleton both directly and indirectly through muscle action. However, the relative contribution of these pathways and the role of the AR in this respect remain largely unknown. Therefore, the aims of our study are to investigate (i) the role of the AR on peak bone mass acquisition, (ii) the role of the AR on muscle mass and function, and (iii) the effects of physical exercise or muscle mass changes on peak bone mass acquisition in male mice. To address these questions, several experiments were performed in growing male mice.

In a first study, the peak bone mass acquisition is compared between mouse models for senile (senescence-accelerated mouse prone 6, or SAMP6) and hypogonadal osteoporosis (orchidectomized control mice). Additionally, the effects of long term DHT and E2 replacement are studied in both models. This way, the role of AR-mediated and/or ER-mediated sex steroid action is investigated in growing male mice (Chapter 4).

The second study employs the AR KO mouse model to focus on the role of the AR in male bone mass acquisition and on the effect of voluntary exercise on bone mass. In this way, the question was addressed whether voluntary exercise is able to reverse the effects of AR deficiency on bone (Chapter 5).

In a third study, a myocyte-specific AR KO mouse (mARKO) was developed to study the role of the AR in muscle. The effect of myocyte-specific AR disruption on muscle mass and performance are compared with the effects observed in mice with ubiquitous AR disruption. Finally, we investigated whether these altered muscle characteristics of mARKO mice affect peak bone mass acquisition (Chapter 6).
Male SAMP6 /Ta mice and their corresponding controls, senescence-accelerated mouse resistant-1 (SAMR1) /Ta mice, were purchased from Harlan (Horst, The Netherlands).

Global androgen receptor KO (ARKO) mice were generated by Dr. Karel De Gendt and kindly provided by Prof. dr. Guido Verhoeven (K.U.Leuven, Leuven, Belgium). ARKO mice were generated using Cre/loxP technology. To disrupt the AR gene, first, female mice (129/Swiss) heterozygous for the floxed AR allele (AR^lox/+) were created by flanking AR exon 2 by loxP sites (floxed). Then, these female AR^lox/+ mice were crossed with male mice (C57Bl/6N) expressing the Cre recombinase ubiquitously under the control of the phosphoglycerate kinase-1 promoter (PGK-Cre^m/). Male and female progeny heterozygous for the PGK-Cre^m allele and inheriting the AR^lox allele from their mothers are expected to undergo exon 2 excision from early zygote stage on and to code for a defective AR (respectively male AR^0/Y and female AR^0/+ mice) (Figure 9). Female AR^0/+ mice were further backcrossed to the C57Bl/6N background for at least 12 generations. Male PGK-Cre^m/;AR^0/Y and PGK-Cre^m/;AR^+/Y littermates are used in our studies. The genotypes of the offspring were identified by PCR.

mARKO mice were generated by crossing female mice (CD1 background) heterozygous for the floxed AR allele (AR^lox/) (provided by Prof. dr. Guido Verhoeven) with transgenic mice expressing the Cre recombinase in heart and skeletal muscle from embryonic day 13, under the control of the muscle creatine kinase promoter (MCK-Cre^+/) (Figure 9). MCK-Cre^+/ mice (129/sv, C57Bl/6, DBA mixed background) (provided by Dr. C. Ronald Kahn from the Joslin Diabeter Center, Harvard Medical School, Boston, MA, USA) were backcrossed to C57Bl/6J for 7 generations prior to crossing with AR^lox/ mice. Male progeny heterozygous for the MCK-Cre allele and inheriting the AR^lox allele from their mothers, MCK-Cre^+;AR^lox/Y mice, are expected to undergo exon 2 excision in heart and skeletal muscle tissue. Male MCK-Cre^+;AR^lox/Y and MCK-Cre^+/;AR^+/Y littermates are used in our studies. The genotypes of the offspring were identified by PCR.
Figure 9: The Cre recombinase (bacteriophage P1) mediates efficient site-specific recombinase between 34 bp recognition sequences known as LoxP sites. To create an ubiquitous or myocyte-specific androgen receptor (AR) knockout (KO) mouse, female mice heterozygous for the floxed AR allele (AR$^{flox/+}$) were crossed with male mice expressing Cre recombinase under the control of phosphoglycerate kinase-1 (PGK) or muscle creatine kinase (MCK) promoter, respectively.

The onset of puberty in mice is around 6 weeks of age. In our experiments animals were investigated longitudinally between 4 and 20 weeks of age (study 1, Chapter 4), 5 and 16 weeks of age (study 2, Chapter 5) or at 16 weeks of age (study 3, Chapter 6).

3.1.2. GENOTYPING

Genotyping was performed by PCR of genomic DNA extracted from tail biopsies. PCR genotyping was done with an appropriate primer pair (forward: 5’-agcctgtatactcagttgggg-3’, reverse: 5’-aatgcatcacattaagttgatacc-3’, accession number: NM 013476, AR exon 2 locus) to identify mice with a wildtype (WT), floxed or excised allele of the AR, revealing bands of 855, 952 and 404 bp, respectively. Also, the presence of the Cre transgene was determined by using appropriate primers (forward 5’-cggtcgatgcaacgagtgatgagg-3’, reverse 5- cagagacggaatccatcgctg-3’, accession number: AJ 627603).
3.1.3. HOUSING AND MANIPULATION OF ANIMALS

All mice were bred in our animal housing facilities (Proefdierencentrum, Leuven, Belgium) and housed under standard conditions of a 12-h light/dark cycle in an air-conditioned room according to our institutional guidelines. During the experiments the mice were fed a standard diet (Muracon G, Nutreco Belgium NV, Gent, Belgium [study 1, Chapter 4 and study 2, Chapter 5] or Sniff, Soest, Germany [study 3, Chapter 6]) and had free access to tap water. Animals were killed under anaesthesia by heart puncture. All experimental procedures were conducted after obtaining formal approval from the ethical committee of the K.U.Leuven or the local Animal Ethics Committee.

Sham-operations, orchidectomy and bone density measurements were performed using pentobarbital anaesthesia (intraperitoneally, 60 mg/kg).

If applicable, food intake was recorded weekly for 4 constitutive weeks during the experimental period and the average was calculated.

3.2. SEX STEROID REPLACEMENT

DHT and E2 were administered as silastic implants subcutaneously in the cervical region immediately after orch (Silclear™ Tubing, Degania Silicone, Jordan Valley, Israel). The silastic implants were prepared from polydimethylsilicone tubing of defined length, filled with crystalline DHT or E2 and sealed at both ends (adhesive type A, Dow Corning Corp., Midland, MI, USA). For the DHT silastics, tubing of 1.5 cm was prepared corresponding with a release of 45µg/day. E2 silastics prepared from 0.5 cm of tubing were filled with trituration of E2 in cholesterol (1/2) corresponding with a release of 0.75 µg/day of E2, when administered in vivo. In order to verify the efficacy of sex steroid replacement, the wet weight of seminal vesicles or uterus was determined immediately after sacrifice.
3.3. VOLUNTARY AND FORCED RUNNING TESTS

3.3.1. VOLUNTARY RUNNING TEST

Mice were able to run voluntarily by placing them in normal mouse cages containing a hamster-sized metal cage wheel with a diameter of 12 cm (Nobby, Bocholt, Germany). The cage wheel was fitted with a digital magnetic counter (M9 Race, Mafac Ertedis, Achères, France) which measured the total distance, total time, and average speed. Every morning, distance, time, and speed of running were monitored and counter was reset.

3.3.2. ENDURANCE TREADMILL

A four-lane treadmill with a speed and inclination adaptable belt was used (Columbus Instruments, Columbus, OH, USA). The first day, a familiarization trial was performed in which the mice had to run three times for 15 minutes with a treadmill speed of 5 m/minute, 10 m/minute and 15 m/minute respectively, (10° inclination) and a rest period of 5 minutes after each run. The experiment was performed the day after the training test. Endurance was tested at a speed of 20 m/minute (10° inclination) until mice stopped running from exhaustion. A mild electric shock was presented to promote running and mice were removed from the experiment after being on the shock grid for 15 consecutive seconds. The time (in seconds) of running until exhaustion was recorded for each mouse.

3.4. RELEVANT SERUM AND URINARY PARAMETERS

Serum was collected by tailbleeding (for longitudinal measurements) or heart punctation at sacrifice. Mouse serum osteocalcin and serum IGF-I were measured by an in-house radioimmunoassay as previously described 226, 227.

Urine was collected by putting the mice overnight in metabolic cages. Urinary collagen cross-links (deoxypyridinolines or DPD) were measured following acid hydrolysis and high-performance liquid chromatography extraction, as previously described 228. The overnight excretion was corrected for creatinine excretion, which was measured kinetically.
3.5. BONE HISTOMORPHOMETRY

One femur and tibia were cleaned from surrounding tissue, immersed in Burckhardt's fixative (24 h, 4°C), kept in 100% ethanol, and embedded in methylmethacrylate. One day and six days before sacrifice, mice were given injections intraperitoneally of the fluorochrome calcein (16 mg/kg, Sigma Chemical Co., St. Louis, MO, USA).

Longitudinal sections of the undecalcified tibia were cut at 4 µm thickness using a rotation microtome (RM 2155 Autocut; Leica, Heidelberg, Germany) with a tungsten carbide blade (Leica, Nussloch, Germany). Sections were stained by a modified Goldner technique and subjected to static histomorphometry. Measurements were performed in the secondary spongiosa of at least three Goldner-stained sections, as previously described. In each section, three consecutive fields were measured along the vertical axis of the central metaphysis, starting at regular distance of the growth plate. Trabecular width and trabecular number were calculated according to the parallel plate model developed by Parfitt et al.

Cross-sections of the undecalcified femur perpendicularly to the long axis were prepared at 200 µm thickness in the mid-diaphyseal region using the contact-point precision band saw (Exakt, Norderstedt, Germany). Sections were ground to a final thickness of 25 µm using a grinding system (Exakt). Sections were left unstained and subjected to dynamic histomorphometry. Three sections in the mid-diaphyseal region were measured by fluorescence microscopy, and the bone formation rate (BFR/B.Pm., μm²/μm/day) was assessed at both the endocortical and periosteal bone surfaces. The BFR was obtained by the product of mineral apposition rate (MAR, μm/day) and mineralizing perimeter per bone perimeter (Min.Pm./B.Pm., %). The mineralizing perimeter was calculated as follows: Min.Pm. = [dL + (sL/2)]/B.Pm., where dL represents the length of the double labels and sL is the length of single labels along the entire endocortical or periosteal bone surfaces. The MAR (μm/day) was calculated as the mean width of double labels, divided by interlabel time (5 days). The mineralizing perimeter and MAR are measures for osteoblast number and osteoblast activity, respectively. Total cross-sectional area, cortical area, cortical thickness, and endocortical and periosteal perimeters were measured on cortical cross-sections. All measurements were performed with a Kontron Image Analyzing computer (KS400 3.00; Kontron Bildanalyse, Munich, Germany) and a Zeiss microscope with a drawing attachment. Specific software was developed in collaboration with the manufacturer. Histomorphometric parameters are reported according to the recommended American Society for Bone and Mineral Research nomenclature.
3.6. PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY (PQCT).

\textit{In vivo} bone densitometry of the femur, \textit{ex vivo} bone densitometry of the femur and \textit{in vivo} calf muscle cross-section was determined by pQCT.

Trabecular BMD and cortical bone parameters were assessed using the Stratec XCT Research M\textsuperscript{+} densitometer (Norland Medical Systems, Fort Atkinson, WI, USA). Slices of 0.2 mm thickness were scanned using a voxel size of 0.070 mm. Three scans were taken from the proximal end of the tibia \textit{in vivo} (at 1.6 \pm 0.1 mm) and the distal end of the femur \textit{ex vivo} (at 2.5 \pm 0.25 mm), using contmode 1, peelmode 20, and a density threshold of 280 mg/cm\textsuperscript{3}. The trabecular bone region was defined by setting an inner threshold corresponding to 30\% of the total cross-sectional area. These three metaphyseal scans were performed to measure the average trabecular volumetric BMD. A second scan was taken 7 mm from the proximal end of the tibia (for \textit{in vivo} measurement) or the distal end of the femur (for \textit{ex vivo} measurement) (an area containing only cortical bone) using separation mode 1 and a density threshold of 710 mg/cm\textsuperscript{3}. These mid-diaphyseal scans were performed to determine cortical volumetric BMD, cortical thickness, cortical area and endocortical and periosteal perimeters.

Calf muscle cross-sectional area was measured by analyzing the \textit{in vivo} pQCT scan of the tibia with its surrounding tissue of the lower leg, using contmode 1, peelmode 2 and separation mode 1. Total soft tissue was measured mid-diaphyseal, at 7 mm from the proximal end of the tibia, and using a density threshold between 35 and 710 mg/cm\textsuperscript{3}.

3.7. MICROCOMPUTED TOMOGRAPHY (µCT)

µCT analysis was performed in the lab of Prof. dr. Claes Ohlsson (Sahlgrenska University Hospital, Göthenborg, Sweden) with a Skyscan 1072 scanner (study 2) (Skyscan N.V., Kontich, Belgium) or in our own lab with a Skyscan 1172 scanner (study 3). Both analyses were performed \textit{ex vivo} on the distal femur.

µCT analysis using the Skyscan 1072 scanner was imaged with an X-ray tube voltage of 50 kV with a 1-mm aluminium filter. The scanning angular rotation was 185° and the angular increment was 0.675°. Pixel size was 4.56 µm and magnification was x60. Reconstructed datasets were segmented into binary images by using adaptive local thresholding \textsuperscript{105}. Trabecular bone distal of the growth plate was selected for analysis within a conforming volume of interest (cortical bone excluded), commencing at a distance of 100 µm from the growth plate and extending a further longitudinal distance of 2.3 mm in the proximal direction. The number of
slices was 500, each with the same thickness as the pixel size, 4.56 µm. Trabecular thickness and separation were calculated by the sphere-fitting local thickness method 105. Trabecular bone thickness measurement was calibrated by using aluminium foils of 20- and 250-µm thickness (Advent Research Materials, Oxford, UK) 105.

µCT analysis using the Skyscan 1172 scanner was imaged using an X-ray tube voltage of 50 kV and current of 200 µA with a 0.5-mm aluminium filter. The scanning angular rotation was 180° and an angular increment of 0.6°, frame averaging of 2 and a pixel size of 5 µm were used. The image slices were reconstructed using the cone-beam reconstruction software (NRecon, v.1.4.4.0, Skyscan) based on the Feldkamp algorithm 230. Reconstructed datasets were segmented into binary images by using simple global thresholding methods. Trabecular bone of the distal femur was selected for analysis by drawing contours with the “CT-analyser” software (Skyscan), commencing at a distance of 500 µm from the growth plate and extending a further longitudinal distance of 1.5 mm in the proximal direction. The number of slices was 300, each with the same thickness as the pixel size, 5 µm. Cortical bone was analyzed starting at a distance of 3.25 mm from the growth plate and extending a further longitudinal distance of 0.5 mm in the proximal direction (100 slices, 5 µm pixel size). Micromorphological information was obtained from image stacks, allowing 3D parameters of bone microstructure to be calculated, including trabecular bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and cortical bone parameters (cortical area [Ct.Ar], cortical thickness [Ct.Th], periosteal perimeter [Ps.Pm], endocortical perimeter [Ec.Pm]). Morphometric parameters measured by CT-analyser have been validated on both virtual objects and aluminium foil and wire phantoms 231. Trabecular thickness was calculated by the sphere-fitting local thickness method 232.

3.8. WHOLE–BODY DUAL-ENERGY X-RAY ABSORPTIOMETRY (DEXA)

Areal total body BMD, lean body mass and fat mass were analyzed in vivo by DEXA (PIXImus densitometer; Lunar Corp., Madison, WI, USA) using ultra-high resolution (0.18 × 0.18 pixels, resolution of 1.6 line pairs/mm) and software version 1.45.
3.9. MUSCLE IMMUNOHISTOCHEMISTRY

3.9.1. AR PROTEIN EXPRESSION IN MYOCYTES

The musculus levator ani (LA) was removed and frozen in isopentane (Sigma-Aldrich, Bornem, Belgium). Transverse sections (5 μm) were cut from the mid-area of the muscle with a cryostat HM560 (Microm International, Walldorf, Germany) at –20 °C and mounted on glass slides. LA was stained for the AR, basal lamina (laminin) and DNA using standard procedures. Sections were fixed for 10 min in 4% paraformaldehyde in PBS (Invitrogen, Merelbeke, Belgium). All rinses were performed with 0.5% BSA (Invitrogen) in PBS. Endogenous binding of biotin and avidin was blocked with the use of an avidin/biotin blocking kit (SP-2001, Vector Laboratories, Peterborough, UK). A rabbit anti-AR antiserum (sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 was utilized in conjunction with a swine anti-rabbit biotinylated second antibody (dilution 1:500, E0353, DakoCytomation). This antibody specifically recognizes the N-terminal domain of the AR. Bound antibodies were incubated by visualizing the sections with ABComplex HRP reagent (K0355, DakoCytomation) followed by color development with 3,3'-diaminobenzidine tetrahydrochloride chromogenic substrate (K3468, Liquid DAB+ kit, DakoCytomation). To visualize the basal lamina, a mouse anti-laminin antiserum (1:500, L8271, Sigma-Aldrich) and secondary antibody conjugated to Alexa Fluor-594 dye (dilution 1:500, Molecular Probes, Leiden, The Netherlands) was used. Sections were stained with Hoechst 33258 (Sigma-Aldrich) to visualize cell nuclei, and images were captured with an inverted microscope (Eclipse E800, Nikon, Brussels, Belgium; Plan Apo 60x/A/1.40 oil objective) connected to a confocal microscope (Bio-Rad Radiance 2100, Bio-Rad Laboratories, Hercules, CA, USA) using LaserSharp 2000 software (version 6.0). Images were processed with Adobe Photoshop and ImageJ software.

The triple-staining of AR (brown), basal lamina (red fluorescent) and nuclei (blue fluorescent) made it possible to distinguish between nuclei staining positive or negative for AR protein and to determine whether these nuclei were located inside or outside the basal lamina. The number of fibers, the number of nuclei inside and outside the basal lamina staining positive for the presence of AR protein (respectively AR⁺ in and AR⁺ out), and staining negative for the presence of AR protein (respectively AR⁻ in and AR⁻ out) were counted in a standardized area in each muscle cross-section. The % AR protein positive cells within the basal lamina was calculated as (AR⁺ in)/(AR⁺ in + AR⁻ in)×100. The % interstitial AR protein positive cells were calculated as (AR⁺ out)/(AR⁺ out + AR⁻ out)×100. Averages of cross-sections were calculated for each mouse and were used to test statistical differences.
3.9.2. FIBER TYPING

Fiber typing was performed in collaboration with Prof. dr. Peter Hespel (the Research Center for Exercise and Health, Faculty of Kinesiology and Rehabilitation Sciences, K.U.Leuven).

Soleus and extensor digitorum longus (EDL) muscles were removed and frozen in isopentane. Transverse sections (4 μm) were cut from the mid-belly area with a Leica CM1850 cryostat (Leica, Nussloch, Germany) at ~20 °C and mounted on glass slides. Cryosections were incubated for 1 h at 37 °C and were shortly rinsed by successive exchanges in 30%, 60%, and 90% acetone in double distilled H₂O, respectively. Next, sections were incubated for 1 h at 4 °C with primary monoclonal antibodies against human MHC type I (A4.840 supernatant, Developmental Studies Hybridoma Bank, Iowa, USA) and IIa (N2.261 supernatant, Developmental Studies Hybridoma Bank), followed by adding (1 h) the appropriate conjugated antibodies (type I: FITC anti-mouse IgM Southern Biotechnology Associates, Birmingham, AL, USA; type IIa: Alexa Fluor-350 anti-mouse IgG1 Molecular Probes, Leiden, The Netherlands). Cover slips were mounted with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA, USA). Slides were examined using a Nikon E1000 fluorescence microscope (Nikon, Boehrvedorp, Germany) equipped with a digital camera. Epifluorescence signal was recorded utilizing a FITC and a DAPI filter for type I and IIa muscle fibers respectively. Captured images (x40 magnification) were processed and analyzed using Lucia G software (LIM, Prague, Czech Republic). The bright-field images were converted post-hoc to grey-scale values and the mean optical density of each fiber was estimated. Mean optical density of a selected region outside the muscle section, which was equal to the optical density of the sections after incubation in a reaction medium without the substrate, was used for background correction. Three to five digital images were taken from each section and the fibers were identified as type I fibers (green staining), type IIa fibers (blue staining) and type IIb/x fibers (no staining). Fiber type distribution as percentage number, fiber-specific proportional surface area, and average fiber size per type were calculated for each of the fiber categories.

3.9.3. SATELLITE CELL DETERMINATION

Satellite cell determination was performed in collaboration with Prof. dr. Peter Hespel.

Soleus and EDL muscles were removed and frozen in isopentane. Immunostaining was performed on transverse sections (4 μm) with Pax 7, a marker of both quiescent and activated satellite cells. Muscle cryosections were fixed in 1% paraformaldehyde, followed by 2 times 5 min washing in 0.5% BSA in PBS. Slides were then quenched with 4% H₂O₂ in methanol and blocked in PBS containing 2% BSA, 5% goat serum and 0.2% Triton X-100. Next, sections were
incubated overnight at 4 °C with primary antibodies directed against Pax 7 (supernatant, Developmental Studies Hybridoma Bank, Iowa, USA) and laminin (polyclonal rabbit anti-laminin, Sigma-Aldrich) diluted in the same blocking solution (1:5 and 1:100 for Pax 7 and laminin, respectively). After overnight incubation, the appropriate secondary antibodies: Alexa Fluor-488 labeled goat anti-mouse IgG1 (dilution 1:200, Molecular Probes) and Alexa Fluor-568 labeled goat anti-rabbit IgG (dilution 1:200, Molecular Probes) were added and incubated for 1 h. Nuclei were counterstained with DAPI (ProLong Gold antifade reagent, Molecular Probes) and cover slips were mounted. Slides were examined using a Nikon E1000 fluorescence microscope (Nikon, Boehrhevord, Germany) equipped with a digital camera. Captured images (x40 magnification) were processed and analyzed using Lucia G software (LIM, Prague, Czech Republic). Epifluorescence signal was recorded using a Texas red excitation filter for laminin, and FITC and DAPI filter for Pax 7 and myonuclei respectively. Satellite cells were identified as positively stained for Pax 7 as well as for DAPI, and located inside the basal lamina. The percentage satellite cells (Pax 7 positive) was calculated as number of satellite cells/number of fibers*100.

3.10. WESTERN IMMUNOBLOTTING

Muscle homogenates were prepared and suspended in reducing SDS sample buffer (80 mM Tris [pH 6.9], 110 mM SDS, 10% glycerol, and 100 mM dithiothreitol). After boiling (95 °C, 3 min), samples (40µg) were separated on a 7.5% polyacrylamide gel and transferred to a Hybond enhanced chemiluminescence membrane (Amersham International). Equal protein loading was confirmed by Ponceau-S staining of membranes after transfer. Membranes were blocked in a Tris-buffered saline solution with 5% nonfatty milk and 0.1% Tween. The membranes were incubated with the primary antibody (rabbit anti-AR antiserum; dilution 1:500, sc-816, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Membranes were washed in Tris-buffered saline solution with 0.05% Tween, and immunoreactive signals were detected by a 1-h incubation at room temperature with goat-anti-rabbit secondary antibody coated with horseradish peroxidase (dilution 1:5000, DakoCytomation, Glostrup, Denmark) and followed by chemiluminescent detection (Western Lightning, PerkinElmer Life Sciences). After exposing the blots to Hyperfilm MP (Amersham Biosciences) band intensities were determined using a sharp JW-330 scanner and Imagemaster 1D software program (Pharmacia Biotech). Equal loading of proteins was confirmed by immunoblotting for α-tubulin (Cell Signaling Technology, Beverly, MA, USA).
3.11. MUSCLE CONTRACTILE PROPERTIES

Measurements of twitch tension, tetanic tension and muscle fatigability of the musculus soleus and EDL was performed in collaboration with Prof. dr. Peter Hespel (the Research Center for Exercise and Health, Faculty of Kinesiology and Rehabilitation Sciences, K.U.Leuven, Leuven, Belgium).

After careful dissection of the soleus and EDL muscles, wires were attached to the tendons. The muscles were mounted vertically in an incubation bath containing Krebs-Henseleit solution (118 mM NaCl, 25 mM NaHCO$_3$, 5 mM KCl, 1 mM MgSO$_4$, 1 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$ and 1 mM glucose) which was continuously gassed with a mixture of 95% O$_2$ and 5% CO$_2$, and maintained at 25 °C. with one tendon attached to a force transducer and were stimulated with capacitor discharges (pulse duration 1 ms; 100 mA) between platinum electrodes. After mounting, a 15-minute stabilization period was allowed and optimal muscle length ($L_0$) was determined by a series of single tetanic contractions (350 ms duration, with 50 and 100 Hz stimulation frequency for soleus and EDL, respectively) administered with 2-minute intervals. Twitch characteristics were assessed during five twitch stimuli (1 ms) interspersed with 1-minute rest intervals. Tetanic tension characteristics were evaluated by three 350 ms stimuli (2-minute rest intervals) in soleus (50 Hz) and EDL (100 Hz). The average maximal specific tension (=force corrected for estimated cross-sectional area) was calculated for twitch contractions and for tetanic contractions. Cross-sectional area was estimated by dividing the wet muscle mass by the product of muscle length ($L_0$) and 1.06 g/cm$^3$, the density of mammalian skeletal muscle. The force-frequency relationship was established by tetanic stimuli (350 ms duration) with 2-min rest intervals between stimuli and with increasing stimulation frequency of 1, 10, 20, 50 and 100 Hz for soleus and 1, 25, 40, 100 and 125 Hz for EDL. The fatigability of the soleus was evaluated as the decrease in tetanic tension and increase in relaxation time during 10 minutes of repeated tetani (350 ms; 100 Hz) with rest-intervals of 1.6 seconds. Average maximal tetanic tension and relaxation time (df/dt) were determined per min of electrical stimulation.

3.12. SUCCINATE DEHYDROGENASE ACTIVITY

Succinate dehydrogenase activity was performed in collaboration with Prof. dr. Peter Hespel and was determined simultaneously with fiber typing to allow fiber type specific succinate dehydrogenase activity.
Transverse sections (4 μm) were cut from the mid-belly area of soleus and EDL muscles with a Leica CM1850 cryostat (Leica, Nussloch, Germany) at −20 °C and mounted on glass slides. Cryosections were incubated for 1 h at 37 °C in a 0.2 M sodium phosphate buffer containing 0.1 M succinic acid (Sigma-Aldrich, Bornem, Belgium) and 1.2 mM nitro-blue tetrazolium (AppliChem; NBT, Darmstadt, Germany). Next, sections were shortly rinsed with double distilled H₂O. Images for succinate dehydrogenase stained sections were examined in bright-field with identical Nikon E1000 microscopy (light intensity: 11.3V; ND filter: 72°; Nikon, Boehravdorp, Germany) equipped with digital camera (exposure time: 1 ms). Captured images (x40 magnification) were processed and analyzed using Lucia G software (LIM, Prague, Czech Republic).

3.13. MUSCLE GLYCOGEN CONTENT

Glycogen content was measured in collaboration with Prof. dr. Peter Hespel on the musculus gastrocnemius. The concentration of glycogen was measured on samples of ± 10 mg and calculated as micromole per gram wet weight. Glycogen was hydrolyzed in 1 M HCl at 100 °C for 2 h and the concentration of glucose residues was assayed with a standard enzymatic fluorometric assay.

3.14. STATISTICAL ANALYSIS

Data analysis was performed using a statistical software program (NCSS, Kaysville, UT, USA). All data are expressed as mean ± standard error (SE) and P<0.05 was accepted as significant. Significance of difference between means of groups was assessed by two-tailed Student’s t-test or one-way analysis of variance (ANOVA), followed by Fisher’s least significant difference multiple comparison test, as appropriate. Two-way ANOVA was used to test difference between means of groups and interaction (significant interaction: P_int<0.05).

When a specific parameter was measured repeatedly over time, a repeated measures ANOVA was performed followed by pre-planned comparisons test when necessary.

Linear regression models were used to test the effect of covariates on correlations between muscle (muscle cross-sectional area) and bone (strength strain index or SSI).
4.1. INTRODUCTION

The SAMP6 mouse model was developed by Takeda et al. 235 by AKR/J mouse inbreeding. Earlier studies demonstrated that SAMP6 mice have a shortened lifespan and show characteristics of senile osteoporosis 236. At older age, SAMP6 mice have severely reduced bone strength and develop spontaneous leg fractures. However, these mice have less trabecular bone volume, thinner but wider cortices and reduced material properties, already at 4-5 months of age 237-239. The SAMP6 bone phenotype appears to be related to deficient osteoblastogenesis although the underlying genetic factors accounting for the osteopenia remain to be elucidated 240-242.

Hypogonadism is also a major risk factor for osteoporosis in men 243. Androgens are essential for the development of a male skeletal phenotype during growth. Similar to humans, hypogonadism in rodents, as induced by orch at puberty, results in low bone mass acquisition as well as low bone strength 67, 71, 214. Orch in male rodents reduces periosteal bone formation and increases bone turnover hereby impairing respectively cortical and trabecular bone mass acquisition 35. Conversely, androgen supplementation in orch growing male rodents stimulates bone gain 35. However, since not only the AR but also the ER as well as the aromatase and 5α-reductase enzymes are present in bone, the relative role of AR-mediated androgen action remains unclear.

This study aimed to characterize the timing as well as the underlying mechanisms of deficient peak bone mass acquisition in models of hypogonadal versus senile osteoporosis. Although deficient peak bone mass acquisition was observed in both models, the mechanisms of osteopenia induced by either sex steroid deficiency or genetic predisposition are probably different and have not been fully addressed. In addition, sex steroids were supplemented to both mouse models to investigate their effects on bone. Not only DHT, a non-aromatisable androgen, but also E2 replacement was evaluated in orch SAMP6 and SAMR1 control mice in order to characterize both AR- and ER-mediated actions.
4.2. EXPERIMENTAL DESIGN

At the start of puberty (4 weeks of age), male SAMP6 and control SAMR1 mice were randomly divided into 5 groups. A baseline group (base) was sacrificed at the start of the experiment. The other mice were either sham-operated (sham) or orchidectomized (orch). Orch mice were treated during an experimental period of 16 weeks with vehicle, DHT or E2. DHT and E2 were administered using subcutaneous silastic implants in the cervical region. Vehicle animals received empty implants. At 20 weeks of age the remaining animals were sacrificed.

At 4, 8, 12, 16 and 20 weeks of age, mice were weighed and placed in metabolic cages to collect urine for measurement of collagen cross-links. Serum was collected by tail bleeding and used for osteocalcin measurement. In vivo pQCT of the left tibia was performed at each time point. Whole body in vivo DEXA was performed at the start and the end of the experiment. Mice were injected intraperitoneally with the fluorochrome calcein at a 5-day interval and were sacrificed 1 day after the second injection. One tibia and femur were dissected for histomorphometric analysis following ex vivo pQCT measurement of the femur.
Male SAMP6 mice are different from their SAMR1 controls at the trabecular and cortical bone compartments, even at early puberty. At 4 weeks of age, trabecular BMD is decreased in the tibia (149.0 ± 5.3 mg/cm³ in SAMR1 [n=33] versus 139.0 ± 1.8 mg/cm³ in SAMP6 [n=55], P=0.04) and tends to be lower in the femur (112.0 ± 6.8 mg/cm³ in SAMR1 [n=13] versus 101.8 ± 5.4 mg/cm³ in SAMP6 [n=8], P=0.31). During growth, trabecular BMD lowers slightly in both SAMP6 and SAMR1, and at 20 weeks of age, no significant difference is noticed between SAMP6 and SAMR1 in tibia (129.7 ± 5.1 mg/cm³ in SAMR1 sham [n=9] versus 123.4 ± 5.1 mg/cm³ in SAMP6 sham [n=17], P=0.44; and Table 2) nor femur (119.6 ± 13.8 mg/cm³ in SAMR1 sham [n=9] versus 94.6 ± 6.3 mg/cm³ in SAMP6 sham [n=16], P=0.07). Accordingly, DPD levels are increased at week 4, but not at week 20 in sham-operated SAMP6 as compared to SAMR1 mice (Table 3). Serum osteocalcin levels are similar between SAMP6 and SAMR1 at week 4 and 20 (Table 3). Overall, serum osteocalcin and urinary DPD declined rapidly in both SAMR1 and SAMP6 during the experimental period, indicating an age-related decrease in bone turnover (Table 3).

Table 2: Trabecular bone parameters of the tibia measured by histomorphometry

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>orch</th>
<th>orch + DHT</th>
<th>orch + E2</th>
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<tbody>
<tr>
<td><strong>Trabecular bone volume (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>4.2 ± 1.5</td>
<td>1.2 ± 0.7</td>
<td>2.1 ± 0.7</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>SAMP6</td>
<td>5.5 ± 1.6</td>
<td>0.3 ± 0.1</td>
<td>5.5 ± 1.1</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td><strong>Trabecular number (mm⁻¹)</strong></td>
<td></td>
<td></td>
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<tr>
<td>SAMR1</td>
<td>1.6 ± 0.5</td>
<td>0.6 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>SAMP6</td>
<td>2.6 ± 0.6</td>
<td>0.2 ± 0.1</td>
<td>3.1 ± 0.5</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td><strong>Trabecular width (μm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>22.1 ± 2.1</td>
<td>16.4 ± 3.0</td>
<td>16.1 ± 1.2</td>
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<tr>
<td>SAMP6</td>
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<td>11.4 ± 1.2</td>
<td>16.7 ± 0.9</td>
<td>14.1 ± 1.9</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SE (n= 5-21 mice per group) for the baseline group (base) and at the end of the experimental period in sham-operated mice (sham), orchidectomized mice treated with vehicle (orch), orchidectomized mice treated with DHT (orch + DHT) and orchidectomized mice treated with E2 (orch + E2).* P<0.05 versus respective SAMR1 group (significant differences tested by Student’s t-test); a, P<0.05 versus sham; b, P<0.05 versus orch; c, P<0.05 versus orch + DHT (significant differences tested by one-way ANOVA). No significant difference between the different treatment groups in SAMR1 was found.
Table 3: serum osteocalcin and urinary DPD

<table>
<thead>
<tr>
<th></th>
<th>base</th>
<th>sham</th>
<th>orch</th>
<th>orch + DHT</th>
<th>orch + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DPD/creatinine (nM/mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>51.8 ± 1.8</td>
<td>11.0 ± 1.0</td>
<td>17.9 ± 3.0</td>
<td>6.8 ± 1.2</td>
<td>11.4 ± 2.7</td>
</tr>
<tr>
<td>SAMP6</td>
<td>69.5 ± 1.8 *</td>
<td>9.3 ± 0.6</td>
<td>22.0 ± 1.7</td>
<td>10.3 ± 1.5</td>
<td>19.6 ± 2.8</td>
</tr>
<tr>
<td><strong>Osteocalcin (ng/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>168.0 ± 7.7</td>
<td>43.4 ± 7.4</td>
<td>67.7 ± 7.4</td>
<td>28.3 ± 2.0</td>
<td>39.2 ± 5.8</td>
</tr>
<tr>
<td>SAMP6</td>
<td>182.1 ± 5.5</td>
<td>42.9 ± 4.2</td>
<td>65.7 ± 6.4</td>
<td>23.5 ± 2.1</td>
<td>50.9 ± 5.3</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SE (n= 5 – 21 mice per group) for the baseline group (base) and at the end of the experimental period in sham-operated mice (sham), orchidectomized mice treated with vehicle (orch), orchidectomized mice treated with DHT (orch + DHT) and orchidectomized mice treated with E2 (orch + E2). *P<0.05 versus respective SAMR1 group (significant differences tested by Student’s t-test); a, P<0.05 versus sham; b, P<0.05 versus orch; c, P<0.05 versus orch + DHT (significant differences tested by one-way ANOVA).

Interestingly, cortical bone is even more impaired. As early as 4 weeks of age, periosteal and endocortical perimeters are significantly increased in SAMP6, resulting in a greater cross-sectional bone area and a larger medullary area (Table 4). Also, cortical thickness and cortical area of the femur of SAMP6 mice are significantly reduced compared with SAMR1 at baseline (Table 4). Accordingly, endocortical bone formation is decreased in SAMP6 at 4 weeks, while periosteal bone formation even tends to be higher in these mice (Table 4).

Cortical bone differences between SAMP6 and SAMR1 become more apparent during growth, resulting in a 22% greater cross-sectional area, 59% wider medullary area and an 11% larger periosteal perimeter of the femur in sham-operated 20-week-old SAMP6 mice (Table 4). Also in the tibia, periosteal and endocortical perimeters are significantly enhanced in SAMP6 at week 4 (Figure 10A). Moreover, the greater cortical cross-sectional area explains the higher polar moment of inertia, although the muscle cross-sectional area mass (a surrogate marker for local muscle mass and loading) is not increased but even decreased in SAMP6 mice (Figure 10D, E). Longitudinal evaluation of tibial growth shows that the excessive expansion of the endosteal perimeter (and compensatory periosteal bone expansion) in SAMP6 mice occurs mainly between 4 and 8 weeks of age (Figure 10A). The endosteal and periosteal expansion is minimal after 8 weeks of age and is not different between SAMP6 and SAMR1 mice (Figure 10A). In line with this finding, periosteal and endosteal bone formation rates are not significantly different between SAMP6 and SAMR1 mice at 20 weeks (Table 4).
Table 4: Static and dynamic cortical parameters of the femur by histomorphometry

<table>
<thead>
<tr>
<th></th>
<th>base</th>
<th>sham</th>
<th>orch</th>
<th>orch + DHT</th>
<th>orch + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortical thickness (µm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>146 ± 3</td>
<td>282 ± 8</td>
<td>261 ± 7</td>
<td>282 ± 10</td>
<td>312 ± 12 a,b,c</td>
</tr>
<tr>
<td>SAMP6</td>
<td>120 ± 1 *</td>
<td>234 ± 5 *</td>
<td>227 ± 3 *</td>
<td>236 ± 2 *</td>
<td>259 ± 5 a,b,c*</td>
</tr>
<tr>
<td><strong>Cortical area (mm²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>0.43 ± 0.01</td>
<td>1.02 ± 0.04</td>
<td>0.85 ± 0.02 a</td>
<td>0.99 ± 0.04 b</td>
<td>1.12 ± 0.03 b,c</td>
</tr>
<tr>
<td>SAMP6</td>
<td>0.40 ± 0.01 *</td>
<td>0.98 ± 0.02</td>
<td>0.87 ± 0.02 a</td>
<td>1.03 ± 0.02 b</td>
<td>1.03 ± 0.04 b</td>
</tr>
<tr>
<td><strong>Medullary area (mm²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>0.59 ± 0.01</td>
<td>0.73 ± 0.02</td>
<td>0.59 ± 0.01 a</td>
<td>0.68 ± 0.01 b</td>
<td>0.67 ± 0.03 b</td>
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<tr>
<td>SAMP6</td>
<td>0.80 ± 0.03 *</td>
<td>1.16 ± 0.05 *</td>
<td>0.93 ± 0.04 a</td>
<td>1.22 ± 0.03 b</td>
<td>0.95 ± 0.04 a,c,*</td>
</tr>
<tr>
<td><strong>Total cross-sectional area (mm²)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>1.03 ± 0.02</td>
<td>1.75 ± 0.05</td>
<td>1.44 ± 0.02 a</td>
<td>1.67 ± 0.04 b</td>
<td>1.79 ± 0.03 b,c</td>
</tr>
<tr>
<td>SAMP6</td>
<td>1.20 ± 0.04 *</td>
<td>2.14 ± 0.05 *</td>
<td>1.80 ± 0.06 a</td>
<td>2.25 ± 0.05 b</td>
<td>1.98 ± 0.07 a,c,*</td>
</tr>
<tr>
<td><strong>Periosteal perimeter (µm)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>4080 ± 66</td>
<td>5158 ± 81</td>
<td>4695 ± 40 a</td>
<td>5096 ± 58 b</td>
<td>5194 ± 52 b</td>
</tr>
<tr>
<td>SAMP6</td>
<td>4365 ± 101 *</td>
<td>5743 ± 65 *</td>
<td>5223 ± 77 a,*</td>
<td>5829 ± 55 b,*</td>
<td>5413 ± 113 a,c</td>
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<tr>
<td><strong>Periosteal bone formation rate per bone perimeter (µm²/µm/day)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>1.73 ± 0.30</td>
<td>0.36 ± 0.11</td>
<td>0.42 ± 0.04</td>
<td>0.14 ± 0.03 a,b</td>
<td>0.45 ± 0.07 c</td>
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<tr>
<td>SAMP6</td>
<td>2.55 ± 0.67</td>
<td>0.53 ± 0.26</td>
<td>0.46 ± 0.07</td>
<td>0.78 ± 0.23 a</td>
<td>0.46 ± 0.10</td>
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<tr>
<td><strong>Endosteal perimeter (µm)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>3238 ± 56</td>
<td>3521 ± 59</td>
<td>3087 ± 43 a</td>
<td>3431 ± 41 b</td>
<td>3439 ± 73 b</td>
</tr>
<tr>
<td>SAMP6</td>
<td>3599 ± 86 *</td>
<td>4437 ± 105 *</td>
<td>3826 ± 71 a,*</td>
<td>4685 ± 69 b,*</td>
<td>3861 ± 152 a,c*</td>
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<tr>
<td><strong>Endocortical bone formation rate per bone perimeter (µm²/µm/day)</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SAMR1</td>
<td>2.48 ± 0.32</td>
<td>0.74 ± 0.09</td>
<td>0.92 ± 0.10</td>
<td>0.51 ± 0.14</td>
<td>1.38 ± 0.2 a,b,c</td>
</tr>
<tr>
<td>SAMP6</td>
<td>1.42 ± 0.21 *</td>
<td>0.60 ± 0.19</td>
<td>0.86 ± 0.15</td>
<td>0.40 ± 0.07</td>
<td>0.76 ± 0.11 *</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SE (n = 5 – 9 mice per group) for the baseline group (base) and at the end of the experimental period in sham-operated mice (sham), orchidectomized mice treated with vehicle (orch), orchidectomized mice treated with DHT (orch + DHT) and orchidectomized mice treated with E2 (orch + E2). *P<0.05 versus respective SAMR1 group (significant differences tested by Student’s t-test); a P<0.05 versus sham; b P<0.05 versus orch; c P<0.05 versus orch + DHT (significant differences tested by one-way ANOVA).
Figure 10: Periosteal (Ps.Pm.) and endosteal perimeter (Ec.Pm.) during the experimental period (A), cortical thickness (B), cortical area (C), polar moment of inertia (D) and calf muscle cross-sectional area (E) at the end of the experimental period as measured by in vivo pQCT in tibia of sham-operated (sham) and orchidectomized (orch) SAMR1 and SAMP6 mice. Data are expressed as mean ± SE, n = 8 – 17 mice per group. Panel A: * , P<0.05 Student’s t-test sham versus orch. Panel B – E: results of 2-way ANOVA of values at 20 weeks are shown in inset. No significant interactions are found. Baseline values are marked in grey.
Table 5: Body composition

<table>
<thead>
<tr>
<th></th>
<th>base</th>
<th>sham</th>
<th>orch</th>
<th>orch + DHT</th>
<th>orch + E2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>18.8 ± 0.5</td>
<td>32.4 ± 0.8</td>
<td>33.6 ± 0.8</td>
<td>35.7 ± 1.2 *</td>
<td>30.8 ± 1 c</td>
</tr>
<tr>
<td>SAMP6</td>
<td>17.7 ± 0.3 *</td>
<td>33.5 ± 0.7</td>
<td>32.6 ± 0.6</td>
<td>38.4 ± 0.7 a,b,*</td>
<td>31.7 ± 0.7 c</td>
</tr>
<tr>
<td><strong>Fat mass (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>1.8 ± 0.1</td>
<td>5.1 ± 0.6</td>
<td>8.7 ± 0.5 a</td>
<td>8.2 ± 0.5 a</td>
<td>6.5 ± 1.3 b</td>
</tr>
<tr>
<td>SAMP6</td>
<td>2.0 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>7.5 ± 0.6 a</td>
<td>7.4 ± 0.5 a</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td><strong>Lean mass (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAMR1</td>
<td>15.3 ± 0.4</td>
<td>24.7 ± 0.7</td>
<td>22.7 ± 0.6</td>
<td>25.1 ± 0.9</td>
<td>23.4 ± 0.3</td>
</tr>
<tr>
<td>SAMP6</td>
<td>14.7 ± 0.2</td>
<td>27.9 ± 0.4 *</td>
<td>23.7 ± 0.3 a</td>
<td>29.0 ± 0.8 b,*</td>
<td>23.4 ± 0.6 a,c</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SE (n= 5 - 16 mice per group) for the baseline group (base) and at the end of the experimental period in sham-operated mice (sham), orchiectomized mice treated with vehicle (orch), orchiectomized mice treated with DHT (orch + DHT) and orchiectomized mice treated with E2 (orch + E2). *P<0.05 versus respective SAMR1 group (significant differences tested by Student's t-test); a, P<0.05 versus sham; b, P<0.05 versus orch; c, P<0.05 versus orch + DHT (significant differences tested by one-way ANOVA).

4.3.2. EFFECT OF ORCHIDECTOMY IN SAMR1 AND SAMP6 MICE

Orch prevents the rise of serum T during puberty. In accordance, seminal vesicle weight is reduced in 20-week-old orch SAMR1 and SAMP6 mice (0.2 ± 0.1 mg/g per body weight and 0.4 ± 0.2 mg/g per body weight in orch SAMR1 [n=8] and SAMP6 [n=13] respectively, as compared to 8.3 ± 0.5 mg/g per body weight and 7.4 ± 0.4 mg/g per body weight in sham SAMR1 [n=9] and SAMP6 [n=17]; P<0.01 orch versus sham). Additionally, orch changes body composition. Indeed, in both models, orch reduces muscle mass gain while increasing fat (Table 5 and Figure 10E).

Trabecular bone growth is also altered by orch. Orch lowers trabecular BMD in femur (to 19.8 ± 4.4 mg/cm³ in orch SAMR1 [n=8] and 21.7 ± 4.0 mg/cm³ in SAMP6 [n=13], both P<0.01 versus sham) as well as tibia (Table 2) by reduction of both trabecular number and width (Table 2). In agreement, serum osteocalcin and urinary DPD are upregulated by orch in SAMR1 and SAMP6 mice (Table 3).

Orch also reduces cortical bone gain in SAMP6 and SAMR1 mice and results in smaller bone size (reduced total, cortical and medullary area, smaller cortical perimeters and reduced polar moment of inertia) and thinner cortex of both femur (Table 4) and tibia (Figure 10A-D) due to an impaired periosteal expansion (Figure 10A). Orch and the SAMP6 phenotype therefore have additive effects on cortical bone: a thinner cortex but wider bone size (effect of SAMP6) with less compensatory periosteal bone expansion (effect of orch) (Figure 10A-C). Longitudinal analysis of tibial growth shows that changes in cortical parameters induced by orch are minimal after 12 weeks of age (Figure 10A). In line with these observations, no significant bone formation differences are detected between 20-week-old sham and orch SAMR1 and SAMP6 mice (Table 4).
DHT improves muscle mass gain in orch SAMR1 and SAMP6 mice, but does not avoid fat mass to increase (Table 5). E2 on the other hand, prevents fat mass gain but has no effect on muscle in orch SAMR1 and SAMP6 (Table 5). Interestingly, DHT prevents orch-induced lower trabecular bone mass gain in SAMR1 and SAMP6 and avoids trabecular BMD loss in tibia (Table 2) as well as femur (95.3 ± 11.9 mg/cm³ in DHT treated orch SAMR1 [n=8], P<0.01 versus orch, and 129.5 ± 7.3 mg/cm³ in DHT treated orch SAMP6 mice [n=13], P<0.01 versus orch, sham and SAMR1). However, trabecular BMD and trabecular number is higher in SAMP6 than in SAMR1 mice following DHT, despite similar seminal vesicle weight (6.3 ± 0.4 mg/g body weight in SAMR1 [n=8] and 5.7 ± 0.3 mg/g body weight in SAMP6 [n=12], P=0.27) (Table 2). Bone turnover markers are also reduced to sham level in both SAMP6 and SAMR1 following DHT (Table 3). In cortical bone, DHT prevents the orchidectomy-induced reduction of cortical thickness and area in SAMR1 and SAMP6 mice (Table 4 and Figure 11A-C) due to more periosteal than endosteal expansion (Table 4 and Figure 11A). The overall increase of perimeters is again greater in DHT treated SAMP6 compared to SAMR1 mice during the experimental period. Moreover, periosteal bone formation is reduced in DHT treated SAMR1 in contrast with SAMP6 (Table 4).

Similarly to DHT, E2 treatment prevents trabecular BMD loss in the femur (133.4 ± 37.4 mg/cm³ in E2 treated orch SAMR1 mice [n=9], P<0.01 versus orch, and 89.2 ± 12.5 mg/cm³ in SAMP6 animals [n=13], P<0.01 versus orch). E2 improves trabecular BMD gain as well as trabecular width and number in the tibia of SAMR1 and SAMP6 mice (Table 2). In orch SAMR1 mice E2 also maintains bone turnover markers at sham level (Table 3). However, in SAMP6 mice, E2 is less effective. Indeed, trabecular BMD loss is not fully prevented, reduced trabecular width not completely avoided and DPD levels were increased as compared to sham level in E2 treated orch SAMP6 mice. As on trabecular bone, E2 also improves cortical bone gain: E2 preserves cortical area and maintains periosteal and endocortical expansion in both tibia and femur of orch SAMR1 mice (Table 4, Figure 11). In addition, E2 stimulates cortical thickness (Figure 11B, Table 4) resulting in 20% and 11% thicker femoral cortex compared to orch and sham SAMR1 mice respectively. In SAMP6 animals, E2 also increases cortical thickness by 11% compared to sham, due to endocortical contraction (Figure 11, Table 4). However, E2 lowers the periosteal and endosteal perimeters compared to sham hereby even partially rescuing the cortical phenotype of SAMP6.
Figure 11: Periosteal (Ps.Pm.) and endosteal perimeter (Ec.Pm.) during the experimental period (A), cortical thickness (B), cortical area (C), polar moment of inertia (D) and calf muscle cross-sectional area (E) at the end of the experimental period as measured by in vivo pQCT in tibia of orchidectomized SAMR1 and SAMP6 mice treated with either DHT (orch + DHT) or E2 (orch + E2). Data are expressed as mean ± SE, n = 8 – 17 mice per group. Panel A: a, P<0.05 Student’s t-test orch + DHT versus orch + E2. Panel B – E: results of 2-way ANOVA of values at 20 weeks are shown in inset. Fisher’s least significant difference multiple comparison test was performed after significant interaction (P<0.05): *, SAMP6 versus respective SAMR1 group; c, orch + E2 versus orch + DHT within genotype. Baseline values are marked in grey.
Bone mass accumulation during puberty determines peak bone mass as well as fracture risk at older age. Genetic background, sex steroid deficiency and muscle loading all affect bone mass accrual and ultimately, bone strength. In this study, trabecular and cortical bone mass acquisition in SAMP6 mice, a senile osteoporosis mouse model, as well as the impact of sex steroid deficiency and replacement during puberty is investigated.

In SAMP6 mice, bone mineral acquisition appears already deficient at early puberty. As early as 4 weeks of age, trabecular BMD is moderately decreased and DPD levels are elevated. In line with some but not all studies, trabecular bone mass also tends to be lower in mature SAMP6 mice. However, bone turnover markers are not significantly lower in 20-week-old SAMP6 in contrast with previous reports of decreased trabecular bone formation.

In addition to these relatively mild changes in trabecular bone, cortical bone of SAMP6 mice is characterized by an excessive medullary expansion and cortical thinning. This results from reduced endosteal bone formation, already apparent in SAMP6 at early puberty. After 8 weeks of age, the endosteal perimeter did not further expand. Furthermore, the endosteal bone formation rate is no longer reduced in mature SAMP6 mice. Therefore, the cortical bone phenotype of SAMP6 mice appears to develop early, even before puberty when endosteal bone formation is highest. In contrast with endosteal bone formation, periosteal bone apposition is not impaired and even enhanced in SAMP6. During the first 8 weeks of age, periosteal bone apposition hereby partially compensates for the excessive medullary expansion in this senile osteoporosis model. The wider cortex also improves bone geometrical properties (as assessed by polar moment of inertia) and balances the well established deficient material properties of SAMP6 mice reported in earlier studies. Interestingly, the cortical bone phenotype of SAMP6, characterized by an early deficit in endosteal bone formation, shows some striking similarities with earlier observations in human case-control studies. Indeed, daughters of female patients with hip fractures have an enlarged femoral neck, suggesting that bone fragility – in humans similar to this senile osteoporosis model - may already become apparent during growth.

In line with earlier studies in rats, early hypogonadism causes a sustained cancellous bone deficit as well as an increase in bone resorption markers in both SAMP6 and SAMR1. Yet, earlier reports in SAMP6 mice could not demonstrate an upregulation of osteoclast formation and activity after orch. These studies however mainly used in vitro evaluations of osteoblasto- and osteoclastogenesis in bone marrow cultures of sham versus orch SAMP6 without prospective in vivo follow-up of bone markers or cancellous bone changes. It is also unlikely that the discrepancy between our findings and these earlier studies is due to the
age at orch, because similar prospective changes in bone markers and cancellous bone mass are seen when mice are orchidectomized at maturity (20 weeks of age, data not shown).

Corresponding to earlier reports, hypogonadism as induced by early orch impairs cortical bone gain in growing mice. Periosteal bone apposition is reduced resulting in smaller and thinner cortices already present 4 weeks after orch. In accordance with earlier cross-sectional studies in orch growing rats, periosteal bone formation was low and no longer significantly reduced at mature age \(^{247}\). However, in contrast, cortical bone deficit induced by orch in mature rodents is mild and induced by increased endosteal bone resorption and not decreased periosteal bone formation \(^{248}\). Therefore, the first weeks of puberty appears to be a critical time window for the development of hypogonadal as well as senile osteoporosis in mice.

In agreement with its well documented anti-resorptive action \(^{84, 249}\), DHT fully prevents the orchidectomy-induced rise in bone resorption and maintains trabecular BMD in both orch SAMR1 and SAMP6 mice. Interestingly, trabecular bone seems even more responsive to DHT in SAMP6 animals, despite similar biological androgen activity (as assessed by seminal vesicle weight). However, androgens and the AR not only maintain trabecular bone mass but also stimulate periosteal bone expansion in growing mice. In accordance, DHT restores periosteal bone expansion, cortical thickness and cortical area in male orch SAMR1 and SAMP6 mice. Interestingly, the effects of DHT on cortical bone mass are only apparent during puberty with no further stimulation (and even reduction in SAMR1) at maturity. Moreover, DHT action is not impaired and even enhanced in SAMP6 compared to SAMR1. Therefore, impairment of androgen action appears not involved in the development of the SAMP6 bone phenotype.

In line with earlier observations, E2 stimulates trabecular bone formation in male mice \(^{250, 251}\). However, stimulation seems less effective in SAMP6 than SAMR1. In agreement, E2 fully restores bone turnover markers in orch SAMR1, but not in SAMP6 mice. It is therefore tempting to speculate that a deficient supply of intramedullary osteoblasts may impair E2 action in trabecular bone in SAMP6. On cortical bone E2 stimulates not only periosteal but also endosteal bone formation, in contrast with DHT. Even more, E2 partially rescues the endocortical cortical deficit which characterizes the SAMP6 phenotype. In addition, endocortical contraction is not observed in E2-treated SAMR1 mice, and does not progress further after 8 weeks in E2-treated SAMP6 mice. Therefore, E2 selectively stimulates net endosteal bone formation between 4 to 8 weeks in animals with severely reduced osteoblast activity such as SAMP6. Similar as seen in DHT-treated mice, these early weeks of puberty therefore appear a critical and vulnerable period not only for endosteal and periosteal bone apposition but also for sex steroid action on
cortical bone acquisition. In this context, and in line with observations in humans, not only androgens but also estrogens appear important for cortical bone growth in males.

Sex steroid deficiency, as well as sex steroid replacement, has not only effects on bone but also on body composition during puberty. Similar as in hypogonadal adolescents, hypogonadism results in increased fat mass and reduced lean mass\textsuperscript{136}. DHT prevents the low lean mass in orch mice, but not the accrual of fat. In contrast, E\textsubscript{2} prevents high fat mass accrual in orch SAMR1 and SAMP6 mice, but is unable to restore the lean mass build-up during growth. These data are in agreement with earlier studies in aromatase deficient mice and AR and ER KO mice showing that AR activation is necessary for optimal acquisition of muscle mass and ER\textalpha{} signaling is involved in lowering fat mass\textsuperscript{183, 184}.

In conclusion, this study shows that bone fragility in both SAMP6 and hypogonadal SAMR1 mice results from deficient bone build-up during early puberty. In SAMP6 mice, deficient endocortical bone formation possibly even present before puberty, results in cortical expansion during growth. In hypogonadal mice on the other hand, reduced periosteal bone expansion results in smaller cortices during the same time window. Moreover, hypogonadism also impairs trabecular bone formation. In addition, this study shows that sex steroids have different effects in a mouse model with intramedullary osteoblast deficiency. Although DHT prevents bone resorption in both mouse models and restores trabecular BMD, it is more effective in SAMP6 animals, suggesting that osteoblast supply is not a limiting factor for this type of anti-resorptive action. However, the action of E\textsubscript{2} on cancellous bone appears impaired in SAMP6 as compared to SAMR1 mice. On cortical bone, DHT and E\textsubscript{2} have similar effects in SAMP6 and SAMR1 animals with E\textsubscript{2} even partially rescuing the failure of endosteal bone addition in SAMP6 mice.
5.1. INTRODUCTION

Androgen action is an important determinant of bone mass acquisition in males. Hypogonadal men have severely reduced BMD and increased risk for fractures. Similarly, castration reduces cortical and trabecular bone growth in rodents (Chapter 4). Androgen therapy rescues bone loss following orch in normal mice, but not in a mouse model with androgen receptor disruption (ARKO). Together with these findings, severely reduced bone mass in mice with defective AR provides compelling evidence that optimal bone mass acquisition in males requires a functional AR.

Physical exercise affects bone mass accrual during growth. According to the mechanostat theory, physical activity creates mechanical stress on bone. Subsequently, bone adaptats to the stress to keep bone strains below a threshold. In line with this concept, several studies have demonstrated that physical activity is positively related with BMD. Both in humans and rodents, exercise intervention studies increase bone mass, at least during growth. In addition, exercise may protect against bone loss induced by gonadectomy. Exercise might therefore potentially be a low-cost bone forming therapy with lifelong benefits to bone strength.

However, the potential interaction of physical exercise with hormonal stimuli, such as androgen action, is unknown and needs further study. The ARKO mouse model provides an opportunity to investigate this interaction. The aim of this study was to explore the bone forming potential of voluntary exercise in the absence and presence of androgen activity.
5.2. EXPERIMENTAL DESIGN

In a separate experiment, the running capacity of WT and ARKO mice was investigated. First, the voluntary running capacity of WT and ARKO mice was investigated from 5 to 16 weeks of age. At weaning (3 weeks of age), male WT and ARKO littermates were placed in a cage with a running wheel for a training period of 2 weeks. At 5 weeks of age, both WT and ARKO mice were put in a cage with a running wheel and running was measured till 16 weeks of age. In addition the endurance capacity of 16-week-old sedentary WT and ARKO mice was investigated by an \textit{in vivo} treadmill running experiment. Since ARKO and WT mice had different running capacity, the effects of voluntary running on bone and muscle was only investigated in ARKO mice.

At weaning (3 weeks of age), male WT and ARKO littermates were placed in a cage with a running wheel for a training period of 2 weeks. At 5 weeks of age, WT mice were put in a cage without a running wheel and ARKO mice were randomly divided in 2 groups: either assigned to a cage with (running group) or without (control group) a running wheel (1 animal/cage) (8-10 animals/group). The ARKO running group was able to run voluntarily with free access to the running wheel 24 h/day from 5 to 16 weeks of age. Food consumption was monitored weekly throughout the entire experiment. \textit{In vivo} pQCT of the tibia was performed and body weight was measured at 5, 8, 12, and 16 weeks of age. At 16 weeks of age, whole body DEXA was performed and mice were put in metabolic cages to collect urine for measurement of collagen cross-links. At sacrifice, serum was collected and used for osteocalcin measurement. Left musculus quadriceps was dissected and weighed. Left femur was dissected and used to perform µCT.
5.3. RESULTS

5.3.1. EFFECT OF AR DISRUPTION ON BONE AND BODY CHARACTERISTICS

Sedentary ARKO mice gained less body weight from 5 to 16 weeks of age compared to WT mice (Figure 12A), due to a lower lean body mass (-17%) without change in fat mass (Table 6). Accordingly, control ARKO mice had reduced calf muscle cross-sectional area and quadriceps weight (Figure 12B, Table 6). Food intake was 12% lower in control ARKO compared to WT mice (Figure 13).

In addition, compared to WT, sedentary ARKO mice achieved a smaller bone size with reduced periosteal and endosteal perimeter, and significantly lower cortical area and cortical thickness from 8 weeks of age on (Figure 12C-F). Linear regression analysis was performed to investigate the correlation between muscle cross-sectional area and bone strength (estimated by SSI), as well as the effect of AR disruption on this correlation. Linear regression analysis showed that the SSI was positively related with the calf muscle cross-sectional area in 16-week-old control WT mice (Figure 13). AR disruption significantly influenced the relationship since no correlation between SSI and calf muscle cross-sectional area could be detected in ARKO mice ($R^2=0.76$, $P<0.01$) (Figure 13, dashed line).

Trabecular bone mass was significantly lower in sedentary ARKO mice at all time points (Figure 12G) and associated with elevated levels of osteocalcin and DPD (Table 7).

Table 6: Body composition and food consumption in 16-week-old WT and ARKO mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ARKO</th>
<th>ARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con</td>
<td>run</td>
<td></td>
</tr>
<tr>
<td><strong>Lean body mass</strong></td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.20 ± 0.55</td>
<td>17.6 ± 0.43 $^a$</td>
<td>18.03 ± 0.13</td>
</tr>
<tr>
<td><strong>Quadriceps</strong></td>
<td>(mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>114.7 ± 19.9</td>
<td>66.0 ± 2.2 $^a$</td>
<td>83.0 ± 12.0</td>
</tr>
<tr>
<td><strong>Quadriceps/weight</strong></td>
<td>(mg/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.2 ± 0.7</td>
<td>2.8 ± 0.1 $^a$</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td><strong>Total body fat mass</strong></td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.20 ± 0.55</td>
<td>5.82 ± 0.40</td>
<td>5.15 ± 0.25</td>
</tr>
<tr>
<td><strong>Food consumption</strong></td>
<td>(g/week)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>30.22 ± 0.66</td>
<td>26.45 ± 0.32 $^a$</td>
<td>33.14 ± 0.71 $^b$</td>
</tr>
</tbody>
</table>

5-week-old male WT and ARKO mice were randomly divided into a sedentary (con) and voluntary wheel running (run) group. Body composition and food consumption were measured at 16 weeks of age. Values are expressed as mean ± SE. $^a$ $P<0.05$ ARKO con vs. WT con, $^b$ $P<0.05$ run vs. con. (n = 8-10 mice/group).
Figure 12: Longitudinal follow-up of (A) body weight, (B) muscle cross-sectional area, and (C) periosteal perimeter, (D) endosteal perimeter, (E) cortical thickness, (F) cortical area and (G) trabecular BMD of the tibia from 5 till 16 weeks of age, as measured by in vivo pQCT in male WT (n=7) and ARKO control mice (n=8). Values are expressed as mean ± SE. *P<0.05 WT versus ARKO at respective time points.
Figure 13: Linear regression analysis of the strength strain index (mm$^3$) in 16-week-old male WT and ARKO control mice with calf muscle cross-sectional area (mm$^2$) and genotype (WT or ARKO) as independent variables. The model was highly significant ($P<0.01$) and $R^2=0.76$.

Table 7: Biochemical markers of bone turnover in WT and ARKO at 16 weeks of age

<table>
<thead>
<tr>
<th></th>
<th>WT (ng/ml)</th>
<th>ARKO (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteocalcin</td>
<td>35.7 ± 3.1</td>
<td>57.0 ± 3.4$^a$</td>
</tr>
<tr>
<td>Deoxypyridinoline</td>
<td>20.8 ± 1.1</td>
<td>28.5 ± 1.6$^a$</td>
</tr>
</tbody>
</table>

5-week-old male WT and ARKO mice were randomly divided into a sedentary (con) and voluntary wheel running (run) group. Values are expressed as mean ± SE. $^a$ P<0.05 ARKO con vs. WT con, $^b$ P<0.05 run vs. con. (n = 8-10 mice/group).

5.3.2. EFFECT OF AR DISRUPTION ON RUNNING ACTIVITY

AR disruption had a significant impact on voluntary wheel running (Figure 14). At all time points, distance, running time as well as average speed was much lower in ARKO mice compared to WT. In contrast, in a forced treadmill experiment, the endurance capacity was significantly (+53%) higher in ARKO mice compared to WT mice (Figure 15).
Figure 14: Effect of AR disruption on voluntary running capacity. Average daily (A) distance, (B) running time and (C) speed ran by WT (n=8) and ARKO (n=9) mice in a cage with running wheel. Values are expressed as means ± SE. * P<0.05 compared with WT.
and their corresponding male WT mice (n=11) was measured in a treadmill experiment. Values are expressed as means ± SE. * P<0.05 compared with WT.

Figure 15: Effect of AR disruption on endurance. Time till exhaustion (in minutes) of male ARKO (n=10) and their corresponding male WT mice (n=11) was measured in a treadmill experiment. Values are expressed as means ± SE. * P<0.05 compared with WT.

5.3.3. EFFECT OF VOLUNTARY WHEEL RUNNING ON BONE

Although food consumption increased (Table 6), voluntary running did not influence body weight (22.9 ± 0.3 g; n=8). Total body fat mass (Table 6), lean mass (Table 6), quadriceps weight (Table 6) and calf muscle cross-sectional area (25.95 ± 0.34 mm²; n=8) are also unchanged in ARKO mice after voluntary running.

Similarly, voluntary running did not affect cortical bone gain in ARKO mice (n=8). Periosteal perimeter (4.258 ± 0.026 mm), endosteal perimeter (3.125 ± 0.043 mm), cortical thickness (180 ± 4 mm²), cortical area (0.67 ± 0.01 mm²) and SSI (0.19 ±0.01 mm³) were not significantly different between ARKO controls and ARKO runners.

Voluntary running changed trabecular bone mass in ARKO mice. Trabecular BMD of the tibia was 14% higher after voluntary running (Table 8). In line with these findings, µCT analysis showed a 31% increase in trabecular bone volume in the femur (Table 8). The enhanced trabecular bone volume resulted from an increase in trabecular number, without difference in trabecular thickness (Table 8). Further evidence for an anti-resorptive effect of voluntary running on trabecular bone in ARKO mice was provided by markers of bone remodeling, with reduced levels of osteocalcin and DPD compared to WT controls (Table 7).
Table 8: Trabecular bone characteristics in 16-week-old ARKO mice

<table>
<thead>
<tr>
<th></th>
<th>ARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>con</td>
</tr>
<tr>
<td>Trabecular bone mineral density (mg/cm³)</td>
<td>84.4 ± 2.8</td>
</tr>
<tr>
<td>Percentage bone volume (%)</td>
<td>3.56 ± 0.29</td>
</tr>
<tr>
<td>Trabecular thickness (µm)</td>
<td>37.52 ± 1.36</td>
</tr>
<tr>
<td>Trabecular number (1/mm)</td>
<td>0.944 ± 0.062</td>
</tr>
</tbody>
</table>

5-week-old male ARKO mice were randomly divided into a sedentary (con) and voluntary wheel running (run) group. Trabecular bone characteristics were measured at 16 weeks of age. Values are expressed as mean ± SE. a P<0.05 Student’s t-test con versus run. (n = 7-9 mice/group)
Physical exercise might potentially be a convenient strategy to optimize bone strength during growth. In this longitudinal study in growing pubertal ARKO mice, we evaluated the effect of voluntary wheel running on mineral acquisition in the absence of androgen action.

Disruption or even a delay of androgen action during puberty represents one of the most serious threats for male skeletal bone mineral accumulation, both in humans and in rodents. In this study, not only bone mass acquisition was reduced, but also body weight and muscle gain were severely impaired in ARKO mice compared to WT controls. Moreover, the SSI – a measure of biomechanical competence – was found to be positively related with the calf muscle cross-sectional area in WT mice. Therefore, it is tempting to speculate that this association may be due to the muscle-associated increase in skeletal loading, which needs to be fully matched by an appropriate adaptation of bone strength (as reflected by the SSI). Absence of androgen action reduced the gain in muscle and impaired skeletal development. Compared to WT, no correlation between SSI and calf muscle cross-sectional area could be found in ARKO mice. These findings suggest that androgens may have a direct anabolic effect on bone strength, independently from their well-documented effects on muscle growth.

Both cortical and trabecular bone mass gain and maintenance were severely reduced in ARKO compared to WT mice. In line with earlier findings, bone turnover parameters were increased in sedentary ARKO mice, reflecting higher cancellous bone remodeling activity. Taken together, these data support the concept that androgens are not only essential to optimize cortical bone mineral acquisition, but even more so for the development and integrity of the cancellous bone network during growth.

An unexpected observation in our study was the severely reduced voluntary wheel running intensity in ARKO compared to WT mice. This observation is in line with earlier findings showing decreased spontaneous activity in ARKO mice. Intriguingly however, this lower voluntary running performance was not reflected by a reduced endurance capacity as measured by a forced treadmill running experiment or changes in muscle function (see Chapter 6). These data suggest that the lower voluntary running intensity, in line with the reduced food consumption in ARKO mice, is due to androgen-related behavioral changes, rather than to differences in running capacity.

In our study voluntary wheel running did not increase puberty-associated cortical bone gain in ARKO mice. This is in agreement with data in humans where high impact physical exercise was more effective than running. Less intense voluntary running in ARKO mice did not impair
body weight gain or muscle mass in androgen disrupted mice and, consequently, did not affect cortical perimeters, thickness or SSI.

Of potential interest from a clinical perspective, moderate physical activity was found to partly prevent trabecular bone loss in ARKO mice. Markers of bone remodeling were reduced in running compared to control ARKO mice, suggesting that moderate physical activity may reduce bone resorption and prevent trabecular bone loss in the context of androgen deficiency. In line with this concept, running was shown to reduce trabecular bone loss and resorption following orchidectomy in rodents in some studies but not all studies. Overall, our data suggest that ARKO mice remain responsive to the beneficial effects of running therapy. It is tempting to speculate that, in hypogonadal men and adolescents, moderate running might potentially also reduce bone turnover and partly prevent osteoporosis.

In conclusion, puberty represents a critical and vulnerable period during which androgen action, muscle mass gain, and physical exercise have both independent and interactive effects on trabecular and cortical bone compartments. In this study, we showed that androgens are an important determinant of cortical and trabecular bone mass. In addition, voluntary physical exercise may reduce bone resorption and prevent trabecular bone loss in the context of androgen deficiency-induced excessive bone turnover.
CHAPTER 6: THE ROLE OF THE AR IN MUSCLE: STUDY IN MOUSE MODELS WITH MUSCLE-SPECIFIC AND UBIQUITOUS ANDROGEN RECEPTOR DISRUPTION

6.1. INTRODUCTION

As life expectancy increases, strategies to reduce age-related sarcopenia receive increasing attention since loss of muscle mass is considered to be an important cause of falls, skeletal fractures and disability in the elderly. Because serum T levels in men decline with age, T supplementation has been considered as a therapeutic intervention to reduce age-related muscle atrophy. Furthermore, many chronic illnesses, such as cardiac or renal failure, chronic obstructive pulmonary disease, neurodegenerative diseases as well as HIV infection, are associated with muscle wasting and impaired muscular functional capacity. It is the prevailing opinion that anabolic therapy, such as the administration of androgens, could improve clinical outcome and quality of life in these patient groups.

The potential of androgens to stimulate muscle hypertrophy is well documented. For instance, T replacement after suppression of endogenous T by a GnRH agonist resulted in a dose-related increase in muscle mass and strength in young as well as older eugonadal men. Furthermore, in hypogonadal men or orchidectomized rats, androgen treatment was found to reverse the detrimental effects of androgen deficiency on muscle volume. Still, some important issues with regard to the clinical and therapeutical potential of androgen therapy remain unanswered.

To date, the mechanism of androgen action in muscle cells is incompletely understood. For instance, it is still uncertain to what extent the anabolic effects of androgens are mediated directly through the AR. In this regard, both mice and humans with a functionally inactive AR have lower lean body mass. In rodent and human skeletal muscle, however, the AR is expressed in various cell types including mesenchymal stem cells, satellite cells, myonuclei and fibroblasts. It has been shown that the AR mediates in vitro myoblast differentiation and myogenic differentiation of pluripotent stem cells. Furthermore, data from in vivo and in vitro studies indicate that T regulates AR number in muscle cells. Thus, T action via an AR-mediated pathway may play a role in the modulation of muscle phenotype.

To further explore the physiological role of androgens in muscle, we developed a mouse model with post-mitotic myocyte-specific loss of the AR (myocyte-specific AR knockout or mARKO mice) using Cre/loxP technology. This model allows us to investigate the physiological role of AR
activation selectively in myocytes. In addition, the role of the AR in muscle development and performance was studied in mice with ubiquitous AR deletion.
6.2. EXPERIMENTAL DESIGN

6.2.1. CREATION AND MUSCLE CHARACTERIZATION OF THE MYOCYTE-SPECIFIC ANDROGEN RECEPTOR KNOCKOUT MOUSE

Myocyte-specific inactivation of the AR was obtained by crossing female AR<sup>−/−</sup> mice (CD1 background) with transgenic mice expressing the Cre recombinase under the control of the MCK promoter (MCK-Cre<sup>+/−</sup>) (C57BL/6J genetic background). Male mice with a myocyte-specific knockout of the AR (MCK-Cre<sup>+/−</sup>;AR<sup>−/−</sup>/Y or mARKO) were compared with their WT littermates (MCK-Cre<sup>−/−</sup>;AR<sup>−/−</sup>/Y or mWT). No statistically significant differences were observed between mWT and other potential controls from the breeding experiments (MCK-Cre<sup>−/−</sup>;AR<sup>−/−</sup>/Y or MCK-Cre<sup>+/−</sup>;AR<sup>−/−</sup>/Y) for body weight, total body fat mass and total body lean mass (measured by in vivo DEXA), bone parameters (measured by in vivo pQCT), heart weight, muscle weights of quadriceps, gastrocnemius and LA, and weights of intra-abdominal and subcutaneous fat pads.

At 16 weeks of age mARKO and mWT mice were weighed and sacrificed after in vivo DEXA and pQCT measurement. On femur was dissected for μCT measurement. In addition, different fat pads (perigonadal, perirenal, mesenteric, inguinal and nuchal white adipose tissue) and muscles (soleus, EDL, quadriceps, gastrocnemius, LA and heart) were dissected and weighed. One soleus and EDL was used for muscle contractile properties and western immunoblotting, and one was used for succinate dehydrogenase activity, fiber typing and satellite cell number determination. Muscle glycogen content was determined on gastrocnemius. Immunohistochemistry was performed on LA.

In a different experiment, the endurance of 16-week-old mARKO and mWT mice was tested by an endurance treadmill experiment.

6.2.2. MUSCLE CHARACTERISTICS OF THE UBIQUITOUS ANDROGEN RECEPTOR KNOCKOUT MOUSE

To have a clearer image on the role of the AR for muscle development, also mice with ubiquitous AR deletion were investigated. To this end, 22-week-old male AR<sup>−/−</sup>/Y (ARKO) and AR<sup>+/−</sup>/Y (WT) littermates (C57BL/6J genetic background) were weighed and sacrificed. Soleus and EDL were dissected, weighed and used for measurement of muscle contractile properties, succinate dehydrogenase activity and fiber typing.
6.3. RESULTS

6.3.1. MARKO MICE SHOW SPECIFIC LOSS OF THE AR IN MYOCYTES

The efficacy and specificity of the myocyte-specific AR inactivation was assessed at the DNA and protein level. Correct excision of the floxed AR exon 2 was demonstrated by PCR analysis of muscle-extracted DNA from 16-week-old male MCK-Cre+;AR<sup>lox/lox</sup> (mARKO) mice. As expected, DNA extraction of striated muscles such as soleus, EDL, quadriceps, gastrocnemius, LA and heart, demonstrated both the excised and floxed allele of the AR, revealing bands of 404 and 952 bp corresponding with the expected excision of exon 2 of the AR in myocytes and the expected presence of the floxed allele in other cell types (such as fibroblasts, adipocytes, blood vessels or nerve cells) present in muscle tissue, respectively. PCR on tissue from tibia, brain, kidney, liver, intestine, testis and intra-abdominal white adipose tissue did not express the excised allele of the AR, but only the floxed AR band of 952 bp. Biological androgen action, as assessed by seminal vesicle weight, was similar in mARKO and mWT mice (334.4 ± 10.9 mg in mARKO [n=13] and 328.5 ± 9.9 mg in mWT mice [n=22]; P=0.71). These data indicate specific AR excision in striated muscle.

Western immunoblotting revealed an 88% decrease in AR protein level in homogenates of soleus muscle of mARKO mice (n=5) as compared to mWT (n=6) (P<0.01) (Figure 16A). Markedly higher AR expression levels, but a less pronounced decrease (54%) was observed in a homogenate of the LA, a highly androgen-responsive perineal muscle. Further immunohistochemical analysis of the LA from male mWT mice (n=3) revealed that 73 ± 2 % of all nuclei inside the basal lamina and 31 ± 1 % of the nuclei of interstitial cells stained positive for the AR (Figure 16B, C). In the LA muscle from mARKO mice (n=3) the percent AR positive nuclei inside the basal lamina dropped to 28 ± 2 % (P<0.01) (an estimated 61% decrease) whereas the number of AR positive nuclei outside the basal lamina were unaffected (32 ± 0.3 %) (P=0.47). It may be noted that not only myocytes, but also myogenic satellite cells are located within the basal lamina (Figure 17). Immunohistochemistry showed that on average 6% of the myofibers (6 ± 1 % in soleus and 6 ± 1 % in EDL muscles of mWT mice; n=5) stained positive for satellite cells. MCK-driven AR ablation altered the number of satellite cells neither in soleus (6 ± 1 %) nor in the EDL (6 ± 2 %) of mARKO mice (n=4).

To evaluate the in vivo functional consequences of AR ablation in myocytes, a treadmill running experiment was set up. Endurance capacity was not significantly different between mARKO and mWT, although there was a trend towards a lower capacity in mARKO (79.1 ± 12.3 min till exhaustion in mARKO mice [n=10] versus 122.4 ± 21.5 min in mWT [n=8], P=0.08)
Figure 16: Androgen receptor protein expression in the mARKO mouse model. AR protein expression was investigated by western immunoblotting of soleus and LA, and immunohistochemistry of the LA. Western immunoblotting of the AR and α-tubulin protein levels were investigated on total protein extracts from soleus of mARKO (n=5) and mWT littermates (n=6) at 16 weeks of age, and compared with protein extracts from the LA (panel A). Confocal microscopy of AR staining (brown) (panel B), and corresponding basal lamina (red fluorescent) and nuclei (blue fluorescent) staining (panel C) of LA muscle cross-section of 16-week-old mARKO and mWT littermates showing loss of AR immunostaining inside, but not outside basal lamina. Nuclei within the basal lamina are indicated by a black arrow, interstitial nuclei by a white arrow. Scale bar, 25 µm.
6.3.2. BODY COMPOSITION OF MARKO MICE

Male mARKO mice showed an interesting change in body composition compared with their mWT littermates. Despite similar food intake (39.2 ± 2.1 g [n=6] in mARKO as compared to 40.0 ± 4.3 g per week in mWT mice [n=5]; \( P=0.63 \)), mARKO mice had lower body weight than mWT mice (Table 9). However, myocyte-specific AR ablation did not alter total heart weight or the weight of the quadriceps, gastrocnemius or soleus (Table 10). In fact, from all muscles studied, only weights of LA (-46%) and EDL (-13%) were lower in mARKO than in mWT mice (Table 10). Still, whole body lean mass, but not percent lean body mass, was significantly reduced in mARKO as compared with mWT littermates (Table 9).
Table 9: Effect of myocyte-specific AR disruption on in vivo body composition.

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
<th>mARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total body weight (g)</td>
<td>36.5 ± 0.7</td>
<td>33.4 ± 0.8*</td>
</tr>
<tr>
<td>Total lean body mass (g)</td>
<td>27.5 ± 0.6</td>
<td>25.2 ± 1.1*</td>
</tr>
<tr>
<td>Total body fat mass (g)</td>
<td>7.6 ± 0.5</td>
<td>5.5 ± 0.6*</td>
</tr>
<tr>
<td>Lean body mass (% of total)</td>
<td>78.5 ± 1.0</td>
<td>82.1 ± 1.7*</td>
</tr>
<tr>
<td>Body fat mass (% of total)</td>
<td>21.5 ± 1.0</td>
<td>17.9 ± 1.7*</td>
</tr>
</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) (n=8) and wildtype littermates (mWT) (n=18) were studied. Data provided are expressed as means ± SE. Student's t-test: * P<0.05 mARKO versus mWT littermates.

Table 10: Effect of myocyte-specific AR disruption on muscle mass.

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
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</tr>
</thead>
<tbody>
<tr>
<td>heart (mg)</td>
<td>160.2 ± 5.3</td>
<td>149.8 ± 5.9</td>
</tr>
<tr>
<td>quadriceps (mg)</td>
<td>190.2 ± 6.6</td>
<td>187.5 ± 6.0</td>
</tr>
<tr>
<td>gastrocnemius (mg)</td>
<td>157.0 ± 3.1</td>
<td>154.8 ± 6.0</td>
</tr>
<tr>
<td>soleus (mg)</td>
<td>9.2 ± 0.4</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>extensor digitorum longus (mg)</td>
<td>10.3 ± 0.4</td>
<td>8.9 ± 0.4*</td>
</tr>
<tr>
<td>levator ani (mg)</td>
<td>22.5 ± 1.7</td>
<td>12.2 ± 1.2*</td>
</tr>
</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) (n=7-13) and wildtype littermates (mWT) (n=7-22) were studied. Data provided are expressed as means ± SE. Student's t-test: * P<0.05 mARKO versus mWT littermates.

Unexpectedly, also total body fat mass and percent body fat mass was reduced in mARKO mice, primarily due to a lower amount of intra-abdominal (perigonadal, mesenteric, perirenal) fat tissue (Table 9, Table 11). Subcutaneous (inguinal and nuchal) fat pads were similar in mARKO and mWT littermates (Table 11).
Table 11: Effect of myocyte-specific AR disruption on fat mass.

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
<th>mARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-abdominal white fat (mg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>perigonadal fat</td>
<td>565.3 ± 61</td>
<td>374.1 ± 26.6*</td>
</tr>
<tr>
<td>perirenal fat</td>
<td>191.8 ± 24.2</td>
<td>109.4 ± 10.9*</td>
</tr>
<tr>
<td>mesenteric fat</td>
<td>234.6 ± 21.8</td>
<td>168.7 ± 19.4*</td>
</tr>
<tr>
<td><strong>Subcutaneous white fat (mg)</strong></td>
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<tr>
<td>Inguinal fat</td>
<td>238.6 ± 20.8</td>
<td>201.2 ± 13.7</td>
</tr>
<tr>
<td>Nuchal fat</td>
<td>146.3 ± 15.7</td>
<td>104.0 ± 6.8</td>
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</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) (n=7-13) and wildtype littermates (mWT) (n=7-22) were studied. Data provided are expressed as means ± SE. Student’s t-test: * P<0.05 mARKO versus mWT littermates.

Myocyte-specific AR ablation did not affect bone mass. Compared to mWT, in mARKO mice trabecular bone (bone volume per total volume, trabecular thickness and trabecular number) and cortical bone parameters (cortical area, cortical thickness, periosteal perimeter and endosteal perimeter) measured in femur with ìCT were not different (Table 12). Also trabecular bone density and cortical bone parameters measured *in vivo* with pQCT on tibia were not significantly different between 16-week-old mARKO and mWT littermates (Table 13).
Table 12: Effect of myocyte-specific AR disruption on trabecular and cortical bone as assessed *ex vivo* by µCT on femur.

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
<th>mARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trabecular bone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>15.72 ± 0.95</td>
<td>17.34 ± 1.11</td>
</tr>
<tr>
<td>Tb.Th (µm)</td>
<td>46.93 ± 0.93</td>
<td>49.27 ± 0.99</td>
</tr>
<tr>
<td>Tb.N (1/mm)</td>
<td>3.34 ± 0.17</td>
<td>3.51 ± 0.18</td>
</tr>
<tr>
<td><strong>Cortical bone</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct.Ar (mm²)</td>
<td>0.78 ± 0.02</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>Ct.Th (µm)</td>
<td>163.6 ± 3.8</td>
<td>170.8 ± 4.0</td>
</tr>
<tr>
<td>Ps.Pm (µm)</td>
<td>5719 ± 134</td>
<td>5733 ± 122</td>
</tr>
<tr>
<td>Ec.Pm (µm)</td>
<td>5365 ± 210</td>
<td>5448 ± 207</td>
</tr>
</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) (n=7) and wildtype littermates (mWT) (n=7) were studied. BV/TV: trabecular bone volume; Tb.Th: trabecular thickness; Tb.N: trabecular number; Ct.Ar: cortical area; Ct.Th: cortical thickness; Ps.Pm: periosteal perimeter; Ec.Pm: endocortical perimeter. Data provided are expressed as means ± SE. Student’s t-test: *P<0.05* mARKO versus mWT littermates. No significant differences between mARKO and mWT littermates were observed.

Table 13: Effect of myocyte-specific AR disruption on trabecular and cortical bone as assessed *in vivo* by pQCT.

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
<th>mARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trabecular BMD (mg/cm³)</strong></td>
<td>237.0 ± 18.1</td>
<td>205.6 ± 7.6</td>
</tr>
<tr>
<td>Ct.Ar (mm²)</td>
<td>0.94 ± 0.02</td>
<td>0.91 ± 0.04</td>
</tr>
<tr>
<td>Ct.Th (µm)</td>
<td>238 ± 4</td>
<td>235 ± 7</td>
</tr>
<tr>
<td>Ps.Pm (mm)</td>
<td>4.71 ± 0.06</td>
<td>4.61 ± 0.11</td>
</tr>
<tr>
<td>Ec.Pm (mm)</td>
<td>3.21 ± 0.04</td>
<td>3.13 ± 0.09</td>
</tr>
</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) (n=8) and wildtype littermates (mWT) (n=18) were studied. Trabecular BMD: trabecular bone mineral density; Ct.Ar: cortical area; Ct.Th: cortical thickness; Ps.Pm: periosteal perimeter; Ec.Pm: endocortical perimeter. Data provided are expressed as means ± SE. Student’s t-test: *P<0.05* mARKO versus mWT littermates. No significant differences between mARKO and mWT littermates were observed.
6.3.3. MUSCLE FIBER CHARACTERISTICS OF MARKO MICE

The fiber composition of soleus and EDL was investigated by immunohistochemistry using specific antibodies directed against MHC type I and type IIa, respectively (Figure 18). Soleus muscles from mARKO mice contained about 10% more type I fibers, versus 10% less type IIa fibers than the corresponding mWT littermates (Table 14). Mean cross-sectional areas of type I and type IIa fibers were similar between mARKO and mWT (Table 14). However, due to the different fiber proportions, the total surface area of type I fibers was greater in mARKO mice, whereas the surface area of type IIa fibers was smaller compared to mWT (Table 14). The number of type IIb/x fibers in soleus was too small to allow a valid quantitative analysis.

In EDL, the proportion of type IIa and type IIb/x fibers was not significantly different between mARKO and mWT (Table 14). Also, the number and mean fiber cross-sectional area of both IIa and IIb/x fibers (Table 14), as well as the fraction of muscle cross-sectional area covered by either fiber type, were not different between mARKO and their mWT littermates (Table 14). The number of type I fibers in EDL was negligible and too small to allow a valid quantitative analysis.

Figure 18: Representative image of muscle fiber type identification by immunofluorescence microscopy in soleus from mARKO and mWT mice. Type I, type IIa and type IIb/x fibers were identified by immunofluorescence microscopy as explained in the material and methods section. Cross-sections from soleus are shown for mARKO mice (panel B) and their mWT male littermates (panel A). Type I fibers are stained green whereas type IIa fibers are stained blue. There were no type IIb/x fibers. On average 100 ± 4 fibers per muscle section were analyzed for each mouse. Notice the higher amount of type I fibers and the lower amount of type IIa fibers in mARKO mice as compared to their mWT littermates.
Table 14: Effect of myocyte-specific AR disruption on fiber type characteristics in mouse soleus and extensor digitorum longus.

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
<th>mARKO</th>
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</thead>
<tbody>
<tr>
<td><strong>SOLEUS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of fibers (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>36.1 ± 1.9</td>
<td>45.7 ± 4.0 $^*$</td>
</tr>
<tr>
<td>Type Ia</td>
<td>62.2 ± 1.8</td>
<td>51.7 ± 3.6 $^*$</td>
</tr>
<tr>
<td><strong>Mean fiber cross-sectional area (µm²)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>4945 ± 356</td>
<td>5735 ± 357</td>
</tr>
<tr>
<td>Type Ia</td>
<td>4678 ± 428</td>
<td>5220 ± 441</td>
</tr>
<tr>
<td><strong>Total surface area (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>37 ± 2</td>
<td>48 ± 3 $^*$</td>
</tr>
<tr>
<td>Type Ia</td>
<td>60 ± 2</td>
<td>50 ± 3 $^*$</td>
</tr>
<tr>
<td><strong>EDL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of fibers (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type Ia</td>
<td>11.4 ± 2.7</td>
<td>10.5 ± 3.8</td>
</tr>
<tr>
<td>Type Iib/x</td>
<td>88.6 ± 2.7</td>
<td>89.5 ± 3.8</td>
</tr>
<tr>
<td><strong>Mean fiber cross-sectional area (µm²)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type Ia</td>
<td>1594 ± 274</td>
<td>1875 ± 373</td>
</tr>
<tr>
<td>Type Iib/x</td>
<td>5436 ± 240</td>
<td>6025 ± 460</td>
</tr>
<tr>
<td><strong>Total surface area (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type Ia</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Type Iib/x</td>
<td>96 ± 1</td>
<td>96 ± 1</td>
</tr>
</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) (n=8) and wildtype littermates (mWT) (n=6) were studied. The proportion and fiber size of type I, IIA and Iib/x fibers was determined by immunofluorescence microscopy on muscle cross-sections incubated with specific antibodies directed against myosin heavy chains type I and IIA. On average 100 ± 4 fibers per muscle section for each mouse were analyzed. Data provided are expressed as means ± SE. Student’s t-test: $^*$ P<0.05 mARKO versus mWT littermates; $^*$ P=0.06 mARKO versus mWT littermates.
Muscle glycogen content reflects the magnitude of muscle energy store in the form of carbohydrates. Glycogen content was 28.6 ± 3.6 mmol.kg wet weight in mARKO mice (n=7) and was similar in mWT mice (32.4 ± 6.1 mmol.kg wet weight; n=8) (P=0.59). Therefore, myocyte-specific AR ablation did not alter muscle glycogen concentration.

Succinate dehydrogenase activity was measured fiber-specifically as an index of muscle oxidative capacity. As expected, succinate dehydrogenase activity was significantly higher in type IIa than in type I fibers in soleus (P<0.01) and on average 2-fold higher than IIb/x fibers in EDL (P<0.01) of mARKO and mWT mice (Table 15). However, no difference in succinate dehydrogenase activity between mARKO and mWT was observed (Table 15).

Table 15: Effect of myocyte-specific AR disruption on fiber-specific succinate dehydrogenase activity in soleus and extensor digitorum longus muscle.

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
<th>mARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLEUS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>5 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Type IIa</td>
<td>8 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>EDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIa</td>
<td>9 ± 1</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Type IIb/x</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) and wildtype littermates (mWT) were studied (n=6-9). Succinate dehydrogenase activity in type I, type IIa and type IIb/x fibers was determined by histochemical staining. Staining intensity was quantified as arbitrary units. Data provided are expressed as means ± SE. No significant differences between mARKO and mWT littermates were observed.

6.3.4. MUSCLE CONTRACTILE PROPERTIES OF MARKO MICE

Resistance of muscles to contraction-induced fatigue was assessed in incubated soleus muscle during 10 min of intermittent electrical stimulation (Figure 19). Tension drop and lengthening of relaxation time, are typical features of muscle fatigue. Initial muscle tension was 199 ± 9 mN/mm² versus 214 ± 10 mN/mm² in mARKO (n=7) and mWT (n=4), respectively (P=0.31). As shown in Figure 19, from the start to the end of the stimulation protocol muscle tension
development in mWT muscles decreased by ±50%, whilst half relaxation time increased by ±60%. In mARKO mice, the fatigue-induced lengthening of relaxation time was largely unchanged as compared to mWT. Also, muscle tensions were similar between mARKO and mWT littermates.

The potential of muscles for maximal tension development was measured in both soleus and EDL during twitch and maximal tetanic stimulation. In mARKO mice, peak twitch and tetanic tensions were affected neither in soleus nor in EDL muscles (Table 16). Furthermore, rates of muscle contraction and relaxation as well as force-frequency curves were similar between mARKO and mWT in either muscle type (data not shown).

Figure 19: Effect of myocyte-specific AR deletion on fatigability of muscle tension and relaxation time in soleus of 16-week-old mARKO (n=7) and mWT (n=4) mice. Muscle tension (panel A) and half relaxation time (panel B) was measured in incubated soleus muscle electrically stimulated to contract during 10 min of supramaximal intermittent tetanic stimulation. Data provided are expressed as means ± SE. * P=0.04 mARKO versus mWT.
Table 16: Effect of myocyte-specific AR disruption on maximal muscle tension in soleus and extensor digitorum longus

<table>
<thead>
<tr>
<th></th>
<th>mWT</th>
<th>mARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLEUS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twitch</td>
<td>33.9 ± 3.9</td>
<td>27.0 ± 2.0</td>
</tr>
<tr>
<td>Tetanic</td>
<td>237 ± 10</td>
<td>220 ± 10</td>
</tr>
<tr>
<td>EDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twitch</td>
<td>25.0 ± 3.3</td>
<td>30.2 ± 2.5</td>
</tr>
<tr>
<td>Tetanic</td>
<td>269 ± 18</td>
<td>296 ± 11</td>
</tr>
</tbody>
</table>

16-week-old male myocyte-specific AR knockout (mARKO) (n=6) and wildtype littermates (mWT) (n=4) were studied. Twitch and tetanic peak tensions (mN/mm²) were measured in incubated soleus and EDL muscles electrically stimulated to contract. Data provided are expressed as means ± SE. No significant differences between mARKO and mWT littermates were observed.

6.3.5. MUSCLE CHARACTERISTICS OF ARKO MICE

Next, the effect of ubiquitous AR deletion on muscle was investigated. ARKO mice have complete absence of AR protein (data not shown). This ubiquitous AR deletion resulted in lower body weight (25.0 ± 0.9 g in ARKO versus 27.6 ± 0.6 g in WT, P=0.04) and EDL muscle mass (9.51 ± 0.31 mg in ARKO versus 10.88 ± 0.43 mg in WT, P=0.02), whilst soleus muscle mass was similar (9.64 ± 0.36 mg in ARKO versus 9.94 ± 0.28 mg in WT, P=0.52). The LA was not detectable in ARKO mice.

Muscle fiber typing showed that soleus muscles from ARKO mice contained about 10% more type I fibers, versus 10% less type IIa fibers than the corresponding WT littermates (Table 17). Since mean cross-sectional areas of type I and type IIa fibers were similar between ARKO and WT (Table 17), the different fiber proportions resulted in higher total surface area of type I fibers in ARKO, and smaller surface area of type IIa fibers (Table 17). The number of type IIb/x fibers in soleus was too small to allow a valid quantitative analysis. Succinate dehydrogenase activity was not different in soleus of ARKO and WT mice (Table 18).

In EDL, the fiber proportion, mean fiber cross-sectional area as well as the fraction of muscle cross-sectional area covered by type IIa and type IIb/x fibers is not significantly different between ARKO and WT (Table 17). The number of type I fibers in EDL was negligible and too small to allow a valid quantitative analysis. As in soleus, succinate dehydrogenase activity of EDL was not different between ARKO and WT mice (Table 18).

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Table 17: Effect of AR disruption on fiber type characteristics in mouse soleus and extensor digitorum longus.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SOLEUS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Number of fibers (%)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>35.9 ± 1.4</td>
<td>45.1 ± 2.7 *</td>
</tr>
<tr>
<td>Type IIa</td>
<td>62.6 ± 1.6</td>
<td>49.0 ± 2.0 *</td>
</tr>
<tr>
<td><em>Mean fiber cross-sectional area (µm²)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>5372 ± 359</td>
<td>6311 ± 550</td>
</tr>
<tr>
<td>Type IIa</td>
<td>5082 ± 407</td>
<td>4792 ± 309</td>
</tr>
<tr>
<td><em>Total surface area (%)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>37 ± 1</td>
<td>50 ± 3 *</td>
</tr>
<tr>
<td>Type IIa</td>
<td>61 ± 2</td>
<td>42 ± 3 *</td>
</tr>
</tbody>
</table>

|                |        |        |
| **EDL**        |        |        |
| *Number of fibers (%)* |        |        |
| Type IIa       | 20.2 ± 3.0 | 13.2 ± 2.5 |
| Type IIb/x     | 79.6 ± 3.0 | 86.7 ± 2.5 |
| *Mean fiber cross-sectional area (µm²)* |        |        |
| Type IIa       | 1766 ± 204 | 1778 ± 237 |
| Type IIb/x     | 5993 ± 596 | 5232 ± 162 |
| *Total surface area (%)* |        |        |
| Type IIa       | 8 ± 2 | 6 ± 1 |
| Type IIb/x     | 92 ± 2 | 94 ± 1 |

22-week-old male AR knockout (ARKO) (n=7) and wildtype littermates (WT) (n=6) were studied. The proportion and fiber size of type I, IIa and IIb/x fibers was determined by immunofluorescence microscopy on muscle cross-sections incubated with specific antibodies directed against myosin heavy chains type I and IIa. On average 100 ± 4 fibers per muscle section for each mouse were analyzed. Data provided are expressed as means ± SE. Student’s t-test: * P<0.05 ARKO versus WT littermates.
Table 18: Effect of AR disruption on fiber-specific succinate dehydrogenase activity in soleus and extensor digitorum longus muscle.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLEUS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>8 ± 3</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Type IIa</td>
<td>14 ± 4</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>EDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IIa</td>
<td>16 ± 3</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Type IIb/x</td>
<td>8 ± 2</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

22-week-old male AR knockout (ARKO) and wildtype littermates (WT) were studied (n=6-9). Succinate dehydrogenase activity in type I, type IIa and type IIb/x fibers was determined by histochemical staining. Staining intensity was quantified as arbitrary units. Data provided are expressed as means ± SE. No significant differences between ARKO and WT littermates were observed.

The resistance to contraction-induced fatigue of muscles of ARKO and WT mice was assessed by measuring tension drop and lengthening of relaxation time. Initial muscle tension was 228 ± 24 mN/mm² versus 192 ± 21 mN/mm² in ARKO (n=8) and WT (n=7), respectively (P=0.26). The relaxation times as well as muscle tensions were similar in the ARKO and WT littermates at any time of stimulation to fatigue (Figure 20).

Also, the potential of muscles for maximal tension development was not affected in ARKO mice. Peak twitch and tetanic tensions were similar in ARKO and WT mice (Table 19). Additionally, rates of muscle contraction and relaxation, and force-frequency curves were similar between ARKO and WT in soleus and EDL (data not shown).
Figure 20: Effect of AR deletion on fatigability of muscle tension and relaxation time in soleus of 22-week-old ARKO (n=8) and WT (n=7) mice. Muscle tension (panel A) and half relaxation time (panel B) was measured in incubated soleus muscle electrically stimulated to contract during 10 min of supramaximal intermittent tetanic stimulation. Data provided are expressed as means ± SE. No significant differences between ARKO and WT littermates were observed.

Table 19: Effect of AR disruption on maximal muscle tension in soleus and extensor digitorum longus

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ARKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOLEUS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twitch</td>
<td>29.0 ± 2.5</td>
<td>25.5 ± 2.6</td>
</tr>
<tr>
<td>Tetanic</td>
<td>204 ± 14</td>
<td>222 ± 16</td>
</tr>
<tr>
<td>EDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twitch</td>
<td>27.3 ± 4.2</td>
<td>26.7 ± 3.8</td>
</tr>
<tr>
<td>Tetanic</td>
<td>232 ± 42</td>
<td>266 ± 29</td>
</tr>
</tbody>
</table>

22-week-old male AR knockout (ARKO) (n=7) and wildtype littermates (WT) (n=5) were studied. Twitch and tetanic peak tensions (mN/mm²) were measured in incubated soleus and EDL muscles electrically stimulated to contract. Data provided are expressed as means ± SE. No significant differences between ARKO and WT littermates were observed.
In skeletal muscles, the AR is expressed in different cell types including mesenchymal stem cells, satellite cells, myonuclei and fibroblasts. In the present study, the AR was successfully, albeit partly (± 60-88%), ablated in myofibers, but not in other cells as demonstrated by western blotting of soleus or LA complemented with immunohistochemical analysis of the LA (a highly androgen sensitive muscle). Male mARKO mice were fertile and had normal seminal vesicle weight, indicating that overall androgen production and activity was not affected. As expected, mice with myocyte-specific AR ablation had lower body weight and reduced lean body mass. This illustrates the importance of a functional AR in myocytes for the development of normal lean body mass. These data are also in accordance with earlier data showing that physiological levels of androgens are necessary to maintain normal muscle mass.

Although total lean body mass was decreased in mARKO mice, this was not consistently reflected in lower individual skeletal muscle weights. Compared with their corresponding mWT littermates, EDL weight in mARKO was slightly decreased (-13%). Soleus, quadriceps and gastrocnemius weights on the other hand were similar in mARKO and mWT mice. However, previous studies have documented that total lean body mass measured by DEXA does not consistently reflect changes in mass of individual peripheral muscles. DEXA measures total body lean mass, adding up the changes in individual skeletal muscles and other organs which may explain the apparent discrepancy between lean body mass and muscle weights. Furthermore, the percent lean body mass was even increased in mARKO mice due to a higher decrease in total fat mass as compared to lean mass.

In contrast to the relatively small changes in skeletal muscle weight, observed in this study, LA muscle weight dropped by 46% in mARKO mice. This diverse response of different muscle types to AR inhibition is in line with earlier observations in mice and rats and possibly reflects differences in AR protein expression and/or androgen sensitivity between muscles. Indeed, it has been demonstrated in rats and mice that the AR density in myonuclei is about 3 to 10-fold higher in LA muscles than in peripheral muscles like EDL. This dissimilar expression of the AR in LA and other muscles is compatible with our immunoblotting data and with the observation that removal of the AR in myocytes induces major atrophy in LA, but considerably smaller or undetectable changes in peripheral skeletal muscles. In pharmacological research the LA is often used as the main or only parameter to evaluate the effects of synthetic androgens on muscle. Our current findings therefore confirm that the LA can be considered as the most sensitive muscle to study responsiveness of myocytes to androgen stimulation. However, responses seen in LA are not representative for effects occurring in other skeletal muscles.
It is generally accepted that, apart from their direct AR-mediated effects on bone, androgens may indirectly affect bone mass and development by inducing changes in muscle mass and body weight and hereby mechanical loading of the skeleton. Support for such a contention comes from observations in ARKO mice, showing that ablation of the AR causes loss of muscle mass as well as diminished bone mineral acquisition (Chapter 5). However, there is evidence to indicate that the absence of AR stimulation at the level of bone tissue results in less bone mineral acquisition, independent from effects mediated via reduced muscular activity (Chapter 5). The present data in mARKO mice confirm these findings and provide further evidence for a direct role of the AR in the acquirement of normal bone mass. Although muscle mass and body weight were lower in mARKO mice, trabecular and cortical bone parameters were comparable in mARKO and mWT mice. Conversely, in ARKO mice both cortical and trabecular bone mass are markedly reduced (Chapter 5). Therefore, these findings suggest that bone mineral gain was not affected by the selective loss of the AR in muscle.

KO of the AR in myocytes increased the fraction of type I fibers (±10%) of soleus in mARKO mice. The number of type IIa fibers decreased proportionally. However, contrary to the expectations, this shift towards a more oxidative muscle type did not result in a reduced rate of fatigue development during sustained intermittent electrical stimulation, as evidenced by unchanged muscle contractile tensions and relaxation times. Myocyte-specific AR ablation caused no major change in muscle relaxation time and no fatigue-induced force drop at any time. Possibly, the shift towards a more oxidative muscle type was too small to significantly alter indices of muscle fatigue as measured in incubated soleus muscles. The higher fraction of type I fibers found in soleus, but not in EDL, of mARKO mice also indicates that AR-signaling may be involved in fiber type differentiation in oxidative, but not in glycolytic muscle types.

In addition, the fiber type switch towards type I in mARKO mice was associated with lowered total body fat mass and reduced intra-abdominal white adipose tissue accumulation. This lower fat mass was not due to decreased food consumption, which was not changed in mARKO mice, or to disruption of the AR in intra-abdominal white adipose tissue or changes in biological androgen action (as assessed by seminal vesicle weight). It has been shown that increasing slow-twitch fibers can lead to improved insulin action and increased lipid oxidation in mice. Also, in humans, obese patients have decreased percentage of type I fibers. Therefore, our data suggest that muscle fiber type composition may be associated with obesity.

Previous studies were inconclusive about the role of androgens and the AR on intrinsic muscle force and muscle fatigue. In humans, some studies reported increased maximal voluntary strength after T administration. However this was mainly, if not entirely due to gain of muscle
Similarly, in hypogonadal men or eugonadal men with androgen deprivation, muscle mass was reduced whereas muscle strength was unchanged or decreased. To investigate the effect of AR ablation in myocytes on muscle function, we measured muscle strength and fatigue resistance in vitro in mARKO and mWT mice. Neither twitch nor tetanic tensions, which express intrinsic contractile muscle force independent of muscle cross-sectional area, were significantly different between groups, either during contraction or relaxation, or after stimulation to fatigue. In accordance with the unchanged fatigue, glycogen content, succinate dehydrogenase activity or in vivo exercise capacity were similar in mARKO and mWT. Similarly, other studies in rodents reported intrinsic contractile muscle force not to be affected by either androgen withdrawal or administration, which indicates that altered muscle force, if any, is only due to changes in muscle volume or decrease. Literature data with regard to the effects of androgens on muscle fatigue are sparse. Both a small improvement of resistance to fatigue, an absence of effect and a decrease have been reported. As a novel finding, the present study now clearly shows that, at least in mice, disruption of normal physiological androgen action throughout growth and development by myocyte-specific AR inactivation does not hamper the development of normal muscular functional capacity at adult age.

Some limitations with respect to the described experiments and conclusions merit to be mentioned. In analogy with other experiments using a similar MCK-Cre+/- mice model, the AR protein was drastically reduced (60-88%) but probably not completely removed from the myocytes in our mARKO mice. The remaining AR protein can be partly explained by AR expressing satellite cells, which are not expected to be affected by MCK-driven AR ablation. Indeed, AR staining by immunohistochemistry could not distinguish between myocytes and myogenic satellite cells, since both are located within the basal lamina. However, in analogy with earlier studies, our data showed that satellite cells represent only a small proportion (6% of the myofibers stained positive for satellite cells) of the total population of myonuclei in adult skeletal muscles. Therefore, androgen action in myocytes could have been partly maintained via the residual fraction of ARs. In addition, the use of the mixed background (50% CD1, 50% C57BL/6J) could be, to some extent, responsible for the relatively small differences in muscle phenotype in mARKO mice. Nevertheless, although the power of our study may have been too low to detect the small differences in muscle mass (<10%) and performance after AR deletion, the power of our study is sufficient (>95%) to exclude a biologically relevant change in muscle mass of 20%, the average difference in muscle mass between mature male and female C57Bl/6 mice.
To circumvent these problems, we also performed muscle analyses in ubiquitous ARKO mice on a homozygous C57BL/6J background. In these mice, the AR is completely absent in all cells. Interestingly however, the complete ablation of the AR in myocytes as well as other cells such as satellite cells in ARKO mice resulted in a completely similar muscle phenotype. Earlier studies showed reduced total body lean mass in ARKO mice. However, muscle mass was decreased in some, but not all skeletal muscles: soleus weight was unchanged while EDL weight was reduced by 13% and the LA muscle even completely absent. These data further indicate the difference between measurement of lean mass and individual muscle weights as well as the importance of the LA for androgen action on muscle.

Also, in accordance with earlier data, intrinsic muscle force and muscle fatigue were not changed in mice with ubiquitous AR ablation. Furthermore, like seen in mARKO mice, ubiquitous AR ablation resulted in a higher proportion of type I fibers in soleus. Opposite to our findings, Altuwaijri et al. reported that the androgen signaling pathway increases expression of slow-twitch-specific skeletal proteins and downregulates fast-twitch-specific skeletal proteins in the quadriceps of ARKO mice, resulting in an increase of slow-twitch muscle fiber cells. The apparent discrepancy in fiber type shift between the two studies could be attributed to the different muscle types investigated (quadriceps versus soleus and EDL) and to the use of different methods for fiber type identification (western immunoblotting on whole muscle versus fiber type specific immunohistochemistry). In accordance with our data, however, microarray analysis in another ubiquitous AR KO mouse model showed an up-regulation of genes encoding slow-twitch muscle contractile proteins. In addition, a higher fraction of type I fibers was found in soleus muscle of female mice as compared to male mice. Accordingly, in humans the proportion of type I fibers in musculus vastus lateralis was lower in males than in females. Although fiber type composition changed towards more slow-type fibers, it was demonstrated that ARKO mice have no change (Chapter 5) or even an increase in fat mass. This was probably due to changes in hormone level or food intake demonstrated in ARKO mice. Available literature data together with our observations thus seem to indicate that a functional AR in muscle is important for the normal distribution of type I and II muscle fibers in some but not all skeletal muscles. In addition, different muscle types may also respond differently to AR disruption.

In summary, this study demonstrates that myocyte-specific AR ablation lowers lean body mass, LA muscle weight and, to a lesser extent, peripheral skeletal muscle mass. In addition selective AR ablation in post-mitotic myocytes may reduce body fat, but not bone mass. Although muscle strength and fatigue are not affected, our results show that the normal activity of the AR pathway is important to fiber type differentiation towards the faster fiber types. Concordant
with the prevailing opinion in preclinical and pharmacological studies, our findings also outline that LA muscle weight is the most sensitive marker of androgen-related effects on muscle. To the best of our knowledge, this study is the first to demonstrate the role of the AR in myocytes on muscle under basal conditions.
CHAPTER 7: GENERAL CONCLUSIONS AND PERSPECTIVES

7.1. GENERAL CONCLUSIONS

7.1.1. INTRODUCTION

Bone is a dynamic tissue; it is continuously modeled throughout childhood, adolescence and later life, and constantly renewed to compensate for daily damage. Bone mass and strength achieved at the end of the growth period play an important role in determining the risk for osteoporotic fractures at later age. Because the effects of T can be either direct (by AR activation) or indirect (by increasing muscle strain or following aromatization into E2 and subsequent stimulation of the ER), the relative contributions of these pathways remain unclear. Therefore, the specific role of the AR in the development of skeleton and muscle mass was assessed by selectively activating and disrupting the AR in male mice. Additionally, the role of increased as well as decreased mechanical strain on bone in growing male mice was investigated.

7.1.2. GROWTH IS A CRITICAL PERIOD FOR BONE DEVELOPMENT

The mass and strength of bone at later age are the result of a combination of bone mass accrual during childhood and adolescence to peak bone mass, and the bone formation and resorption taking place during the following years. Therefore, it is generally accepted that enhancing bone mass during growth, increases bone mass at later age. Several studies seem to support this concept. Bone density seems to track along a percentile throughout life. At the end of a longitudinal follow-up period lasting up to 22 years, areal BMD in women ranging in age from 20 to 94 years showed a high correlation (r=0.83) with baseline femoral neck areal BMD. Furthermore, skeletal size, bone volume and cancellous bone density increased linearly during pubertal maturation without overlapping. Consequently, prepubertal children at the high end of the bone mass distribution remained at the high end of this distribution at the end of sexual maturity, suggesting that osteoporosis-prone individuals can be identified even before the onset of puberty. The study we performed on SAMP6 mice (which spontaneously develop fractures at later age), supports this idea and demonstrates deficient cortical bone size and thickness as soon as early puberty (Chapter 4). In addition, by longitudinal evaluation of cortical growth in SAMP6 and SAMR1 control mice, we demonstrated that rapid growth between 4 and 8 weeks of age...
(early and late puberty, respectively) is important for cortical bone development. Similarly, in humans, 60% of peak bone mineral mass is attained during puberty, a critical period for bone mass development. Clearly, growth during childhood and adolescence is crucial for bone development, and can possibly modulate susceptibility for fractures later on in life.

Although genetic factors account for a large portion of the variance in adult BMD (60%-80%), they do not determine all of the variance, opening a window to treatment modalities such as enlarged mechanical loading or sex steroid administration. In this respect, androgen treatment is of special interest. Indeed, age-related decrease in serum T has been associated with increased risk for bone fractures. Androgens are considered to protect against fractures by their direct actions on bone. Additionally, they enlarge muscle mass which in turn elevates mechanical strain on bone, resulting in concomitant increased bone strength. Moreover, the elevated muscle mass reduces the risk for falls by improving maintenance of balance and finally, also helps to decrease force of impact when a fall does take place. For these reasons, a compound which could selectively modulate the AR (SARM) in muscle and bone without adversely affecting tissues such as the prostate could have major therapeutic potential. However, because both the effects of androgens as well as the role of their receptor in muscle and bone remain uncertain, investigation into the function of the AR in these tissues is paramount.

7.1.3. TRABECULAR BONE MASS DEVELOPMENT IS AR-DEPENDENT, WHILE CORTICAL BONE MODELING REQUIRES BOTH AR AND ERA ACTIVATION

AR-mediated androgen action is clearly important for normal peak bone mass accrual. In man, their role in normal bone mass acquisition during puberty is supported by reports of decreased areal BMD of spine and hip in caIS patients as well as in men with a history of delayed puberty. Similarly, in the hypogonadal SAMR1 mouse which was orchidectomized at early puberty, both trabecular and cortical bone mass acquisition are impaired. Decreased trabecular and cortical bone mass are also observed in the testicular feminized male mouse model and in mice with ubiquitous AR deletion (Chapter 5).

Trabecular bone development is AR-dependent. Orch at early puberty (Chapter 4) as well as AR inactivation (Chapter 5) result in reduced trabecular bone mass development and in line with these findings, bone turnover markers are increased in these mice. Supplementation of androgens (both aromatizable and non-aromatizable like DHT) improves trabecular bone mass acquisition and lowers bone turnover markers in orch mice (Chapter 4), but not in AR KO mice. Moreover, the trabecular bone sparing effect of T in orch mice or rats could not be impaired.
by inhibition of aromatase activity or by administration of the estrogen receptor antagonist ICI 182,780 \textsuperscript{67, 286}. Together, these data indicate that AR activation is essential for normal development of trabecular bone in growing male mice and that neither residual peripheral aromatization nor remaining ERα activation are able to compensate for the trabecular bone loss. In agreement with this reasoning, male ERα KO mice do not present with decreased trabecular bone mass. Conversely, these mice show an elevated mass of trabecular bone \textsuperscript{68, 271, 287, 288}, which is normalized following administration of an anti-androgen \textsuperscript{288}. This suggests that their enhanced trabecular bone mass is the result of higher endogenous androgen levels acting via the AR. In ERα KO mice, orch reduces trabecular bone volume to the same levels as WT control animals, but this is fully prevented by T, again demonstrating the important role of AR-mediated androgen action in trabecular bone growth \textsuperscript{68, 288}. However, similar to our observations in the SAMP6 and SAMR1 mouse models, in male WT mice, E2 treatment after orch increases trabecular bone volume and even seems to have an osteoanabolic effect \textsuperscript{68, 287, 288}. Several research groups showed that this effect is regulated through ERα, although the doses used in their experiments are pharmacological and not representative of the significantly lower physiological levels (±5 pg/ml) present in male mice \textsuperscript{105, 287, 288}.

Androgen action via the AR is also important for normal periosteal bone formation. Males have higher and wider bones than females, as shown by their larger cross-sectional area \textsuperscript{289}. Androgen deficiency, as induced by orch in SAMR1 and SAMP6 mice, lowers both periosteal bone formation \textsuperscript{67} and expansion during growth (Chapter 4). Reduced bone perimeter (Chapter 5) and periosteal bone formation rate \textsuperscript{67} are also observed in mice with ubiquitous AR ablation. Administration of non-aromatizable DHT to orch mice or rats was shown to stimulate periosteal bone formation \textsuperscript{67, 290} and resulted in larger bone perimeters (Chapter 4). Androgen insensitive ARKO mice on the other hand, are unresponsive to T or DHT \textsuperscript{67}. However, there are several indications that the aromatization of androgens into estrogens and subsequent ER activation may also be important for radial bone growth. E2 treatment increased bone perimeter in orch SAMP6 and SAMR1 mice (Chapter 4). Adult men incapable of producing (due to aromatase deficiency) or responding to E2 (lack of ERα) are osteopenic \textsuperscript{95-97}. A similar phenotype was observed in aromatase \textsuperscript{98, 100} and ERα KO mice \textsuperscript{68, 101}. Likewise, in growing male rats and mice, administration of an aromatase inhibitor reduced bone width and periosteal bone formation \textsuperscript{67}. \textsuperscript{291} Importantly, this reduced radial growth was associated with reduced serum IGF-I levels \textsuperscript{67, 99, 101}, indicating that it may also be an indirect effect mediated by the GH/IGF-I axis.
Androgens regulate muscle mass development during growth by acting via the AR. During puberty, boys gain more muscle mass than girls and thus attain higher muscle mass at maturity \(^54, 122\). T treatment in prepubertal boys \(^292\) or adolescents \(^141, 153\) increases lean mass by improving protein synthesis and reducing protein breakdown. Similarly, in mature SAMP6 and SAMR1 mice in early puberty, orch reduces both cross-sectional area and lean mass of muscle, while DHT supplementation prevents this (Chapter 4). Further evidence for the role of the AR in normal muscle mass accrual during growth comes from studies performed on ARKO mice. In analogy to what was seen in other studies \(^277\), AR deletion resulted in reduced lean mass and muscle weight \(^67\) (Chapter 5). Additionally, mARKO (muscle-specific AR deletion) mice show decreased muscle mass buildup (Chapter 6). However, not all muscle types react to AR disruption in the same manner. LA, an androgen-sensitive muscle in the pelvic floor, is severely reduced in weight in mARKO mice and is even completely absent in ARKO mice, confirming its value for a sensitive assay for anabolic androgenic steroid activity \(^262\). The effects on other skeletal muscles are minor and seem to correlate with AR expression levels \(^171, 267, 277\).

The role of the AR in muscle performance is less clear. Several studies indicate that androgens can improve muscle strength. In hypogonadal or eugonadal men with androgen deprivation, voluntary muscle strength was unchanged \(^143, 151\) or decreased \(^137\). On the other hand, T administration increased maximal voluntary strength. However, gain of muscle strength was proportional to increase in muscle mass and seemed not due to improvement of intrinsic contractile muscle function \(^145, 147, 154, 156, 264, 265\). Similarly, in mature rodents, maximal tetanic force is decreased after orch and can be restored by androgen treatment \(^163-165\), but reduction of strength did not always persist after correction of muscle mass \(^164\). Intrinsic muscle force was also unchanged in mice with muscle-specific (Chapter 6) or ubiquitous AR deletion \(^277\) (Chapter 5). Therefore, most data seems to indicate that androgens and the AR influence muscle strength by increasing muscle mass but not by improving intrinsic contractile function.

The role of the AR in muscle fatigability remains largely unknown. In humans, female muscles are more resistant to fatigue than male muscles \(^133, 134, 293\). Similarly, female mice show more endurance during treadmill running than their male counterparts \(^299\)(unpublished data). Muscle fatigue is the combined result of different processes both extrinsic (blood supply and neuromuscular activation activity) and intrinsic to muscle tissue (metabolism and fiber type) \(^134\). Electric stimulation of isolated muscles of myocyte-specific and ubiquitous AR KO mice shows reduced \(^277\) to slightly reduced (Chapter 6) soleus fatigue. In accordance, the soleus contains an elevated proportion of type I fibers in mARKO and ARKO mice (Chapter 6). Genes encoding slow-
twitch muscle contractile properties are up-regulated in gastrocnemius of ubiquitous AR KO mice as well \(^\text{277}\). Therefore, AR deletion seems to influence the development of muscle fatigability resistance. However, on a treadmill, ARKO mice (Chapter 5) but not mARKO mice (Chapter 6) ran longer than their male (m)WT littermates. Therefore, the changes in muscle fatigability do not always reflect alterations in running endurance. So, the increased fatigability resistance of ARKO mice is probably due to altered processes extrinsic to muscle tissue.

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**7.1.5. Changes in Mechanical Strain Have Modest Effects on Bone**

There are several indications that bone can adapt to increased load by increasing its strength. During puberty, an increase in muscle mass precedes gain of bone mass \(^\text{109}\). Boys acquire more bone mass but also have higher loads on bone due to increased body weight and muscle strength \(^\text{122}\). Besides its direct osteogenic effect, physical activity also indirectly affects bone by enhancing muscle mass and hence the tension generated on bone \(^\text{295}\). Interestingly, exercise seems most effective during prepuberty and early puberty given the fact that immature bones are more responsive to mechanical stress \(^\text{295, 296}\). In accordance, voluntary running in growing ARKO mice (Chapter 5) or treadmill running in young orch male rats \(^\text{258}\) increased trabecular bone mass. Similarly, both in young mice and rats, running or resistance exercises increased femoral bone mass in some \(^\text{257, 258, 297-299}\) but not all studies \(^\text{260, 261}\). The differences observed may depend on the type and the intensity of the exercise. Indeed, excessive changes in bone strain (paralysis or unloading) were shown to cause significant loss of bone, while strenuous physical activity (such as gymnastics or racquet sport players) was demonstrated to augment its mass \(^\text{203, 204, 300}\). Less strenuous exercising (such as swimming) or more physiological exercises (such as daily walking) have less effect \(^\text{301}\). Similarly, it was calculated that osteogenesis may require an increase in lean mass of >10%, equivalent to ±4 kg of muscle mass in a 60-kg woman \(^\text{186}\). This may explain why the highly increased muscle mass (40%-100%) in mice with myostatin deficiency \(^\text{302}\) improves bone strength, while the relatively small reductions in muscle mass in our mARKO mice (−8% lean mass) have no measurable effects on bone (Chapter 6).

When compared to genetic factors or sex steroids, mechanical strain seems to have the least pronounced effect. In SAMP6 mice, tibial periosteal perimeter and polar moment of inertia are increased despite reduced calf muscle cross-sectional area (Chapter 4). Interestingly, jumping resulted in an adaptive response on bone in C57Bl/6J mice, but not in C3H/HeJ mice \(^\text{303}\), demonstrating that the adaptive response of bone to loading can differ between genetic strains. There are also indications that sex steroid deficiency prevents the ability of exercise or muscle
mass loading to improve bone mass. Although muscle mass is reduced in both muscle-specific and ubiquitous AR deletion mice, bone mass is severely reduced only in ARKO mice (Chapter 6). Additionally, the positive correlation between muscle cross-sectional area and polar moment of inertia observed in WT mice is lost in mice with ubiquitous AR disruption (Chapter 5). This suggests that the absence of AR stimulation at the level of bone tissue results in less bone mineral acquisition and may be, at least partly, independent from effects mediated via reduced muscular activity. Voluntary running reduces bone loss in ARKO mice but can not restore bone mass to WT levels (Chapter 5). Similarly, treadmill running in orchidectomized mice and rats was able to restore bone mass in some but not all studies. However, these data do not exclude physical exercise as a possible therapy for improving bone mass. Despite the modest effects of physiological changes in mechanical loading on bone mass, physical exercise remains an easy and low-cost way to improve bone strength, especially during growth.

7.1.6. CONCLUSION

In conclusion, it seems that androgen action mediated by the AR is essential for normal trabecular and cortical bone accrual during growth. However, aromatization and subsequent ER activation are also involved in cortical bone development. In addition, the AR is important for normal gain of muscle mass, but less so for muscle performance. Finally, our data show only limited effects of changes in mechanical loading on bone. However, they do not exclude a possible role of physical exercise on bone during growth.
7.2. FUTURE PERSPECTIVES

The important role of the AR for male skeletal development is clearly illustrated in the previous chapters: AR activation is necessary for the development of a normal trabecular and cortical phenotype. Moreover, AR inactivation increases bone resorption resulting in osteopenia in male mice \(^9\) (Chapter 4, Chapter 5). Further research is however warranted to elucidate if the target cells of this AR-mediated effects are the osteoblasts, osteoclasts or both. In addition, the molecular working mechanism remains to be elucidated. This objective could be approached by \textit{in vitro} cell culture experiments derived from ARKO and WT mice. Primary osteoblast cultures can be used to investigate the effect of AR deletion on osteoblast proliferation, differentiation and mineralization. Cocultures of primary osteoblasts with osteoclasts allow determination of changes in the supportive role of osteoblasts in the formation of osteoclast and also allow to evaluate changes in osteoclast characteristics after AR deletion. Furthermore, RT-PCR analysis of gene expression in osteoblasts or microarray analysis of RNA prepared from long bones can give insight into androgen-regulated gene expression involved in cortical bone expansion.

The study of mice with myocyte-specific AR deletion provides insight into the role of the AR in muscle and its indirect effect on bone. However, additional research is necessary to further elucidate the role of direct and indirect androgen action. To this end, an osteoblast-specific AR KO mouse can be developed by mating floxed AR mice with transgenic mice expressing Cre recombinase selectively in osteoblasts (collagen I promoter or Runx promoter). This will not only provide insight in the action of androgens mediated by AR in osteoblasts but also shed light on the direct role of androgens on bone.

In the previous chapters it was demonstrated that changed mechanical loading by voluntary running may influence bone mass accrual during growth. However, the regulation of bone growth by mechanical loading is more complex. It has been shown that the adaptive response to loading is less effective in the absence of ER\(\alpha\) in female mice \(^304\). The role of the AR in the bone’s osteogenic response to loading is however unknown. This question could be answered by an \textit{in vivo} ulna loading experiment in ARKO mice \(^305\). In this experiment the left ulna is subjected to dynamic axial loading and the right ulna is used as an unloaded control. Bone responses to loading can be determined by dynamic histomorphometry. If bone responses to loading are changed in ARKO mice, it can be speculated that the protective effect of androgens are partly exerted by changing sensibility of bone to mechanical loading. Next, in a second experiment, the role of osteocytes (which are thought to mediate the response to mechanical loading) can be investigated. By subjecting osteocytes of ARKO and WT mice to shear stress \textit{in vitro} \(^306\), the
difference in response can be studied. This will allow us to determine whether the loading-
induced effects observed in ARKO and WT are mediated through osteocytes.

This knowledge can be used to design new drugs with a more specific and tissue-selective mode
of action (selective AR modulators or SARMs) which may lead to more effective treatment
modulators for osteoporosis.
Osteoporosis is an important health problem which affects not only women but also men. Despite the lower incidence of osteoporotic fractures in men, male osteoporosis is associated with even greater morbidity, mortality and medical costs than postmenopausal osteoporosis. Although osteoporotic fractures are most commonly observed among the elderly, the pathogenesis of osteoporosis starts early in life and involves the interaction of multiple genetic and environmental factors. Sex steroids and muscle loading are associated with bone strength and are believed to be largely responsible for the difference in bone strength between men and women. A better understanding of the mechanisms involved in the construction of the male skeleton may therefore lead to more effective treatment modalities for osteoporosis in men.

T is the main sex steroid in men and has been proposed to be the key regulator of pubertal growth. The effects of T can be mediated directly via the AR on bone or indirectly via the AR on skeletal muscle hereby affecting mechanical loading. However, the relative importance of these pathways and the role of the AR in this respect remains largely unknown. To elucidate the role of the AR in this muscle-bone interaction, several experiments were performed in growing male mice.

First, we investigated the difference in peak bone mass acquisition in a mouse model for senile (SAMP6) and hypogonadal osteoporosis (orchidectomized control SAMR1) by a longitudinal study from 4 to 20 weeks of age. Additionally, the effects of long term DHT and E2 replacement are studied in both models. This way, the role of AR-mediated and/or ER-mediated sex steroid action is investigated in growing male mice (Chapter 4). Interestingly, cortical bone mass acquisition was significantly lower in SAMP6 mice compared to their controls, SAMR1. Already at 4 weeks of age (before puberty) SAMP6 mice had significant thinner cortex due to cortical expansion. SAMP6 mice had significant lower endocortical bone formation (-43%) and increased periosteal bone formation (+47%) resulting in increased periosteal and endosteal perimeters. Cortical differences between SAMR1 and SAMP6 became more apparent during growth and resulted in severely reduced cortical bone thickness in 20-week-old SAMP6 mice. This difference in cortical bone mass could not be explained by the equal weight or lower muscle cross-sectional area in SAMP6 compared to SAMR1. Early orchidectomy, on the other hand reduced periosteal bone apposition during growth with limited reduction of endocortical bone expansion. Interestingly, the defects in cortical bone development occurred before 8 weeks of age, with no
or little progress thereafter. As expected, muscle growth was impaired in hypogonadal mice as well.

At the cancellous bone compartment, trabecular bone formation is not different between SAMP6 and SAMR1. In contrast, orchidectomy was characterized by high bone turnover (increased serum osteocalcin and urinary DPD) and less trabecular bone gain due to decreased trabecular number. Next, sex steroid action was investigated in orchidectomized SAMP6 and SAMR1 mice.

Interestingly, both DHT and E2 stimulated periosteal expansion in orchidectomized SAMP6 and SAMR1. In addition, cancellous bone was improved by DHT and E2 treatment. However, while DHT treatment was more effective, E2 was less effective in SAMP6. Interestingly, E2 stimulated endosteal bone apposition in SAMP6, partly restoring its cortical phenotype. Moreover, the sex steroid action occurred mainly during the short time period of puberty (4-8 weeks of age) and not later on. Overall, these data indicated that puberty, and even before puberty, is a vulnerable period for cortical bone development. Both DHT and E2 improved bone mass acquisition in orchidectomized animals, suggesting a role for the AR as well as the ER in male skeletal development.

Secondly, the role of the AR in male bone mass acquisition and the effect of voluntary exercise on bone mass was investigated in ARKO mice. During an experimental period of 11 weeks, 5-week-old ARKO and WT mice were followed-up and one group of ARKO mice was allowed to run voluntarily in a cage with running wheel (Chapter 5). Cortical bone gain (cortical thickness, area, perimeters and SSI) was reduced in mice with AR deletion. In addition, body weight and muscle mass gain was reduced in ARKO mice. However, in contrast to WT, no correlation between SSI and calf muscle cross-sectional area could be found in ARKO mice. Also trabecular bone mass gain was severely reduced in ARKO compared to WT mice. Bone turnover parameters were increased in sedentary ARKO mice, reflecting higher cancellous bone remodeling activity. Voluntary running reduced markers of bone remodeling and was found to partly prevent trabecular, but not cortical bone loss in ARKO mice. Taken together, these data support the concept that androgens are not only essential to optimize cortical bone mineral acquisition, but even more so for the development and integrity of the cancellous bone network during growth. In addition puberty represents a critical and vulnerable period during which androgen action, muscle mass gain, and physical exercise have both independent and interactive effects on trabecular and cortical bone compartments.
Finally, a myocyte-specific AR knockout mouse (mARKO) was developed to study the role of the AR in muscle. In addition, the effect of myocyte-specific AR disruption on muscle mass and performance are compared with the effects seen in mice with a ubiquitous AR disruption (Chapter 6). mARKO mice displayed a marked reduction in AR protein levels (60-88%). Interestingly, body weights (-8%) and lean body mass (-8%) were lower in mARKO mice than in their WT littermates. The weight of the highly androgen-sensitive LA was significantly reduced (-46%). In contrast, no or only minor reductions in the weight of other peripheral skeletal muscles were observed in mARKO mice. Surprisingly mARKO mice had lower intra-abdominal fat than their respective control littermates. In contrast with ARKO, mARKO mice had no cortical and trabecular bone phenotype, indicating that selective ablation of the AR in myocytes does not affect male skeletal homeostasis. Furthermore, muscle contractile performance in mARKO mice did not differ from that observed in their WT littermates; myocyte-specific AR ablation resulted in a conversion of fast towards slow fibers, without large affects on muscle intrinsic strength or fatigue. Importantly, the muscle phenotype of mARKO mice was similar to mice with ubiquitous AR deletion. Also in ubiquitous ARKO mice, muscle mass was decreased in some, but not all skeletal muscles. Soleus weight was unchanged while EDL weight was reduced by 13% and the LA muscle even completely absent in ARKO mice. These data further indicate the importance of the LA for androgen action on muscle. Again parallel with the changes in mARKO mice, a higher fraction of slow fibers was also found in soleus in ARKO mice, while intrinsic muscle force and muscle fatigue were not changed. Taken together, our findings show that, under basal conditions, myocyte AR signaling contributes to the maintenance of muscle mass and fiber type regulation but not or less to muscle strength or fatigue. The LA weight remains the most sensitive and specific marker for detection of AR-mediated anabolic action. In addition myocyte-specific AR deletion influences body composition, without influencing bone mass acquisition.

In conclusion, these data indicate that the AR is essential for normal trabecular and cortical bone development in the male skeleton. Furthermore, the AR is important for normal muscle mass acquisition, but less for muscle mass performance. Voluntary running is able to improve trabecular bone mass gain, although small changes in muscle mass do not seem to influence bone gain during growth.
SAMENVATTING

Osteoporose is een belangrijk gezondheidsprobleem, niet enkel bij vrouwen, maar ook bij mannen. Ondanks de lagere incidentie aan osteoporotische fracturen bij mannen, gaat osteoporose bij de man gepaard met grotere morbiditeit, mortaliteit en medische kost in vergelijking met postmenopausale osteoporotische vrouwen. Alhoewel osteoporotische fracturen vooral voorkomen bij ouderen, ontstaat osteoporose reeds tijdens de jonge levensjaren door een interactie van zowel genetische als omgevingsfactoren. Geslachtshormonen worden hierbij gezien als belangrijke spelers verantwoordelijk voor het verschil in botsterkte tussen mannen en vrouwen. Een beter inzicht in de rol van mannelijke geslachtshormonen voor de opbouw van het skelet kan daarom leiden tot betere behandelingsmethoden voor osteoporose.

Testosteron, het voornaamste androgeen bij mannen, wordt beschouwd als de belangrijkste determinant van mannelijke botgroei tijdens de puberteit. De botgroei kan echter ook verklaard worden door de hogere toename van spiermassa in mannen, waarbij het bot zich aanpast aan de grotere krachten die erop uitgeoefend worden. Bovendien kunnen de effecten van testosteron op bot zowel rechtstreeks gemediëerd worden via de androgeen receptor in botweefsel als onrechtstreeks, via de androgeen receptor aanwezig in spier. Om de rol van de androgeen receptor in deze spier-bot interactie te verduidelijken, werden verschillende experimenten uitgevoerd in mannelijke muizen tijdens de groei.

Eerst werd tijdens een longitudinale studie de botmassaopbouw vergeleken in een muismodel voor seniele osteoporose (SAMP6) met een muismodel voor hypogonadale osteoporose (gecastreerde controle SAMR1) van 4 tot 20 weken. Vervolgens werd ook het effect van langdurige dihydrotestosteron (DHT) en oestradiol (E2) substitutie onderzocht in deze beide muismodellen. Aangezien DHT een niet-aromatiseerbaar androgeen is en E2 een oestrogeen, werd op deze manier de rol van zowel de androgeen als de oestrogeen receptor onderzocht (Chapter 4).

Een interessante bevinding van deze studie was de significant lagere opbouw van corticale botmassa in SAMP6 muizen in vergelijking met hun controlemuizen, SAMR1. Reeds voor de puberteit (voor de leeftijd van 4 weken) hadden SAMP6 muizen een merkably dunnere cortex als gevolg van een verhoogde medullaire botexpansie en gedaalde endocorticale botformatie (-43%). De periostale botformatie was echter toegenomen (+47%) in SAMP6 muizen, wat resulteerde in toegenomen endocorticale en periostale botomtrekken. Dit verschil in corticale
botmassa kon echter niet verklaard worden door het gelijke lichaamsgewicht of kleinere spierdiameter in SAMP6 in vergelijking met SAMR1 muizen. In tegenstelling tot deze bevindingen, resulteerde castratie op jonge leeftijd vooral in een vermindere periostale botaanmaak en een vermindere toename in spiermassa tijdens de groei. Bovendien ontstonden de cortical botdefecten voornamelijk tijdens de puberteit (4 tot 8 weken). Castratie resulteerde ook in een vermindere opbouw van trabeculaire botmassa en een hoge botturnover, terwijl de trabeculaire botmassa niet verschillend was tussen SAMP6 en SAMR1.

Vervolgens werd ook de werking van verschillende geslachtshormonen onderzocht in gecastreerde SAMP6 en SAMR1 muizen. Zowel DHT als E2 stimuleerde periostale botexpansie in gecastreerde SAMP6 en SAMR1 muizen. Bovendien werd ook de trabeculaire botvorming verbeterd door DHT en E2 toediening. Opmerkelijk hierbij was dat E2 de endocorticale botformatie stimuleerde in SAMP6, waardoor het corticale fenotype gedeeltelijk hersteld werd. Aangezien ook hier het effect van beide geslachtshormonen zich voornamelijk beperkte tot de puberteit (4 tot 8 weken bij de muis) wordt de de puberteit, en zelfs de periode voor de puberteit, aanzien als een zeer kritische periode voor de corticale botontwikkeling. Bovendien was zowel een niet-aromatiseerbaar androgeen als een oestrogeen in staat om de botmassa te verbeteren bij hypogonadale muizen. Daarom lijkt niet alleen de androgeen receptor maar ook de oestrogeen receptor belangrijk voor de botopbouw bij de man.

Ten tweede werd de rol van de androgeen receptor en het effect van vrijwillig lopen onderzocht tijdens de opbouw van botmassa in muizen die androgeen resistent zijn door een volledige afwezigheid van de androgeen receptor (ARK0). Gedurende een experimentele periode van 11 weken werden 5 weken oude ARKO en wildtype (WT) muizen opgevolgd, waarbij 1 groep ARKO muizen vrijwillig kon lopen in een kooi met een loopwiel (Chapter 5).

Hierbij zagen we dat de corticale bottoename (corticale dikte, oppervlakte, omtrek en ‘strength strain index’) verminderd was in muizen zonder androgeen receptor. Bovendien werd er, in tegenstelling tot WT muizen (controle muizen), geen correlatie gevonden tussen de ‘strength strain index’ (een parameter voor botsterkte) en de kuitspierdiameter. Ook de opbouw van trabeculaire botmassa was ernstig verminderd in ARKO muizen ten opzichte van WT muizen. De botturnover parameters waren toegenomen bij de ARKO, een weerspiegeling van hun hoge trabeculaire bot hermodellering. Vrijwillig lopen reduceerde de bot turnovermarkers in ARKO muizen en was in staat om het trabeculair botverlies, maar niet het corticale botverlies, in ARKO muizen gedeeltelijk tegen te gaan. Bijgevolg suggereerden deze bevindingen dat androgenen niet alleen essentieel zijn om de corticale botmassa te optimaliseren, maar vooral van belang zijn.
voor de ontwikkeling van het trabeculaire botnetwerk tijdens de groei. Bovendien is de groei een kritische en kwetsbare periode gedurende dewelke androgenen, spiermassa en lichaamsbeweging trabeculair en corticaal bot kunnen beïnvloeden.

Zoals hoger aangegeven, stimuleren androgenen niet enkel de botmassa maar ook de spiermassa. Daarom werd ook de rol van de androgeen receptor in de opbouw van de mannelijke spiermassa bestudeerd. Hiervoor werd een spiervezel-specifieke androgeen receptor knockout muis (mARKO) ontwikkeld. In tweede instantie werd het effect van de spiervezel-specifieke androgeen receptor disruptie op spiermassa en -functie ook vergeleken met de effecten in muizen met een globale androgeen receptor knock-out (ARKO) om een beter inzicht te krijgen in de rol van de androgeen receptor in spier (Chapter 6).

mARKO muizen vertoonden een belangrijke reductie in androgeen receptor in spier (-60-80%). Dit resulteerde in een lager totaal lichaamsgewicht (-8%) en lager vetvrij lichaamsgewicht (-8%) in mARKO dan WT muizen. Bovendien was ook het gewicht van de androgeen-gevoelige levator ani spier significant verminderd (-46%). Het gewicht van andere perifere skeletspieren was echter slechts weinig of niet gedaald in mARKO muizen. Opmerkelijk was ook dat mARKO muizen minder intra-abdominaal vet hadden dan hun WT controlemuizen. Ook hadden de mARKO muizen, in tegenstelling tot de ARKO muizen, geen corticaal en trabeculair botfenotype, wat aantoont dat de deletie van de androgeen receptor in spiervezels de skeletontwikkeling in de man niet beïnvloedt. De selectieve disruptie van de androgeen receptor in spiervezels resulteerde evenwel in een verschuiving van snelle naar trage spiervezels, echter zonder grote gevolgen op intrinsieke spiersterkte of vermoeidheid zoals gemeten in vitro. Bovendien was het spierfenotype van mARKO muizen gelijkaardig aan dat van muizen met een globale androgeen receptor deletie (ARKO muizen). Ook in ARKO muizen was het gewicht gedaald in sommige, maar niet alle skeletspieren. De levator ani was zelfs compleet afwezig. De levator ani lijkt daarom dus veel gevoeliger voor androgenen dan perifere skeletspieren. In overeenstemming met de veranderingen in mARKO muizen, werd er in de soleus van ARKO muizen ook een hoger percentage aan trage spiervezels gevonden terwijl de intrinsieke spiersterkte en -vermoeibaarheid grotendeels onveranderd waren. Alles bij elkaar genomen, toonden de bevindingen in deze studie aan dat, onder basale omstandigheden, de androgeen receptor in spiervezels bijdraagt tot de ontwikkeling van spiermassa en de spiervezelverdeling, maar niet of nauwelijks tot de intrinsieke spiersterkte of -vermoeibaarheid. Het gewicht van de levator ani bleek de meest gevoelige parameter voor androgeen receptor gemediéerde anabole effecten.
Bovendien verandert de specifieke deletie van androgeen receptor in spiervezels de globale lichaamssamenstelling maar niet de botopbouw.

Ter conclusie, de androgeen receptor is essentieel voor een normale trabeculaire en corticale botontwikkeling in het mannelijk skelet. Ook is de androgeen receptor belangrijk voor een normale opbouw van spiermassa, maar minder voor spierfunctie. Bovendien kan een veranderde mechanische belasting door vrijwillig lopen of verandering in spiermassa de opbouw van trabeculaire en corticale botmassa gunstig beïnvloeden.
REFERENCES


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29. Tabensky A, Duan Y, Edmonds J, Seeman E. The contribution of reduced peak accrual of bone and age-related bone loss to osteoporosis at the spine and hip: Insights from the daughters of women with vertebral or hip fractures. *J Bone Miner Res*. 2001;16:1101-1107


64. Horowitz M, Wishart JM, O’Loughlin PD, Morris HA, Need AG, Nordin BE. Osteoporosis and klinefelter’s syndrome. *Clin Endocrinol (Oxf).* 1992;36:113-118


101. van der Eerden BC, van Til NP, Brinkmann AO, Lowik CW, Wit JM, Karperien M. Gender differences in expression of androgen receptor in tibial growth plate and metaphyseal bone of the rat. Bone. 2002;30:891-896


112. Hofbauer LC, Ten RM, Khosla S. The anti-androgen hydroxyflutamide and androgens inhibit interleukin-6 production by an androgen-responsive human osteoblastic cell line. J Bone Miner Res. 1999;14:1330-1337


dehydroepiandrosterone sulfate, and the ratio of insulin-like growth factor 1 to growth hormone. *Proc Natl Acad Sci U S A*. 1997;94:7537-7542


156. Storer TW, Maglione L, Woodhouse L, Lee ML, Dzekov C, Dzekov J, Casaburi R, Bhasin S. Testosterone dose-dependently increases maximal voluntary strength and leg power, but does not affect fatigability or specific tension. *J Clin Endocrinol Metab*. 2003;88:1478-1485


166. Singh R, Artaza JN, Taylor WE, Gonzalez-Cadavid NF, Bhasin S. Androgens stimulate myogenic differentiation and inhibit adipogenesis in c3h 10t1/2 pluripotent cells through an androgen receptor-mediated pathway. *Endocrinology*. 2003;144:5081-5088


177. Liu JP, Baker J, Perkins AS, Robertson EJ, Efstatiadis A. Mice carrying null mutations of the genes encoding insulin-like growth factor i (igf-1) and type 1 igf receptor (igf1r). *Cell*. 1993;75:59-72


237. Silva MJ, Brodt MD, Ettner SL. Long bones from the senescence accelerated mouse samp6 have increased size but reduced whole-bone strength and resistance to fracture. J Bone Miner Res. 2002;17:1597-1603


240. Silva MJ, Brodt MD, Ko M, Abu-Amer Y. Impaired marrow osteogenesis is associated with reduced endocortical bone formation but does not impair periosteal bone formation in long bones of samp6 mice. J Bone Miner Res. 2005;20:419-427


255. Valdimarsson O, Linden C, Johnell O, Gardsell P, Karlsson MK. Daily physical education in the school curriculum in prepubertal girls during 1 year is followed by an increase in bone mineral accrual and bone width--data from the prospective controlled malmo pediatric osteoporosis prevention study. Calcif Tissue Int. 2006;78:65-71


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276. Tanner CJ, Barakat HA, Dohm GL, Porres WJ, MacDonald KG, Cunningham PR, Swanson MS, Houmard JA. Muscle fiber type is associated with obesity and weight loss. Am J Physiol Endocrinol Metab. 2002;282:E1191-1196


286. Vandenput L, Swinnen JV, Van Herck E, Verstuyf A, Boonen S, Bouillon R, Vanderschueren D. The estrogen receptor ligand 182,780 does not impair the bone-
sparing effects of testosterone in the young orchidectomized rat model. *Calcif Tissue Int.* 2002;70:170-175


LIST OF PUBLICATIONS

PUBLICATIONS IN PEER-REVIEWED JOURNALS


ORAL PRESENTATIONS AT INTERNATIONAL MEETINGS

Ophoff J, Vanderschueren D 2006 Sustained Running Reduces Cancellous Bone Resorption in Growing Male Mice with Androgen Receptor Disruption. (oral presentation, 11th Paulo Symposium on Preventing Bone Fragility & Fractures, Tampere, Finland)


ABSTRACTS AT INTERNATIONAL MEETINGS


Mice with Liver-Specific Overexpression of IGF-I. J Bone Miner Res 22(Suppl 1):S169 (poster presentation, 29th Annual Meeting of the American Society for Bone and Mineral Research, Honolulu, HI, USA)

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