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ANDROGENS AND THE MOLECULAR REGULATION OF SKELETAL MUSCLE MASS

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It always seems impossible until it's done ~ Nelson Mandela

Hélène

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LIST OF ABBREVIATIONS

4EBP1	factor 4E binding protein 1
AIS	androgen insensitivity syndrome
ALS	amyotrophic lateral sclerosis
AR	androgen receptor
ARE	androgen response element
ARKO	androgen receptor knockout
ATP	adenosine triphosphate
AIDS	acquired immunodeficiency syndrome
AMPK	5'-adenosine monophosphate-activated protein kinase
BC	bulbocavernosus muscle
COPD	chronic obstructive pulmonary disease
CSA	cross-sectional area
CYP	cytochrome P450-dependent enzyme
DBD	DNA-binding domain
DHT	dihydrotestosterone
DXA	dual-energy x-ray absorptiometry
E1	ubiquitin-activating enzyme
E2	ubiquitin-carrying enzyme
E_2	17β-estradiol
E3	ubiquitin-ligase enzyme
EDL	extensor digitorum longus muscle
eIF2B	eukaryotic initiation factor 2B
eIF4E	eukaryotic initiation factor 4E
ER	estrogen receptor
Fbxo40	F-box protein 40
FoxO	forkhead box containing proteins, O-subclass
FT	free testosterone
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	gastrocnemius muscle
GH	growth hormone
GLUT4	glucose transporter 4
GnRH	gonadotropin-releasing hormone
GSK3β	glycogen synthase kinase 3β
HIV	human immunodeficiency virus
HSD	hydroxysteroid dehydrogenase-dependent enzyme
HPG	hypothalamo-pituitary-gonadal axis
HPLC	high-performance liquid chromatography
IGF1	insulin-like growth factor 1
IκB	inhibitor of nuclear factor of kappa B
IKK	IκB kinase
IL-6	interleukin-6

IRS	insulin receptor substrate
KO	knockout
LA	levator ani muscle
LBD	ligand-binding domain
LC-MS/MS	liquid chromatography tandem mass spectrometry
LH	luteinizing hormone
mARKO	myocyte-specific ARKO
MHC	myosin heavy chain
mTOR	mammalian target of rapamycin
MAFbx	muscle atrophy F-Box
MuRF1	muscle RING (really interesting new gene) finger-1
ND	nandrolone decanoate
NF-κB	nuclear factor kappa B
NTD	NH_2-terminal region
ORX	orchidectomy
$p70^{S6K}$	p70-ribosomal S6 kinase
PGC1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	phosphatidylinositol-3-phosphate kinase
PIP3	phosphatidylinositol-3,4,5-triphosphate
PLT	plantaris muscle
pQCT	peripheral quantitative computed tomography
QUADR	quadriceps muscle
qPCR	quantitative polymerase chain reaction
REDD1	development and DNA Damage responses 1
SARM	selective androgen receptor modulator
SCI	spinal cord injury
SHBG	sex hormone binding globulin
SNP	single nucleotide polymorphism
SOCS-3	suppressor of cytokine signalling-3
SOL	soleus muscle
T	testosterone
TA	tibialis anterior muscle
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor alpha
TP	testosterone propionate
TS	tail suspension
UPP	ubiquitin-proteasome pathway
VL	vastus lateralis muscles
WT	wild type

SAMENVATTING

Spieratrofie of het onvrijwillig verlies van spiermassa kan optreden als gevolg van pathologische aandoeningen zoals spierdystrofieën, chronische aandoeningen (cachexie), ondervoeding en immobilisatie (spier inactiviteit), of als gevolg van veroudering (sarcopenie). Gezien de vitale rol van spieren in het behoud van de lichaamshouding, de controle van bewegingen en de regulatie van het eiwitmetabolisme, kan een verlies van spiermassa ernstige gevolgen hebben voor de patiënt. Dit leidt vaak tot een verminderde levenskwaliteit. Onderzoek naar de preventie of behandeling van skeletspieratrofie is daarom van groot belang voor zowel de klinische geneeskunde als voor ouderen en atleten.

Fysieke activiteit, al dan niet gecombineerd met voedingssupplementen, is een effectieve en veilige manier om spieratrofie te verminderen of te voorkomen. Deze aanpak is echter niet altijd haalbaar voor ernstig zieke patiënten of ouderen. Wanneer men de klinische uitkomst van patiënten met skeletspieratrofie wil verbeteren, is het essentieel om inzicht te verwerven in de etiologie en de onderliggende mechanismen van verschillende aandoeningen geassocieerd met spieratrofie, alsook om de determinanten van spiermassa bij gezonde volwassenen te identificeren.

Androgenen zijn de belangrijkste geslachtshormonen die de lichaamssamenstelling regelen en zijn vooral gekend voor hun anabool effect op de spieren. Hun klinische toepassingen op de behandeling van spieratrofie is echter beperkt omwille van de ernstige bijwerkingen. Het is daarom belangrijk om therapeutische middelen te ontwikkelen die anabole effecten uitoefenen op de skeletspieren, zonder androgene activiteit op andere perifere weefsels. Een betere kennis over de androgene regulatie van de skeletspiermassa en van de signalisatiewegen die gereguleerd worden door androgenen, kan helpen om nieuwe spier-specifieke therapeutische doelwitten te identificeren voor de behandeling of preventie van spieratrofie.

Een eerste doel van dit proefschrift was om meer inzicht te krijgen in de interindividuele variatie in spiermassa. De determinanten van spiermassa en -functie werden uitgebreid onderzocht in een cross-sectionele studie (**studie 1**) bestaande uit een cohorte van 677 gezonde mannen (25-45 jaar). Er werd bovendien getracht om genetische variaties in de androgeenreceptor (AR), die geassocieerd zijn met serum testosteron (T) concentraties en spiermassa, te identificeren. Onze resultaten bevestigden dat spiermassa en –kracht sterk erfelijk bepaald zijn, en beïnvloed worden door leeftijd, antropometrie, lichaamssamenstelling, fysieke activiteit en geslachtshormonen. Naast het aantal CAG-repeats vonden we 2 single nucleotide polymorphisms (SNPs) (rs5965433 en rs5919392) in de AR die geassocieerd zijn met serum T concentraties. Echter, er werd geen bewijs geleverd dat deze genetische variaties in de AR ook een invloed hebben op de spiermassa of -functie.

In een tweede deel van deze thesis werden de effecten van T en estradiol (E_2) onderzocht op de signalisatiewegen, die degradatie van spiereiwit reguleren, in een *"androgeen deprivatie geïnduceerd spieratrofie muismodel"* (**studie 2**). De gen- en eiwitexpressie van drie verschillende spieratrofie inducerende targets nl. Atrogin-1, MuRF1 and myostatine werden op 3 verschillende tijdsstippen (1, 7 en 30 dagen) en in 3 verschillende spiertypes [extensor digitorum longus (EDL), soleus (SOL) en levator ani/bulbocavernosus spieren (LA/BC)] van gecastreerde muizen onderzocht. Uit onze resultaten bleek dat er belangrijke verschillen in atrofiesignalisatie bestaan tussen de LA/BC en de locomotorische spieren. In de LA/BC resulteerde androgeen deprivatie in een snelle en aanhoudende opregulatie van Atrogin-1 en MuRF1 mRNA, en in een neerregulatie van myostatine mRNA gedurende een periode van 30 dagen. Deze effecten waren reversibel na toediening van T. In de SOL en EDL was er een minder uitgesproken opregulatie van beide atrogenen kort na castratie (na 1 dag), terwijl de myostatine genexpressie enkel in de EDL werd opgereguleerd. Expressies van de eiwitten Atrogin-1, MuRF1 of myostatine in de EDL bleven ongewijzigd op elk gemeten tijdstip na orchidectomie. Deze resultaten stellen de rol van deze atrogenen in de androgene regulatie van de locomotorische spiermassa in vraag. Onze resultaten toonden bovendien aan dat het anabool effect van E_2-behandeling op de LA/BC tijdens androgeen deprivatie gepaard ging met een partiële onderdrukking van Atrogin-1 en MuRF1 genexpressie, wat het belang van verder onderzoek naar de effecten van E_2 op de spiermassa aanduidt.

In **studie 3** werd de androgene regulatie van atrofie en hypertrofie signaalmoleculen onderzocht in een *"inactiviteit spieratrofie muismodel".* Na 1, 5 en 14 dagen 'staartophanging' werden gen- en eiwitexpressies van IGF1/Akt/$p70^{S6K}$, alsook van myostatine, REDD1, atrogin-1 and MurF1 in de SOL en EDL onderzocht. Gezien eerdere studies aantoonden dat inactiviteit spieratrofie geassocieerd is met verlaagde serum T spiegels, veronderstelden we dat het therapeutisch gebruik van androgenen in dit model anabole effecten zou hebben. Staartophanging resulteerde in een verhoging, gevolgd door een tijdelijke daling in T spiegels. IGF1 genexpressie was neergereguleerd tijdens 1 dag en 5 dagen staartophanging, gevolgd door een daling in gefosforyleerd Akt na 14 dagen staartophanging. Atrogin-1, MuRF1, myostatine en REDD1 genexpressies waren tijdelijk opgereguleerd al vanaf 1 dag immobilisatie, nog voordat spieratrofie werd waargenomen. T behandeling tijdens staartophanging was echter niet in staat om de spiermassa te verbeteren, noch om de expressieniveaus van de catabole en anabole signaalmoleculen te herstellen. Dit wijst erop dat de anabole effecten van T niet voldoende zijn om spiermassa tijdens spier inactiviteit te behouden of te verbeteren.

Deze thesis draagt bij tot een betere kennis van de moleculaire regulatie van skeletspiermassa door T en E_2 door gebruik te maken van 3 verschillende modellen, nl. een cohorte van eugonadale mannen, een androgeen deprivatie geïnduceerd spieratrofie muismodel (orchidectomie), en een

spier inactiviteit muismodel (via staartophanging). Onze resultaten leverden bewijs voor een complex mechanisme waarbij T de spiermassa reguleert, met belangrijke tijds- en spiertype afhankelijke verschillen.

SUMMARY

Muscle atrophy or the unintended loss of skeletal muscle mass can occur as a consequence of pathological disorders e.g. muscular dystrophies, chronic diseases (cachexia), malnutrition, immobilisation (disuse), as well as from normal ageing (sarcopenia). As skeletal muscles play vital roles in maintaining body posture, controlling movement and regulating whole body protein metabolism, muscle atrophy can have debilitating consequences for the patient, often leading to a reduced quality of life. The prevention or therapy of skeletal muscle atrophy has been a topic of interest for many years and its applications are spreading from sport sciences to ageing and clinical medicine.

Physical exercise, whether or not combined with nutritional supplements, is an effective and safe countermeasure to attenuate or prevent muscle wasting. However, this approach is not always feasible in seriously ill patients, elderly people or those who have suffered severe injuries. Therefore, a thorough understanding of the etiology and underlying mechanisms of different muscle wasting conditions, together with the identification of factors determining muscle mass in healthy adults, is important to improve the clinical outcome for people suffering from skeletal muscle atrophy.

Androgens are considered to be the main sex steroids regulating body composition, with decades of research highlighting their anabolic effect on muscle mass. However, their clinical application in the treatment of muscle wasting is limited because of severe side effects. Therapeutic agents that could achieve anabolic effects on skeletal muscle without androgenic activities on other pheripheral tissues are of great clinical interest. Enhancing our understanding of the androgenic regulation of skeletal muscle mass and of the molecular factors and signalling pathways modulated by androgens, may help in the identification of novel muscle-specific therapeutic targets to combat the devastating effects of muscle wasting.

A first aim of this thesis was to gain more insight into the interindividual variation in skeletal muscle mass. In a population-based cross-sectional study (**study 1**), we extensively investigated the determinants of muscle mass and function in a cohort of 677 healthy young men (25-45 years). Moreover we tried to indentify genetic variations in the androgen receptor (AR) that are associated with serum testosterone (T) levels and muscle mass and function. Our results mainly confirmed previous findings that skeletal muscle mass and strength are highly heritable and are influenced by age, anthropometrics, body composition, physical activity and sex steroid levels. Next to the number of CAG repeats, we identified two single nucleotide polymorphisms (SNPs) (rs5965433 and rs5919392) in the AR gene that are associated with serum T levels. However, we could not provide evidence that these genetic variations in the AR gene also affect muscle mass or function.

In a second part of this thesis, we investigated the effects of T and estradiol (E_2) administration on the signalling pathway regulating muscle protein degradation in an *"androgen deprivation-induced muscle atrophy mice model"* (**study 2**). The gene and protein expression levels of muscle atrophy-inducing targets including Atrogin-1, MuRF1 and myostatin were measured at 3 different time-points (1, 7, and 30 days) and in 3 different muscle types [extensor digitorum longus (EDL), soleus (SOL) and the levator ani/bulcocavernosus muscles (LA/BC)] following orchidectomy of male mice. Our results showed important differences in atrophy signalling response between the LA/BC and the locomotor muscles. In the LA/BC, androgen deprivation resulted in a rapid and persistent upregulation of Atrogin-1 and MuRF1 mRNA and a downregulation of myostatin mRNA during the 30 day period, effects which were fully reversed by T. In the SOL and EDL muscle, a less pronounced upregulation of both atrogenes was only detectable early after orchidectomy (day 1), while myostatin mRNA levels were upregulated in the EDL only. No changes in the protein levels of Atrogin-1, MuRF1 and myostatin in EDL were found at any time point following orchidectomy, questioning their role in the androgenic regulation of the locomotor muscle mass. Furthermore, our results demonstrated that E_2-treatment during androgen deprivation has anabolic effects on the LA/BC, which was associated with a partial suppression of Atrogin-1 and MuRF1 gene expression, indicating that further experiments examining the effects of E_2 on skeletal muscle mass are of potential significance.

In **study 3,** the regulation of atrophy and hypertrophy signalling molecules by T were examined in a "*disuse atrophy mice model".* Following 1, 5 and 14 days of tail suspension, gene and protein expression levels of IGF1/Akt/$p70^{S6K}$ as well as myostatin, REDD1, Atrogin-1 and MuRF1 were examined in the SOL and EDL muscle. Because previous studies have shown that disuse atrophy is associated with reductions in serum T levels, we hypothesized that the therapeutic use of androgens in this setting would be beneficial. Tail suspension resulted in an increase, followed by a transient drop, in T levels and a decrease in muscle mass. IGF1 mRNA levels were downregulated during 1 and 5 days of tail suspension, and a subsequent reduction in the phosphorylated levels of Akt after 14 days of tail suspension was observed. Atrogin-1, MuRF1, myostatin and REDD1 gene expression levels were rapidly and transiently upregulated as early as 1 day following immobilization, even before muscle atrophy was observed. However, T treatment during tail suspension was not able to ameliorate muscle mass and did not restore the alterations in expression levels of catabolic and anabolic signaling molecules, indicating that the known anabolic effects of T are not sufficient to maintain muscle mass during muscle disuse.

In conclusion, this thesis contributed to the better understanding of the molecular regulation of skeletal muscle mass by androgens and estrogens using 3 different models: a cohort of eugonadal men, an androgen deprivation-induced muscle atrophy mice model (orchidectomy), and a disuse

atrophy mice model (tail suspension). Our results provided evidence for a complex mechanism by which T regulates muscle mass, with important time- and muscle type dependent differences.

I. GENERAL INTRODUCTION

1. BACKGROUND

Skeletal muscle comprises about 40% of the human body, and the maintenance of skeletal muscle mass is crucial for human health. Next to its vital function in maintaining body posture and controlling movement, it also plays an important role in thermogenesis and whole body protein metabolism (Wolfe 2006; Karagounis and Hawley 2010). By serving as a protein reservoir, skeletal muscle is able to provide amino acids for other tissues in which the preservation of protein content is essential for survival (i.e. skin, brain, heart, and liver), especially in response to stress. In addition, skeletal muscle functions in energy homeostasis via insulin stimulated glucose uptake. It is therefore not surprising that the maintenance of adequate skeletal muscle mass is not only important for physical independence but also for disease prevention (especially diabetes and heart diseases), and thus quality of life (Wolfe 2006).

The prevention or therapy of skeletal muscle loss or muscle atrophy has been a topic of interest for many years in athletes, elderly people, astronauts and patients suffering from metabolic and neuromuscular diseases. Until now, only physical exercise, whether or not combined with nutritional supplements, appears to be an effective and safe countermeasure to attenuate or prevent muscle wasting (Mallinson and Murton 2013). However, this approach is not always feasible in seriously ill patients, elderly people or those who have suffered severe injuries. Therefore, a thorough understanding of the etiology and underlying mechanisms of different muscle wasting conditions, together with the identification of factors determining muscle mass in healthy adults, may lead to the development of effective strategies to prevent muscle wasting and to improve the clinical outcomes for patients suffering from skeletal muscle atrophy.

In this introducing chapter, a summary of the current knowledge regarding muscle wasting conditions and its countermeasures is described in the first part. In the second part, the recent advances in our knowledge regarding the mechanisms regulating muscle mass are discussed, and a more detailed overview of the hypertrophy and atrophy signalling pathways is given in part three. Finally the regulation and synthesis of sex steroids in men as well as their androgenic action on muscle mass and force are presented in the last part.

2. SKELETAL MUSCLE ATROPHY AND COUNTERMEASURES

2.1 Interindividual variation in skeletal muscle mass

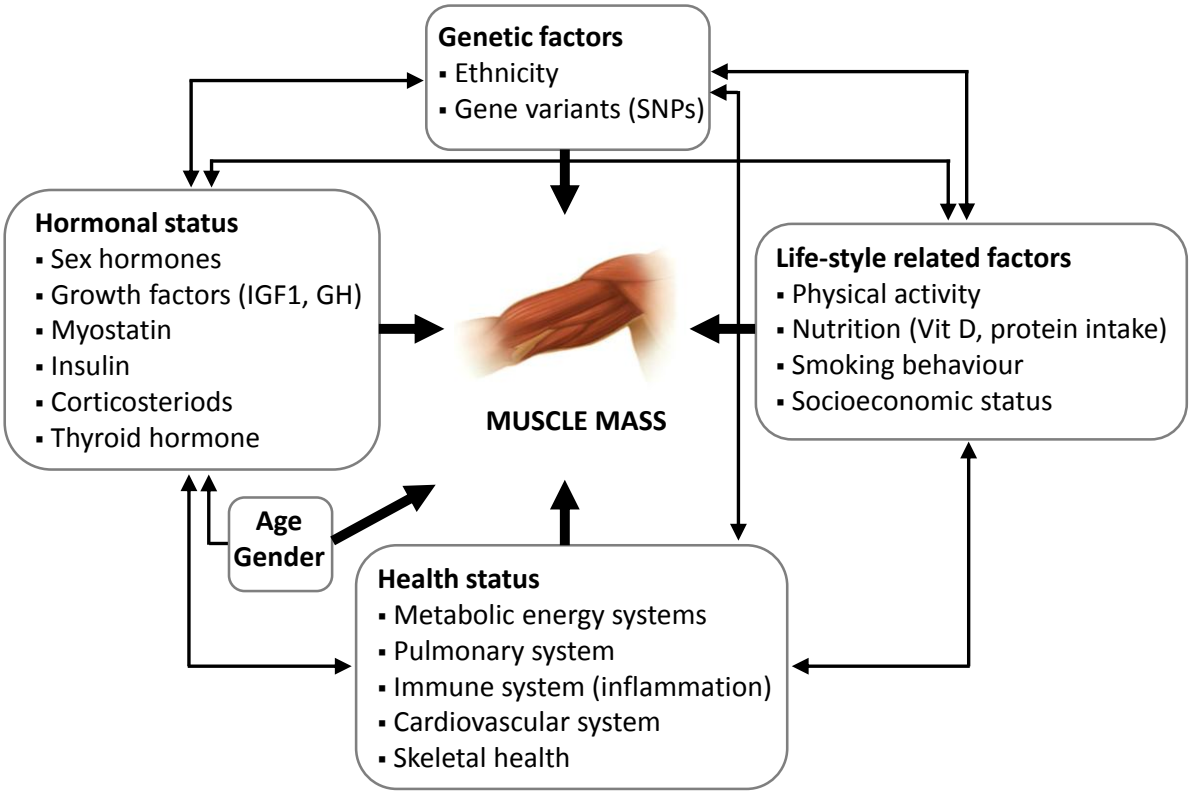

Figure 1. Scheme illustrating the determinants of skeletal muscle mass and function and their interactions. SNPs: single nucleotide polymorphisms, GH: growth hormone, IGF1: insulin-like growth factor 1. Based on data from Gerace et al. 1994; Gallagher et al. 1997; Arden and Spector 1997; Gallagher and Heymsfield 1998; Baumgartner et al. 1999; Rankinen et al. 2002; Szulc et al. 2004b; Beunen and Thomis 2004; Freitas et al. 2007; Geirsdottir et al. 2012.

The determinants of skeletal muscle mass and function and their interactions are illustrated in figure 1. It is well documented that individual differences in muscle cross-sectional area (CSA) and strength performance exist within a population (Hortobágyi and Katch 1990; Hulens et al. 2001). Gender and age are obvious determinants of muscle mass (Loos et al. 1997; Gallagher et al. 1997; Gallagher and Heymsfield 1998). The physical appearance of males is clearly different from females. Healthy young men are taller and heavier, with relatively more lean- and less fat mass. This sexual dimorphism and the profound changes in body composition during lifetime suggest a substantial impact of sex steroids in the determination of muscle mass (Vermeulen et al. 1999; Wells 2007). From cross-sectional and longitudinal studies, lean mass is known to be relatively stable during adult life. Muscle

mass generally peaks in the 20s or 30s and then declines (Baumgartner et al. 1999; Janssen et al. 2000), a process which might ultimately lead to sarcopenia (see section 2.2.5).

Studies in twins have suggested that both muscle mass and muscle strength are largely determined by ***genetic factors***, explaining 30-50% of their total variance (Arden and Spector 1997; Peeters et al. 2009). Genomic factors causing individual variation in muscle mass and strength are determining factors in predicting health status, but are also of interest in athletic populations. Over the last decade, several (genome-wide) linkage and association studies have screened the human genome for chromosomal regions where candidate genes causing variation in muscle mass (Karasik et al. 2009) and strength are located (De Mars et al. 2008; Bray et al. 2009; Windelinckx et al. 2011; Puthucheary et al. 2011). Within these genes, more than 200 single nucleotide polymorphisms (SNPs) have been identified, each representing a small portion of the total genetic component underlying the variability in health-related fitness and physical performance. Many of these genes encode proteins directly involved in the regulation of musculature such as muscle strength, muscle mass, fiber type, and muscle collagen. Also variations in genes encoding proteins with a functional role in the anatomical, biochemical and physiological systems e.g. in hormonal status, vitamin D metabolism, cardiovascular-, skeletal-, and pulmonary health, glucose-, lipid- and protein metabolism, inflammation and anthropometry (stature, weight), have been associated with muscle fitness. Besides, ethnic differences in muscle mass exist, with African-American subjects having significantly greater skeletal muscle mass compared with Caucasian subjects (Gerace et al. 1994; Gallagher et al. 1997). A more detailed overview of recent genetic work on muscular strength phenotypes can be found in Thomis & Aerssens (2012).

The majority of the remaining variance in muscle mass and strength can be explained by ***environmental factors***. Physical exercise and adequate protein intake are probably the most important environmental factors increasing muscle mass at all ages (Larsson 1982; Geirsdottir et al. 2012). On the other hand, chronic diseases and life-style related factors including tobacco smoking (Szulc et al. 2004b), malnutrition and low socioeconomic status (Freitas et al. 2007) are associated with lower lean mass. Like all variation in human phenotypes, muscle mass and strength in each individual result from an interaction between environmental stimuli and the individual's unique genotype (Beunen and Thomis 2004; Beunen and Thomis 2006). A collaborative research group of the FAMuSS study (Pescatello et al. 2013) has recently identified 17 genes that are associated with muscle strength and size at baseline and in response to resistance training. Further investigation of gene-training interaction effects are thus of potential interest.

2.2 Conditions associated with skeletal muscle atrophy

Muscle atrophy can occur as a consequence of pathological disorders (muscular dystrophies) (Emery 2002), chronic diseases (cachexia) (Evans et al. 2008), malnutrition or starvation (Pasiakos et al. 2010), hormonal deficiencies (androgen and growth hormones (GH) deficiency), immobilisation (disuse) (Urso 2009) as well as from normal ageing (sarcopenia) (Baumgartner et al. 1999). Ageing and several chronic diseases, e.g. chronic obstructive pulmonary diseases (COPD) and chronic heart failure, are accompanied with exercise intolerance and malnutrition, which in turn contribute to the loss of muscle mass (Glass 2003; Evans et al. 2008).

2.2.1 Muscle disuse

Muscle disuse atrophy is a consequence of long periods of inactivity as seen following limb immobilization, prolonged bed rest, and trauma to the neuromuscular apparatus i.e. denervation, paraplegia, amyotrophic lateral sclerosis (ALS), spinal cord injury (SCI), as well as in astronauts staying in microgravity environments (Glass 2003). All these muscle disuse atrophy conditions are caused by reduced neuromuscular activity or external loading, and are characterized by a rapid and extensive loss of skeletal muscle mass (up to 30%) and function within 2 days of disuse onset (Vandenburgh et al. 1999; Clark 2009; Narici and de Boer 2011). Age, muscle fiber type, and the degree of inactivity influence the rate and amount of muscle loss. It is well described that muscle disuse induces more pronounced atrophy in slow-twitch muscles when compared to fast-twitch muscles (Gardetto et al. 1989; Okamoto et al. 2011; Miokovic et al. 2012). A fiber type transition towards a more fast-twitch phenotype can explain the increased susceptibility to fatigue that goes along with the muscle atrophy (Kachaeva and Shenkman 2012).

2.2.2 Cachexia and muscular dystrophy

Muscle atrophy is also a serious complication of many chronic diseases such as heart-, liver- and renal failure, COPD, cancer and acquired immunodeficiency syndrome (AIDS). This loss of muscle is also known as cachexia, and is caused in part by an increase of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Evans et al. 2008).

Muscular dystrophies, e.g. Duchenne muscular dystrophy, are hereditary muscle disorders characterized by progressive degeneration of muscle fibers and thus muscle weakness. Genetic defects in genes encoding proteins involved in the organization of the muscle membrane, such as dystrofine, sarcoglycans and laminins, are the causes of the observed muscle atrophy (Emery 2002).

2.2.3 Hormonal deficiency syndromes and female-to-male transsexuality

Male-to-female transsexual persons or trans women often undergo estrogen therapy, together with anti-androgen treatment, to induce the secondary sex characteristics of females and to suppress endogenous testosterone (T) production (Hembree et al. 2009). Next to their role in sexual development, androgens are well-known regulators of muscle mass (see section 5). Androgen deficiency in trans women, as well as in hypogonadal men in which androgen deficiency results from testicular dysfunction or defects at the pituitary, is therefore often associated with a signifant loss in muscle mass (Bhasin et al. 1997). Although this loss in muscle mass is desired in trans women, it may result in important metabolic consequences (Wolfe 2006).

Adult patients with GH deficiency are also characterized by a reduced lean body mass. GH is considered to be the main hormone regulating tissue anabolism during stress and fasting. By direct action or by indirect stimulation of insulin growth factor-1 (IGF1) and insulin, GH increases lipolysis and free fatty acid levels during fasting, hereby preserving glucose and protein synthesis. Lack of GH or diminished GH secretion during stress and fasting are associated with increased protein breakdown and thus reduced lean body mass (Moller et al. 2009; Reed et al. 2013).

2.2.4 Food deprivation and malnutrition

Malnutrition in industrialized countries is a major concern in elderly people and during chronic illness, and often results from changes in appetite, chewing or swallowing problems, or malabsorption (Bolin et al. 2010). An adequate daily intake of proteins is important in the maintenance of muscle mass (Phillips 2011). In fasting conditions, skeletal muscles serve as a protein reservoir that is able to provide amino acids for other tissues such as brain, heart, and liver, in which the maintenance of protein content is essential for survival (Wolfe 2006; Pasiakos et al. 2010). Moreover, amino acids are important for maintaining the blood glucose concentration at appropriate levels during starvation, as they supply precursors for the hepatic gluconeogenesis (Felig et al. 1969). When muscle protein breakdown becomes inadequate, death from starvation may occur (Winick 1979).

2.2.5 Ageing

The term ‘sarcopenia’ was introduced by Rosenberg and Roubenoff (1995) and refers to the age-related decline in muscle mass and muscle strength. Sarcopenia is generally diagnosed when the percentage of skeletal muscle mass is more than two standard deviations below the mean of young healthy adults (Baumgartner et al. 1999). This age-related decrease in muscle mass and strength is

greatest in the appendicular skeletal muscles (i.e. the limb muscles), with preferential loss at the lower extremities (Gallagher et al. 2000; Visser et al. 2003). The rate of muscle loss is estimated to be 10% per decennium after the age of 50 (Hughes et al. 2002), and is related to a higher risk of falls, bone fractures, loss of independency and increased mortality. This functional loss, together with the age-related decline in maximal oxygen uptake (VO_2max) (Astrand et al. 1973), contribute to the development of frailty in the elderly. Considering demographic evolutions with ageing of the population, sarcopenia has emerged as a relevant public health issue (Janssen et al. 2004).

The exact mechanism underlying sarcopenia is not entirely understood, but the pathogenesis is probably multifactorial and complex (Narici and Maffulli 2010; Lang et al. 2010). Diminished physical activity and neuropathic (neurodegeneration), hormonal (sex steroids, GH, IGF1), immunological (inflammation) and nutritional (malnutrition) defects contribute to the etiology of sarcopenia (Rudman 1985; Dutta and Hadley 1995; Evans 1997; Lexell 1997). In men, age-related muscle loss has been attributed in part to the decline of androgen levels with age (Kaufman and Vermeulen 2005; Orwoll et al. 2006). There is evidence that muscle atrophy in post-menopausal women is associated with the decline in estrogen levels (Poehlman et al. 1995), but no consistency exists (see section 5.5). In contrast to muscle disuse, there is an apparent selective atrophy of the fast-twitch fibers in ageing muscles (Andersen 2003; Léger et al. 2008). This is explained by the age-related degeneration of α-motor units, especially of those innervating type II fibers, leading to muscle fiber denervation (Brown 1972; Doherty et al. 1993; Faulkner et al. 2007).

2.3 Therapeutic approaches counteracting muscle atrophy

2.3.1 Exercise

The anabolic effects of exercise, and in particular resistance exercise, on muscle is well established and is currently the most efficient and safe intervention to treat skeletal muscle atrophy (Swift et al. 2010; Nicastro et al. 2011). The efficiency of exercise training depends on the type, the duration and the intensity of the exercise. Physical exercise with high resistance loading appears efficient to prevent or counteract muscle atrophy during human bed rest (Shinohara et al. 2003; Trappe et al. 2007) and during hindlimb suspension in animal models (Herbert et al. 1988; Chopard et al. 2009). The beneficial effects of exercise on muscle mass are also well established in the elderly (van der Bij et al. 2002; Delecluse et al. 2004). However, it is clear that personalized exercise programs are necessary to ensure the feasibility and, more important, the maintenance of regular training in ageing people (Delecluse et al. 2004).

2.3.2 Nutrition

Food intake stimulates net muscle protein synthesis (Phillips 2011). However, it is not the higher energy intake per se, but the protein and amino acid ingestion in particular that is responsible for the increased rate of muscle protein synthesis. Much of the knowledge regarding the beneficial effects of protein intake has been derived from studies assessing nutritional interventions in athletes (Phillips 2004; Phillips and Van Loon 2011). However, recent studies also provided evidence regarding the efficacy of dietary protein intake on disuse muscle atrophy during bed rest (Paddon-Jones et al. 2004). During disuse, a reduction in energy requirement and/or appetite results in an inadequate dietary protein intake. Therefore, an adequate intake of proteins or essential amino acids during periods of disuse is important to attenuate muscle atrophy (Wall and van Loon 2013).

The current dietary reference intake for protein is 0.8 g/kg/day. However, the extent of protein synthesis is determined by the amount of protein (Bohé et al. 2001), the type of protein or amino acid (Churchward-Venne et al. 2014), and the timing of protein ingestion (Esmarck et al. 2001). Moreover, physical activity (resistance exercise) increases energy expenditure and food intake, and facilitates muscle protein anabolism (Aguirre et al. 2013).

Studies suggest that early post-exercise ingestion of 20–25 g (or 1.3-1.5 g/kg/day consumed over 4 meals) of high-quality protein (i.e. high in leucine content, e.g. whey protein), maximizes the response of muscle protein synthesis following resistance exercise in young, resistance-trained males. However, more protein may be required during periods of high frequency training (Phillips and Van Loon 2011). In elderly people or during periods of inactivity, a state of 'anabolic resistance' of the muscle, i.e. loss of the ability to build protein, may occur, and a protein dose twice as great as that in young persons is recommended for these people to minimize total body protein loss (Tipton and Phillips 2013).

Other nutritional supplements beneficial in promoting muscle mass include vitamin D (Ceglia and Harris 2013) and E (Servais et al. 2007), antioxidants (Arbogast et al. 2007) and creatine (Deldicque et al. 2005).

2.3.3 Pharmacological interventions

Several studies have aimed at testing pharmacological interventions to counter muscle wasting; however, conclusive results are limited. Available anabolic treatments for attenuating muscle wasting, were mostly tested in AIDS wasting and include cytokine modulators (anti-TNF-α, IL-6 inhibitors), appetite stimulants, clenbuterol (beta-2 adrenoreceptor agonist), growth hormones and anabolic steroids (reviewed by Glass and Roubenoff et al. (2010)). However, the effects of most of these treatments are not very large and are often accompanied by undesirable side effects.

Recombinant human GH and IGF1 have shown efficacy in manipulating skeletal muscle mass (Clemmons 2009) and are approved by the U.S. Food and Drugs Administration (FDA) for the treatment of AIDS wasting (Serostim, Merck Serono). However, next to its role in the anabolic pathways, IGF1 plays a major role in other biological processes including cell survival (Datta et al. 1999), cell differentiation (Florini et al. 1993; Coleman et al. 1995) cell proliferation (Sato et al. 2002), and regeneration (Musarò et al. 2001) as such that it can promote tumorigenesis. Another effective strategy that has been approved to stimulate muscle mass in AIDS-wasting and hypogonadal and elderly men is androgen therapy (Bhasin et al. 1996; Snyder et al. 1999). As will be discussed further, androgens are well-known for their anabolic effects, but because of their effects on multiple tissues, caution must be taken when treating patients with androgens.

"Factors determining muscle mass give the potential for lifestyle modifications and other clinical interventions, such as pharmacotherapy, to modify muscle mass and strength."

3 CELLULAR MECHANISMS REGULATING SKELETAL MUSCLE MASS

The maintenance of skeletal muscle mass is regulated by protein turnover and cell turnover, by which muscle fibers adapt in CSA and in fiber number respectively. The turnover rate of skeletal muscle protein is approximately 1–2% per day (Wall and van Loon 2013). As discussed in more detail below, cellular turnover mainly plays a role during embryonic and postnatal growth (Sartorelli and Fulco 2004), whereas skeletal muscle mass in adults is mainly determined by processes controlling muscle protein synthesis and muscle protein breakdown (section 4). By altering the net balance between protein synthesis and protein degradation, skeletal muscle is able to quickly adapt to changing environmental conditions such as mechanical stress, physical activity, nutrients, growth factors, hormones and pathophysiological states. A chronic imbalance results in either muscle loss (atrophy) or muscle growth (hypertrophy) (Phillips 2004) (Figure 2). In the next paragraphs, our current knowledge regarding the molecular events regulating skeletal muscle hypertrophy as well as muscle atrophy is summarized.

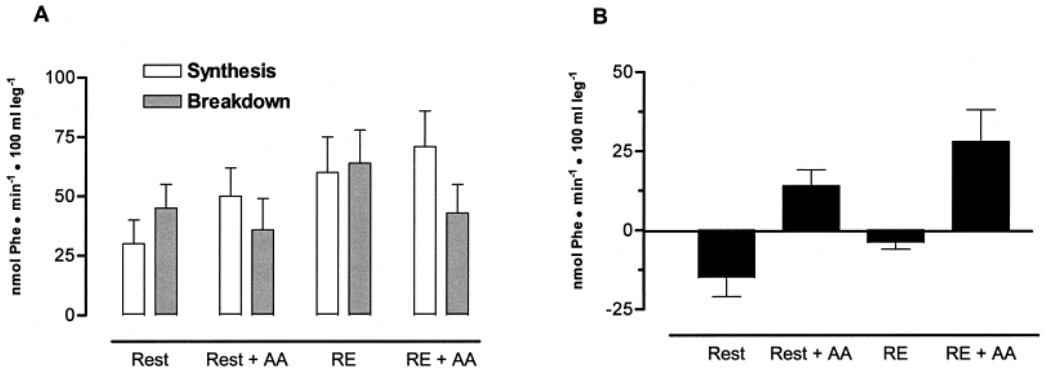

Figure 2. Adaptation of skeletal muscle to changing environmental conditions. (A) Influences of amino acid (AA) consumption at rest, performance of resistance exercise (RE), and AA consumption after RE on muscle protein synthesis and breakdown. (B) Net protein balance (synthesis minus breakdown) under the same conditions. Values are means ± standard deviation. Adopted from Phillips et al. (2004).

3.1 Skeletal muscle hypertrophy

3.1.1 The role of satellite cells

During the embryonic development of skeletal muscle, muscle progenitor cells originate from the mesodermal lineage and differentiate into myoblasts. These myoblasts subsequently become terminally differentiated myocytes and fuse to form multinucleated myotubes, which can mature into contracting muscle fibers. A distinct population of myoblasts fails to differentiate and remains

associated to the surface of the myofiber as satellite cells, where they play an important role in postnatal growth (Hawke and Garry 2001).

In adults, these satellite cells may become activated during muscle regeneration. Upon inflammatory stimulation after myotrauma from injury or exercise (Ciciliot and Schiaffino 2010; Macaluso and Myburgh 2012), quiescent satellite cells ($Pax7^+/MyoD^-$) become activated and enter an intermediate progenitor stage. Subsequent expression of MyoD and Myf5, transcriptional activators of the myogenic regulatory factor family, promotes progenitor cells to proliferate into myoblasts ($Pax7^+/MyoD^+$). Later on, these proliferating myoblast express p21, a cell cycle arrest protein, and Myogenin and MRF4, two myogenic regulatory factors, resulting in terminal differentiation into myotubes ($Pax7^-$/Myogenin). Fusion of the myotubes with the damaged fibers results in the addition of new myonuclei and increased fiber size. Activated satellite cells that do not express MyoD but continue to express Pax7 are able to self renew by returning to a quiescent state for the maintenance of a satellite cell pool (Figure 3) (Kadi and Thornell 2000; Chargé and Rudnicki 2004).

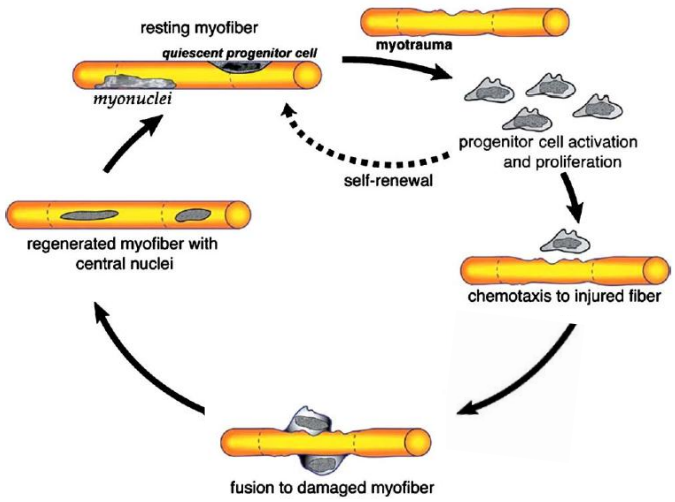

Figure 3. Satellite cell response to myotrauma in adult muscles. In response to injury, satellite cells become active and proliferate. Some of the satellite cells will re-establish a quiescent satellite cell pool via self-renewal. Satellite cells will migrate to the damaged region and fuse to the existing myofiber. Adopted from Hawke and Garry (2001).

Two signalling pathways, namely Notch and Wnt, play a critical role in muscle development and postnatal myogenesis. While Notch promotes the transition of activated satellite cells to highly proliferative myogenic precursor cells and myoblasts, Wnt plays a main role in myoblast differentiation and myotube fusion during muscle regeneration. Upon muscle injury, Notch signalling is activated which ultimately leads to the expression of its target genes Hey and Hes, both encoding

proteins involved in myogenesis such as Delta-1 and Notch-1. As long as Notch signalling is active, the differentiation of myoblasts into myotubes is prevented trough inhibition of Myogenin transcriptional activity. Cessation of Notch activity by Numb results in activation of Wnt signalling and subsequently upregulation of Wnt downstream target genes c-myc and Cyclin D1 (Tsivitse 2010; Buas and Kadesch 2010). Recently, also the Hippo pathway member Yap has been identified as an important promotor of satellite cell proliferation (Judson et al. 2012).

Although satellite cells play a role in adult muscle homeostasis, their contribution in cellular turnover during muscle hypertrophy in adulthood remains equivocal (McCarthy and Esser 2007; Rehfeldt 2007; Amthor et al. 2009; Blaauw et al. 2009; McCarthy et al. 2011). However, there is growing evidence supporting the idea that satellite cells play a key role in determining the potential for skeletal muscle hypertrophy. As each single myonucleus controls cellular processes for a certain amount of cytoplasm within a muscle fiber (i.e. myonuclear domain), an increased incorporation of new myonuclei seems to be necessary to allow muscle fiber hypertrophy (Zammit et al. 2006; Petrella et al. 2008; Snijders et al. 2009; Bruusgaard et al. 2010; Verdijk et al. 2013).

3.2 Skeletal muscle atrophy

Muscle atrophy is characterized by shrinkage of the muscle fibers caused by a loss of contractile proteins. Next to its role in controlling muscle mass, muscle protein breakdown is also an essential mechanism that prevents the accumulation of damaged or misfolded proteins (Wolfe 2006). Although Lexell et al. (1997) provided evidence for a loss in fiber number during sarcopenia, the contribution of cellular turnover during muscle atrophy seems unlikely.

Up to date, four proteolytic systems involved in protein degradation are described: the adenosine triphosphate (ATP)-dependent ubiquitin-proteasome pathway (UPP), the autophagy-lysosomal pathway, the Ca^{2+}-dependent calpain system, and the caspase system, with the first two being the most important cell proteolytic systems that control protein turnover in muscle (Figure 4). Because this thesis only focuses on the UPP, a more detailed overview of this pathway will be given.

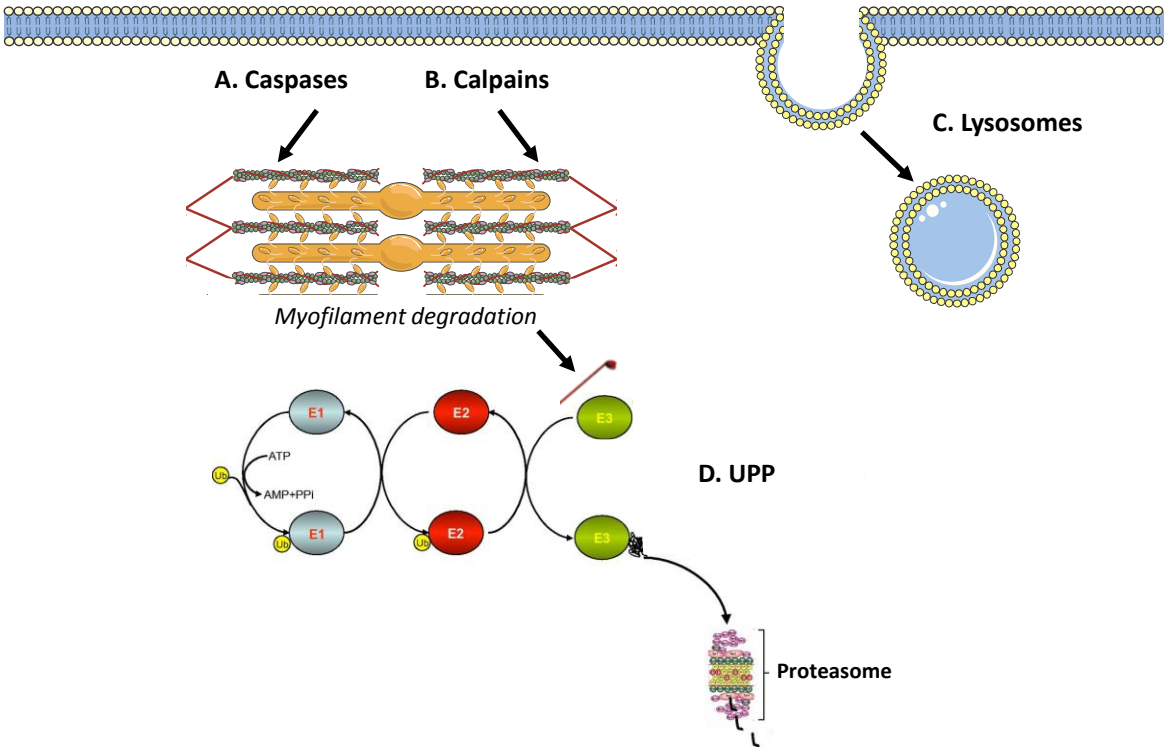

Figure 4. Four main proteolytic systems implicated in muscle atrophy. The caspase system (A), the Ca^{2+}-dependent calpain system (B), the autophagy-lysosomal system (C), and the ubiquitin-proteasome system (UPP) (D). Recent evidence points toward interactive involvement of these 4 systems in proteolysis. Adapted from Jackman and Kandarian (2004) and Bonaldo and Sandri (2013).

3.2.1 The ubiquitin-proteasome pathway (UPP)

In 2004, Aaron Ciechanover won the Nobel Prize in Chemistry for his discovery of the involvement of ubiquitin in protein degradation (http://www.nobelprize.org/nobel_prizes/chemistry/laureates/) (Ciechanover et al. 1980b; Ciechanover et al. 1980a). Wing et al. (2005) were the first to describe increased levels of ubiquitinated proteins in rat skeletal muscles during denervation and starvation, implicating a role for the ubiquitin system in atrophying skeletal muscle. Ubiquitin is a small conserved protein that functions as a signalling molecule for protein degradation. Substrates covalently bound to a polyubiquitin chain are recognized and degraded into small peptides by the proteasome. This process involves a series of sequential reactions (Figure 5).

Ubiquitin-activating enzymes (E1) mediate the first step in the ubiquitin-proteasome-dependent proteolysis process by activating ubiquitin monomers using ATP. This is subsequently followed by the transfer of the ubiquitin to a ubiquitin-carrying enzyme (E2). Finally, a ubiquitin-ligase enzyme (E3) transfers the ubiquitin from the E2 enzyme to the protein substrate (Solomon and Goldberg 1996; Jagoe and Goldberg 2001). The repetition of these reactions leads to the formation of a

polyubiquitin chain that allows recognition of the protein substrate by the proteasome 26S (Pickart 2000; Jagoe and Goldberg 2001; Wing 2005). The 26S proteasome is a large complex (2000kDa) made up of at least 50 subunits (Jagoe and Goldberg 2001). The most important subunits are 19S and 20S. 19S is a regulatory subunit that recognizes, unfolds and translocates the protein substrate to the 20S subunit, the central proteolytic core of the proteasome. The polyubiquitin chain is subsequently broken into free ubiquitin molecules, by specific deubiquitinating enzymes, which can then be re-used in the degradation of other proteins. The rate-limiting step in the degradation process is believed to be determined by the action of E3s, which are responsible for the specificity of substrate recognition. The human genome encodes more than 650 ubiquitin ligases (E3), from which Muscle Atrophy F-Box (MAFbx, also known as Atrogin-1 and Fbxo32), Muscle Really interesting new gene Finger-1 (MuRF1, also known as Trim63) (Bodine et al. 2001a; Gomes et al. 2001), and recently F-box protein 40 (Fbxo40) (Shi et al. 2011) were identified to be muscle-specific (see section 4.3.2). Nedd4, E3α, Mdm2, CHIP, XIAP, Trim32, TRAF6, Mul1 and ASB2β are other ubiquitin ligases which seem to play an important role in a variety of atrophy conditions including denervation, hindlimb unloading and starvation (Cao et al. 2005; Bello et al. 2009; Paul et al. 2010; Cohen et al. 2012; Lokireddy et al. 2012).

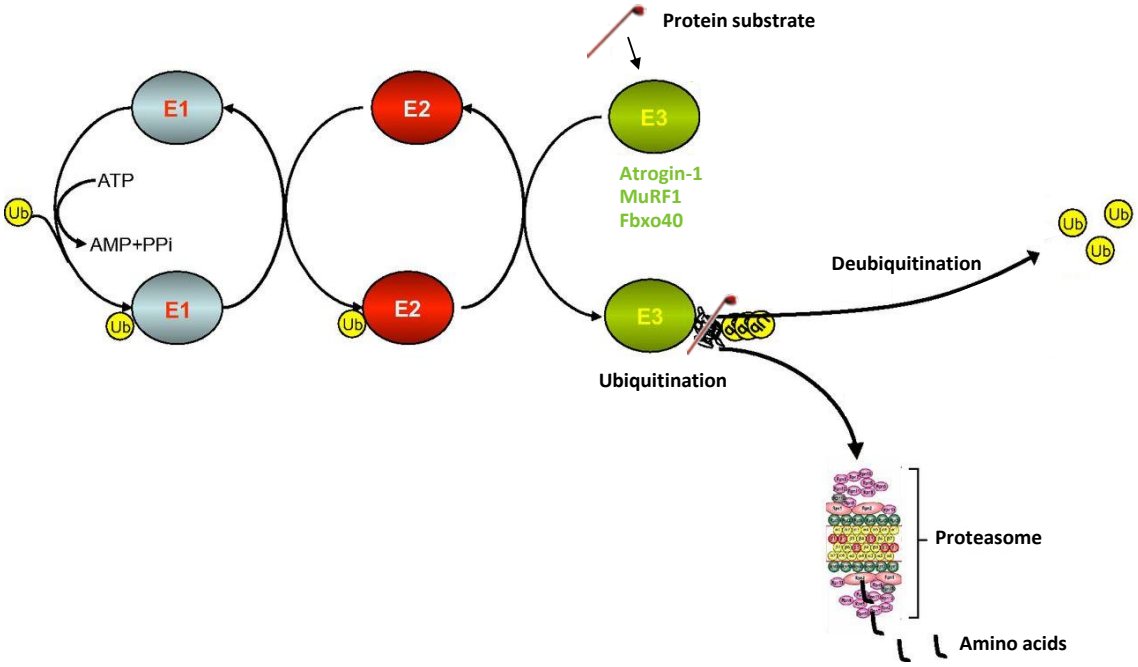

Figure 5. The Ubiquitin-proteasome system for degrading target protein. Ubiquitin-activating enzymes (E1) activate ubiquitin proteins (Ub) after the cleavage of ATP. The ubiquitin is then moved from E1 to members of the ubiquitin-carrying enzyme (E2) class. The final ubiquitylation reaction is catalysed by members of the ubiquitin-ligase enzyme (E3) class. E3 binds to E2 and the protein substrate, inducing the transfer of ubiquitin from E2 to the substrate. Once the substrate is polyubiquitylated, it is docked to the proteasome for degradation. Adapted from Bonaldo and Marco (2013).

The UPP system is believed to degrade mainly unbound myofibrillar proteins, and most soluble short-lived proteins. Under normal physiological conditions, the ATP-dependent UPP constantly degrades damaged or misfolded proteins to mediate normal protein turnover. Moreover, it is involved in a variety of cellular processes such as inflammatory response, immune surveillance, degradation of transcriptional regulators and cell cycle regulation (Solomon and Goldberg 1996; Clarke et al. 2007; Fielitz et al. 2007; Cohen et al. 2009).

3.2.2 The autophagy-lysosomal pathway

Autophagy is a catabolic mechanism through which intracellular components are delivered to lysosomes for their breakdown (Figure 4C). These lysosomes are cellular organelles that contain hydrolases necessary for degradation. The autophagy-lysosome system was described many years ago during denervation-induced muscle atrophy in rats (Schiaffino and Hanzlíková 1972; Furuno et al. 1990), but its involvement in muscle catabolism has only recently been recognized. Autophagy is constitutively active in skeletal muscle and plays a crucial role in cellular remodelling by removal of long-lived soluble proteins, damaged organelles and protein aggregates (Bechet et al. 2005; Kirkin et al. 2009). Three different forms of autophagy have been described in mammals: macroautophagy, chaperone-mediated autophagy and microautophagy, each form having its own substrate selectivity and mechanism for delivering its target to the lysosomes. Macroautophagy is believed to be the main mechanism involved in lysosomal degradation (Bejarano and Cuervo 2010). Recent studies have shown that the autophagy-lysosome and ubiquitin-proteasome systems are coordinately regulated during muscle wasting (Mammucari et al. 2007; Zhao et al. 2007).

3.2.3 The calpain pathway

The Ca^{2+}-dependent calpains are implicated in many physiological functions, including apoptosis, cytoskeleton organization, signal transduction and proteolysis of proteins involved in cell cycle. The role of Ca^{2+}-mediated protein degradation during skeletal muscle atrophy was firstly reported by Tischler et al. (1990), who showed that inhibitors of Ca^{2+}-dependent proteolysis were able to attenuate protein degradation rates in skeletal muscle of unloaded rats. However, inconsistency exists about whether there rather is a change in mRNA or in activity of the calpains during different atrophy conditions (Taillandier et al. 1996; Spencer et al. 1997; Ikemoto et al. 2001; Haddad et al. 2003; Stevenson et al. 2003). Nevertheless, the calpains remain likely involved in myofibrillar disassembly by selective cleavage of sarcomere anchoring proteins, hereby providing muscle proteins, such as actin and myosin, to the ubiquitin proteasome system (Jackman and Kandarian 2004; Wing 2005) (Figure 4B).

3.2.4 The caspase system

The caspases, member of the family of cysteine proteases, are enzymes which cleave substrates preferentially after aspartic acid residues. They play an essential role in programmed cell death, but are also involved in several non-apoptotic functions (Kuranaga 2012). Caspase-3 was shown to cleave the actomyosin complex in skeletal muscles and may therefore, like calpains, be involved in providing substrates to the ubiquitin proteasome system (Du et al. 2004) (Figure 4A).

> *"More insight into the muscle-specific molecules involved in the regulation of muscle mass could lead to the identification of potential novel muscle-specific therapeutic targets for the prevention and treatment of muscle atrophy"*

4 HYPERTROPHY AND ATROPHY SIGNALLING PATHWAYS

Below, the current identified proteins involved in hypertrophy and atrophy signalling regulating muscle mass will be introduced, with emphasis on those regulating the UPP and those relevant to understand this thesis. It has recently become clear that muscle atrophy and hypertrophy signalling pathways are coordinately regulated by crosstalk and modulation at different levels, demonstrating that skeletal muscle atrophy is not simply the converse of hypertrophy (Hoffman and Nader 2004; Russell 2010) (Figure 6). The commonly up- or downregulated genes in different catabolic states are believed to regulate the loss of muscle components and are termed atrophy-related genes or 'atrogenes' (Sacheck et al. 2007).

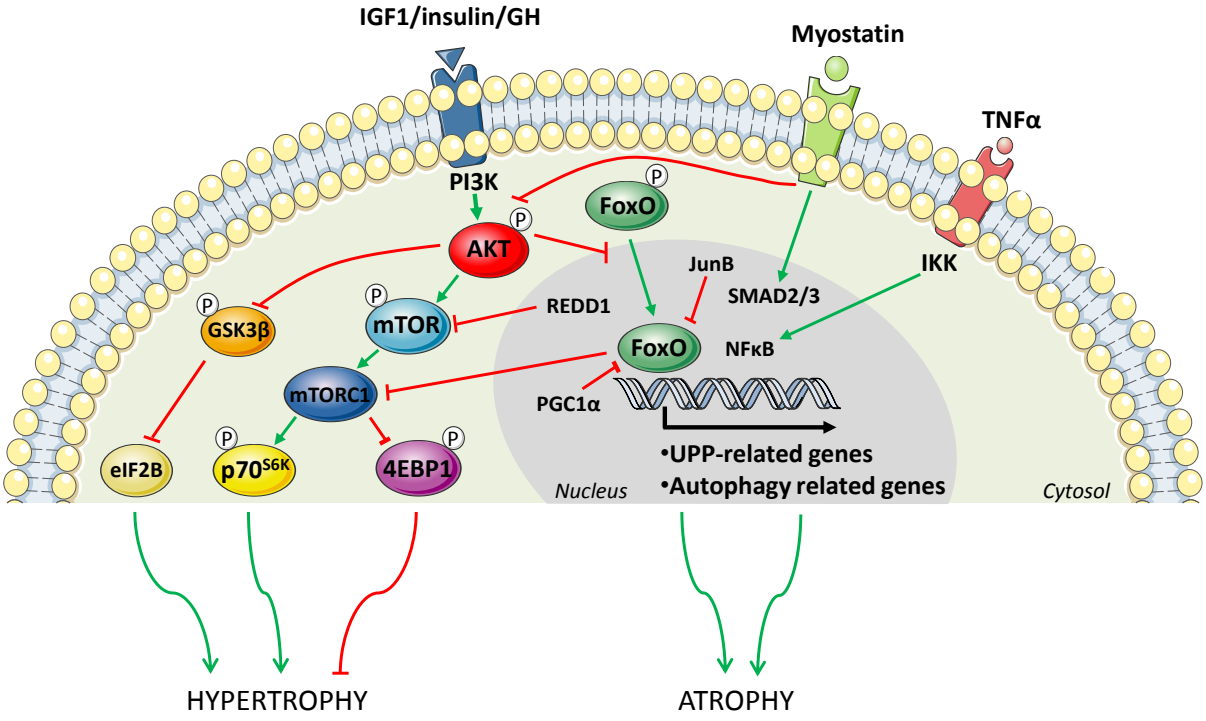

Figure 6. Main signalling pathways regulating muscle mass. Protein synthesis and degradation are regulated by multiple signalling pathways which are co-ordinately regulated. Green lines depict activation, red lines depict inhibition. Based on data from Bonaldo and Sandri (2013).

4.1 The pivotal role of Akt

IGF1 is one of the most well-characterized muscle growth-promoting factors and is produced under influence of GH in many tissues including the skeletal muscle itself. After binding to its receptor, phosphatidylinositol-3,4,5-triphosphate (PIP3) is generated by the recruitment of phosphoinositide 3-kinase (PI3K) which, in turn, recruits Akt (also known as protein kinase B) to the membrane where

it is activated by phosphorylation (Schiaffino and Mammucari 2011). Akt plays a role in muscle hypertrophy by both increasing protein synthesis and repressing protein degradation:

1. Akt promotes muscle hypertrophy via its phosphorylation and hence activation of the mammalian target of rapamycin (**mTOR**) (section 4.2).
2. Another downstream pathway of Akt is the glycogen synthase kinase 3β (**GSK3β**) pathway which regulates eukaryotic initiation factor 2B (eIF2B), a translation initiation factor involved in protein synthesis (Welsh et al. 1997; Jefferson et al. 1999).
3. Akt interacts with the atrophy signalling pathway by phosphorylating and inhibiting the forkhead box containing proteins, O-subclass (**FoxO**) family of transcription factors (Sandri et al. 2004) (section 4.3).

4.2 IGF1-Akt-mTOR signalling pathway

In vivo studies have shown that muscle hypertrophy can be blocked by rapamycin, an inhibitor of mTOR, demonstrating the rapamycin-dependent role of mTOR in muscle growth (Bodine et al. 2001b; Pallafacchina et al. 2002). In fact, mTOR is a major sensor of nutritional status, cellular stress and growth factor signals (Rivas et al. 2009; Shimizu et al. 2011; Xu et al. 2012). The serine/threonine kinase mTOR is part of two different complexes: mTORC1 and mTORC2 (Laplante and Sabatini 2012). mTORC1 regulates protein synthesis through the posphorylation of its downstream targets p70-ribosomal S6 kinase ($p70^{S6K}$) and factor 4E binding protein 1 (4EBP1). Phosphorylated $p70^{S6K}$ is able to activate S6, a ribosomal protein which is involved in protein translation (Ohanna et al. 2005), whereas phosphorylation of 4EBP1 releases its inhibitory effect on eukaryotic initiation factor 4E (eIF4E), which is involved in protein translation (Hara et al. 1997). Active mTORC1 is also believed to inhibit autophagy by phosphorylation and subsequenlty suppression of autophagy-related proteins such as ULK1 (Kim et al. 2011; Sanchez et al. 2012).

Regulated in Development and DNA damage responses 1 (REDD1), is believed to play a role in muscle atrophy by inhibiting mTOR activity (Brugarolas et al. 2004). REDD1 was first identified in response to hypoxia (Shoshani et al. 2002) and DNA damage (Ellisen et al. 2002) and its gene expression was subsequently found to be upregulated in different cell types in response to other stress stimuli such as endoplasmic reticulum stress, serum deprivation, and treatment with glucocorticoid, hydrogen peroxide or dexamethasone (Wang et al. 2003; Lee et al. 2004; Whitney et al. 2009). In skeletal muscles, REDD1 mRNA was recently shown to be upregulated in hindlimb immobilized rats (Kelleher et al. 2013) and following glucocorticoid treatment (Wang et al. 2006).

4.3 IGF1-Akt-FoxO signalling pathway

4.3.1 FoxO transcription factors

In skeletal muscle, three isoforms of FoxO have been described: FoxO1, FoxO3 en FoxO4, with FoxO3 being the best known isoform. The role of FoxO3 in the control of proteolysis was shown by Sandri et al. (2004) and Stitt et al. (2004) who demonstrated that blocking of FoxO3 function prevented muscle atrophy in mice and mouse myoblast cell line C2C12 respectively. Activation of FoxO3, on the other hand, induced loss of muscle mass.

The transcriptional activity of FoxO is determined by its phosphorylation state (Van Der Heide et al. 2004). When phosphorylated, FoxO is retained in the cytosol, whereas dephosphorylation promotes its translocation to the nucleus where it binds to the promoter region of its target genes and subsequently activates transcription. Studies in C2C12 cells and rodents have demonstrated that FoxO controls the expression of ubiquitin ligases of the UPP (Sandri et al. 2004) and key genes involved in the autophay-lysosomal pathway, e.g. LC3, Atg12 and ULK2 (Zhao et al. 2007). The role of two muscle-specific ubiquitin protein ligases Atrogin-1 (Sandri et al. 2004) and MuRF1 (Stitt et al. 2004) in skeletal muscle atrophy will be discussed in more detail below (section 4.3.2). FoxO also represses the hypertrophy-promoting protein mTOR and subsequently upregulates the hypertrophy-inhibiting protein 4EBP1 (Southgate et al. 2007; Demontis and Perrimon 2010), hereby mediating another crosstalk between protein breakdown and protein synthesis. As previously mentioned, a main negative regulator of FoxO function is Akt (Sandri et al. 2004).

FoxO transcriptional activity is also inhibited by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), a critical cofactor for mitochondrial biogenesis (Puigserver and Spiegelman 2003). Finally, the transcription factor JunB has been reported to block atrophy by interacting with FoxO on the one hand, and by inhibiting the myostatin signalling pathway on the other hand (section 4.4) (Raffaello et al. 2010).

4.3.2 The role of Atrogin-1 and MuRF1 in skeletal muscle atrophy

In 2001, Gomes et al. (2001) and Bodine et al. (2001a) identified two muscle-specific ubiquitin protein ligases, Atrogin-1 and MuRF1, which genes were found to be commonly upregulated in different models of muscle atrophy. Mice knocked out (KO) for Atrogin-1 were subsequently shown to be partially protected against muscle atrophy induced by denervation (Bodine et al. 2001a) and fasting (Cong et al. 2011), whereas MuRF1 KO mice were shown to be resistant to dexamethasone-induced (Baehr et al. 2011) and denervation-induced muscle atrophy (Bodine et al. 2001a). From

then on, extensive progress has been made in understanding the molecular mechanisms of the UPP-dependent muscle atrophy system. Increased Atrogin-1 gene expression has been observed in more than 13 in vitro and in vivo rodent models of muscle atrophy including uremia, ageing, denervation, diabetes, burn injury, underweighting, sepsis, hindlimb suspension, cancer, renal failure, and treatment with dexamethasone, cytokines and TNF-α (Sacheck et al. 2007).

Although both ubiquitin protein ligases appeared to be promising therapeutic targets, a number of reports were unable to show a correlation between Atrogin-1 gene expression levels and rates of protein breakdown (Krawiec et al. 2005; Fareed et al. 2006; Dehoux et al. 2007). Moreover, few studies have measured changes in protein levels under atrophic conditions (Doucet et al. 2007; Léger et al. 2009; Nedergaard et al. 2012), or have investigated the actual functional role of Atrogin-1 and MuRF1. The identification of substrates of Atrogin-1 and MuRF1 are still under intense investigation, and will certainly aid in our knowledge of their regulation and function.

4.4 Myostatin, a negative regulator of muscle growth

Myostatin is a member of the transforming growth factor-β (TGF-β) and acts as a negative regulator of myogenic differentiation (Rios et al. 2002) and postnatal muscle growth (Taylor et al. 2001). Myostatin is secreted by the skeletal muscle itself, and binds to its activin recepor II B (Lee et al. 2005). Several studies in animals and humans have demonstrated that loss-of-function and other mutations in the myostatin gene result in a hypertrophic phenotype (McPherron and Lee 1997; Schuelke et al. 2004; Clop et al. 2006; Li et al. 2010). In addition, blocking of myostatin in adult muscles has been shown to increase muscle mass (Whittemore et al. 2003). Although there is evidence that myostatin treatment of muscle cell cultures activates FoxO and subsequently upregulates the ubiquitin ligases Atrogin-1 and MuRF1 (McFarlane et al. 2006), its role in atrophy in vivo is less clear as muscle-specific overexpression of myostatin in transgenic mice and rats resulted in only 20% of muscle loss in males, but not in females (Reisz-Porszasz et al. 2003; Durieux et al. 2007). The mechanisms of its action on the hypertrophy and atrophy signalling cascades are thus far from elucidated, but recent reports suggest that myostatin-induced muscle atrophy in adulthood is established via blockade of Akt signalling (Trendelenburg et al. 2009) and may depend on the transcription factors Smad2 and Smad3 (Sartori et al. 2009).

4.5 NF-κB signalling pathway

The nuclear factor kappa B (NF-κB) is involved in a variety of cellular processes including immunity and inflammation. In skeletal muscle, NF-κB plays a major role in mediating the effect of

inflammatory cytokines on muscle wasting (Peterson et al. 2011), which was primarily described during TNF-α cytokine-mediated atrophy (Li and Reid 2000). Under basal conditions, NF-κB is retained in the cytosol by the inhibitor of nuclear factor of kappa B (IκB). TNFα is able to activate NF-κB by phosphorylation of IκB kinase (IKK) which subsequently degradates IκB, allowing NF-κB to translocate into the nucleus. Inhibition of NF-κB during denervation has shown to inhibit atrophy (Judge et al. 2007), whereas muscle-specific overexpression of IKK in transgenic mice resulted in severe muscle wasting (Cai et al. 2004). The atrophic effect of NF-κB is believed to be partly mediated by activation of MuRF1 (Cai et al. 2004) and by upregulation of autophagy-related genes (Mofarrahi et al. 2012).

TNFα is also known to regulate the transcription of suppressor of cytokine signalling-3 (SOCS-3) (Emanuelli et al. 2001), hereby inhibiting GH signalling which possibly results in a reduction of IGF1 transcription (Hansen et al. 1999; Ram and Waxman 1999).

4.6 Signalling pathways involved in disuse muscle atrophy

Several rodent and human models have been developed in order to study muscle disuse atrophy including (head-down) bed rest, casting, denervation, joint immobilisation and hindlimb unloading. Tail suspension in rodents, in which the hindlimbs are unloaded by caudal elevation via the tail, has been frequently used to mimic microgravity (Morey-Holton and Globus 2002). New insights in the molecular and cellular mechanisms of disuse-induced muscle loss have been derived from those models, as reviewed by Urso et al. (2009) and Chopard et al. (2009). The majority of them have provided evidence that muscle disuse atrophy results from a decreased rate in protein synthesis. Decreased signalling through PI3K-Akt-mTOR and consequently decreased phosphorylation of S6K1 and 4EBP1 have been reported in rodent models of immobilization and hindlimb unloading (Hornberger et al. 2001; Haddad et al. 2003; Kelleher et al. 2013; Bodine 2013). In addition, increased myostatin gene and protein expression (Wehling et al. 2000) and increased activation of NF-κB (Hunter et al. 2002) have been described in rats subjected to hindlimb unloading. Although some researchers believe that a reduction in protein synthesis is the only cause for the disuse-induced muscle atrophy (Phillips et al. 2009; Rennie et al. 2010), there are some arguments for a role of protein degradation. Increases in the mRNA levels of Atrogin-1 and MuRF1 have been reported in rodents and humans following immobilization (Jones et al. 2004; Krawiec et al. 2005; Haddad et al. 2006; Okamoto et al. 2011). Moreover, Labeit et al. (2010) have shown an attenuated atrophic response in MuRF1 KO mice subjected to unloading, whereas two other studies (Bodine et al. 2001a; Gomes et al. 2012) were able to modulate denervation-induced muscle atrophy in Atrogin-1- and MuRF1-null mice. Nevertheless, further research is needed to understand the regulation of MuRF1

and Atrogin-1 under disuse conditions and future studies looking for additional genes affecting both protein degradation and protein synthesis are required.

4.7 Promising therapeutic targets

The recent advances in our knowledge of the molecular regulation of skeletal muscle mass have led to promising pharmacological interventions to counteract muscle wasting. Targeting the myostatin pathway has become of major interest in the search for novel treatments. Different anti-myostatin compounds that block myostatin's function have been tested in different models (Wagner et al. 2008; Watt et al. 2010; Kawakami et al. 2011). However, blocking the signalling pathways downstream the activin receptor seems to be a better strategy (Tsuchida et al. 2009). Molecules that inhibit the activin II receptors have shown their positive effect on muscle mass (Sun et al. 2008; Ohsawa et al. 2012) and are currently being investigated in early phase clinical trials (Acceleron Pharmaceuticals and Novartis). Another class of promising drugs showing successful results in blocking atrophy in different animal models (Supinski et al. 2009; Caron et al. 2011; Jamart et al. 2011) are proteasome inhibitors. However, as mentioned earlier, protein breakdown is an important mechanism that prevents the build-up of damaged or misfolded proteins (Wolfe 2006), as such that prolonged inhibition of protein degradation can be detrimental for muscle cells.

A consideration in the development of muscle atrophy counteracting agents, is that there is growing evidence that the regulation of each individual skeletal muscle atrophy model depends on unique atrophic stimuli, as such that apparent differences exist concerning transcriptional and translational adaptations (Urso 2009). Moreover, studies in rodents and humans have suggested that muscles with differences in fiber type distribution respond differently in the same atrophic conditions (Degens and Alway 2006; Goodman et al. 2012).

> *"A thorough understanding of the specific molecular alterations involved in each atrophic condition and in each muscle fiber type may be important for the development of effective therapies to attenuate muscle atrophy."*

5 SEX STEROIDS AND THE MALE SKELETAL MUSCULATURE

In the following section, we will describe the regulation and synthesis of sex steroids in men as well as their androgenic action on muscle mass and force. Further, the molecular mechanisms of the androgenic and estrogenic action on skeletal muscle will be discussed in detail.

5.1 Sex steroid metabolism

Androgens are male sex hormones of the steroid family which are involved in sexual and reproductive functions. These male sex steroids are also essential for the development and maintenance of secondary sexual characteristics, including muscular development (Wilson et al. 1995). Furthermore, androgens are known for their metabolic effects on protein, carbohydrate, and fat metabolism and therefore contribute to the determination of muscle mass and strength, next to that of bone and fat mass (Mårin 1995; Urban et al. 1995; Köhn 2006). The major biologically active male sex steroid is T, which can be further metabolized into several biologically active hormones, such as dihydrotestosterone (DHT) (Horton and Tait 1967) and 17β-estradiol (E_2) (Gooren and Toorians 2003). Only 1 to 2% of the circulating T is free (FT), and together with the albumin bound T (40-50%), it represents the bio-available T, which has rapid access to the target cells (Vermeulen et al. 1971; Hammond et al. 1980). The remaining T is strongly bound to sex hormone binding globulin (SHBG) (50-60%) which is believed not to be readily available for biological action (Dunn et al. 1981).

In men, T is produced and secreted almost exclusively by the testes (95%) and to a lesser extent by the adrenals (5%). The synthesis of T is mediated through sequential cytochrome P450-dependent (CYP) and hydroxysteroid dehydrogenase-dependent (HSD) enzymatic reactions (Figure 7). The first and rate-limiting step is the conversion of cholesterol to pregnenolone, which is regulated by a negative feedback mechanism involving the hypothalamo-pituitary-gonadal axis (HPG). Gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and stimulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary. LH and FSH are responsible for the stimulation of T production and for spermatogenesis respectively. In turn, circulating T exerts a negative feedback at hypothalamic and pituitary level by inhibiting the release of GnRH and LH (Stocco and Clark 1996).

From the daily produced amount of T, approximately 1% is converted into E_2 by the aromatase enzyme (CYP19). This enzyme is mainly present in adipose tissue, but can also be found in other peripheral tissues such as liver, brain, muscle, bone and gonads. The remaining E_2 (20%) existing in the circulation is directly secreted by the testes (Thompson and Siiteri 1973). In peripheral tissues, T can also be irreversibly converted into DHT by the enzyme 5α-reductase. DHT is a biologically more

potent androgen which is able to locally activate the androgen receptor (AR). Next to the testes, 5α-reductase is also present in the brain, skin and prostate gland, and its gene expression has been found in muscle tissue (Sato et al. 2008), although its activity in the latter remains uncertain. 20% of the circulating DHT originates from direct conversion of T in the testes, whereas the remaining 80% is the result of 5α-reduction of T in peripheral tissues (Russell and Wilson 1994; Aizawa et al. 2007).

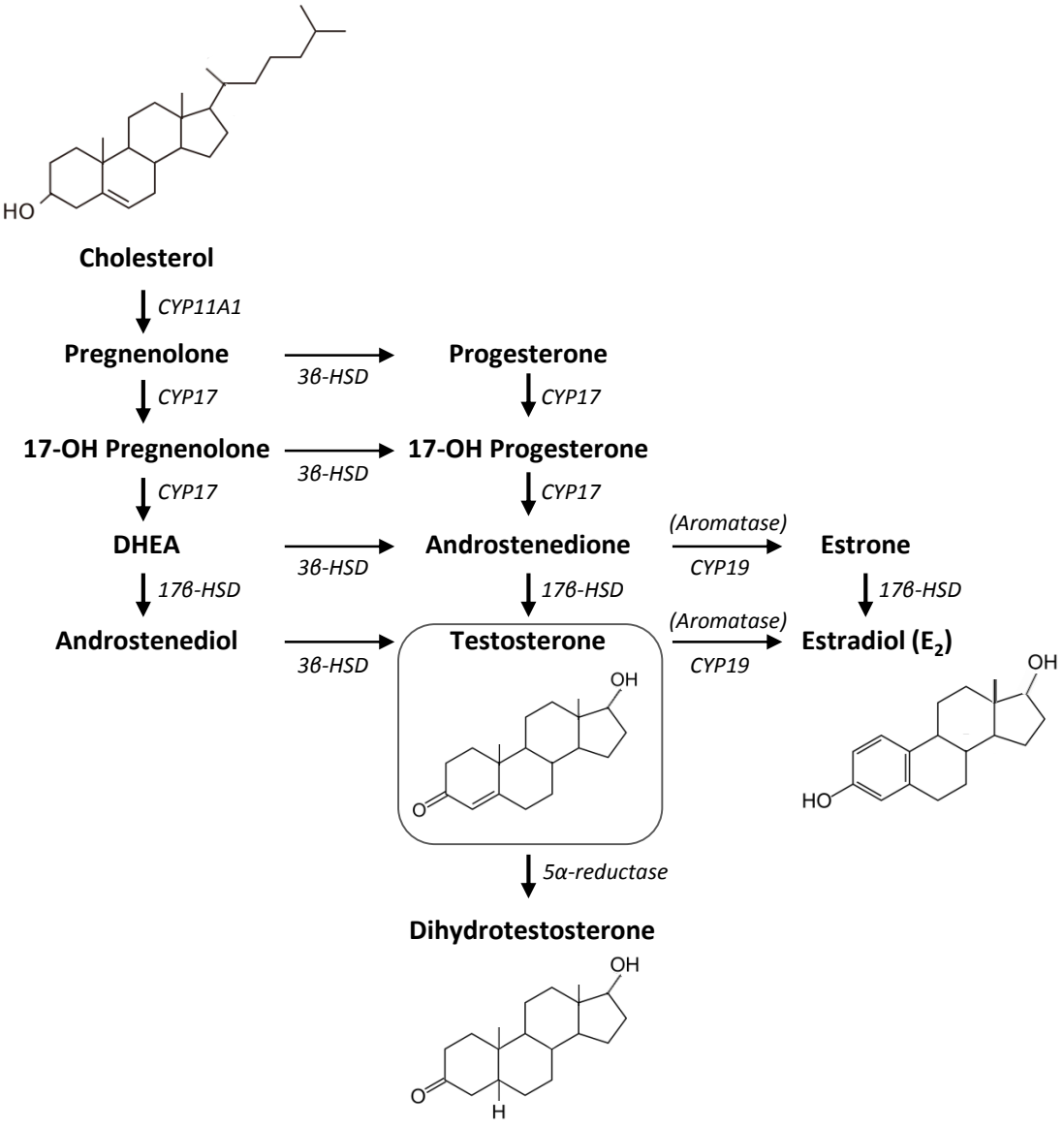

Figure 7. The major sex steroid hormone biosynthesis pathways. CYP11A1: Cholesterol side chain cleavage enzyme, 3β-HSD: 3β-hydroxysteroid dehydrogenase, CYP17: 17α-hydroxylase, 17β-HSD: 17β-hydroxysteroid dehydrogenase, DHEA: Dehydroepiandrosteron. Based on data from Federman et al. (2006).

5.2 Mechanism of androgen action

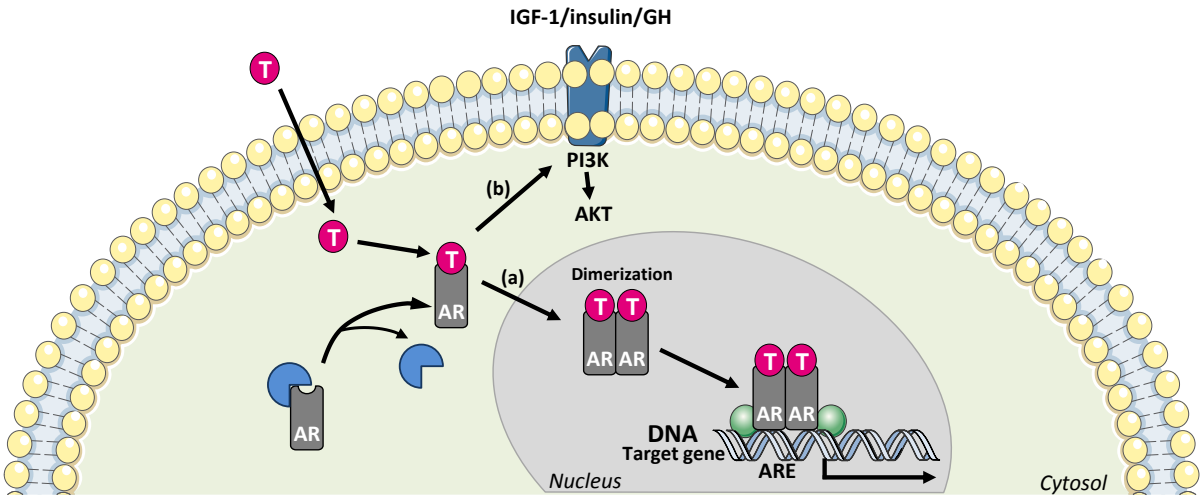

Figure 8. The mechanisms of action of sex steroids. In the classical genomic action (a), androgens (T) diffuse into the cell and bind to their respective steroid hormone receptor (AR). This steroid-receptor complex translocates to the nucleus where it dimerizes with another steroid-bound receptor, which stimulates gene transcription of target genes by binding to an androgen respons element (ARE). In the non-transcriptional mechanism (b), the steroid-AR complex cross-talks with other signalling molecules. Based on data from Chang et al. (1995) and Baron et al. (2004).

5.2.1 The classical mechanism of action

The classical genomic action of sex steroids on tissues occurs through binding to their respective steroid hormone receptor, located in the cytoplasmic compartment in the form of a multicomplex protein. When activated, this family of nuclear receptors is able to translocate into the nucleus where they can stimulate gene transcription of target genes (Figure 8a). According to this classical mechanism of sex steroid action, androgens first diffuse into the cell where they bind with high affinity to their intracellular AR. This causes a conformational change in the receptor protein structure, resulting in activation and subsequently translocation of the steroid-receptor complex into the nucleus, where it dimerizes with another hormone-bound receptor. This complex can then bind to an androgen response element (ARE), a specific DNA sequence present within the promoter region of the target gene. In this way, it affects transcription through direct binding with the transcriptional machinery and via recruitment of chromatin structure modifying enzymes and interaction of additional transcriptional factors (Giorgi and Stein 1981; Chang et al. 1995). Tissue-specific coregulators can further affect the transcriptional activity of the AR, by which transcription may be up- (coactivators) or downregulated (corepressors) (Tsai and O'Malley 1994; Tenbaum and Baniahmad 1997). A considerable number of AR coregulators have been identified to date (Heinlein

and Chang 2002; Heemers and Tindall 2007; van de Wijngaart et al. 2012), but the exact mode of action remains unclear for most of them. The corepressor Ankrd1 (Wu et al. 2013), the coactivators PGC-1 (Knutti et al. 2000) and some actin-binding proteins (Gelsolin, supervillin, filamin A, ARA55 and Paxillin) (Nishimura et al. 2003; Ting and Chang 2008) were found to be predominantly active in skeletal muscle.

Alternatively, androgens can indirectly affect gene expression of target genes by regulating microRNA transcription by binding to the ARE in microRNA-encoding genes (Wyce et al. 2010). MicroRNAs are small non-coding RNAs which are able to degrade mRNA and inhibit protein translation of target mRNAs (Hamilton and Baulcombe 1999; Bartel 2004). They are highly present in skeletal muscle and a large number of miRNAs in rat levator ani (LA) muscle were shown to be reduced by orchidectomy (Narayanan et al. 2010), suggesting that microRNAs are targeted by androgens in skeletal muscle. Complete description of the role and regulation of microRNA in skeletal muscle is beyond the scope of this thesis, hence we refer to the review of Guller et al. (2010).

5.2.2 The non-canonical mechanisms of action

As further described (section 6.2), the T-AR complex can also mediate fast non-transcriptional actions through cross-talking with other signalling molecules such as IGF1 (Weissberger and Ho 1993; Hobbs et al. 1993) and Akt (Baron et al. 2004). Akt can be activated and subsequently phosphorylated by a direct interaction of the T-bound AR with the regulatory subunit of PI3K (Baron et al. 2004) (Figure 8b).

5.2.3 The androgen receptor gene and structure

As mentioned previously, androgens act on various organs due to the presence of the AR in these tissues. Expression of the AR has been found in the hypothalamus, adrenal gland, epididymis, prostate, pituitary gland, skeletal muscle, kidney, liver, heart and bone (Sar et al. 1990; Burgess and Handa 1993; Abu et al. 1997). Evidence exists that androgens can autoregulate AR expression as such that the presence of its ligand downregulates or upregulates AR mRNA (Gonzalez-Cadavid et al. 1991; Burgess and Handa 1993). In skeletal muscle, resistance exercise (Ratamess et al. 2005) and administration of T (Ferrando et al. 2002) resulted in a transient increase in AR protein expression.

The AR gene is located on the X-chromosome, and its sequence is more than 90kbp long (Figure 9). The protein structure of a steroid receptor consists of several regions including (1) an NH_2-terminal domain (NTD), which is entirely encoded by the first exon and is involved in transcriptional activation

and protein-protein interactions, (2) a DNA-binding domain (DBD), consisting of two zinc fingers directing target gene specificity, (3) a hinge region, important for nuclear localization, and (4) a highly conserved ligand-binding domain (LBD) which has several functions such as hormone binding, receptor dimerization and nuclear translocation in addition to transcriptional activation (Evans 1988; Freedman 1992).

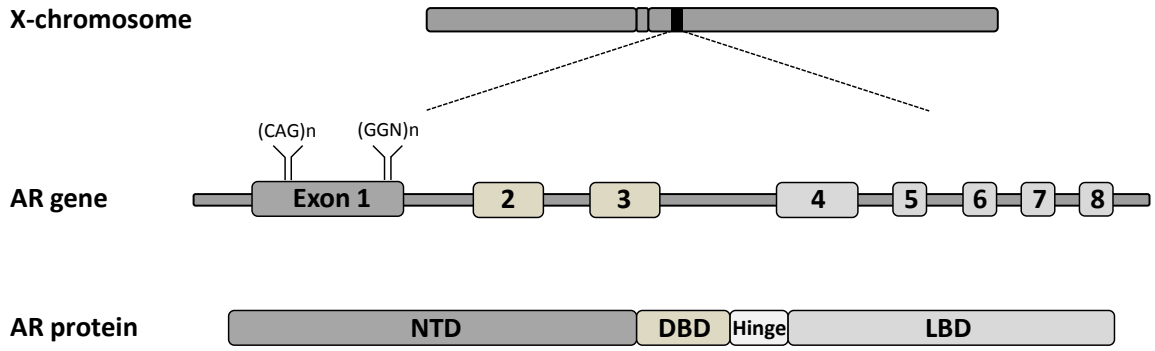

Figure 9. Schematic view of the androgen receptor gene and protein. NTD: NH_2-terminal domain DBD: DNA-binding domain, LBD: ligand-binding domain. Based on data from Lonergan et al. (2011).

5.3 Inter- and intraindividual variations in testosterone levels in healthy men

In healthy men, large ***interindividual variations*** in serum T and FT levels exists (Crabbe et al. 2007). This between-subjects variability in T levels has been related to age, body mass index and environmental conditions such as smoking (Ukkola et al. 2001), and is considerably influenced by genetic factors (Meikle et al. 1988; Ring et al. 2005).

It is well-known that T secretion increases at the onset of puberty in boys. In fact, there is an earlier transient production of T during the prenatal period which drives the differentiation of primary reproductive tissues. After puberty, T secretion stabilizes until the fourth decade of life, after which T levels start to decline (Kaufman and Vermeulen 2005). Next to these age-related changes in androgen levels, the between-subject variation in T levels appears to be genetically determined, explaining 60% of the total variation of T levels, as shown by family, twin and sib-pair studies (Meikle et al. 1982; Meikle et al. 1986; Harris et al. 1998; Bogaert et al. 2008). A large number of candidate genes contributing to the genetic determination of T levels have been identified. Next to many enzymes involved in biosynthesis and degradation, also the AR gene plays a role in the determination of serum androgen levels. Polymorphisms in the AR gene have been described to modify its activity, resulting in an altered sensitivity to T and thus influencing the hypothalamic-pituitary feedback regulation. Diminished androgen feedback, and consequently higher serum T

concentrations have been associated with the CAG repeat length, and to a lesser extent with the GGN repeat length (Crabbe et al. 2007; Bogaert et al. 2009). Both repeats are located in exon 1 encoding the NTD of the AR (Figure 9). Variability in the repeat length imposes changes in the absolute number of amino acids in the AR protein, hereby altering its transcriptional activity and thus androgen sensitivity (Sleddens et al. 1992). Furthermore, several SNPs located in the AR gene have been linked with the androgen insensitivity syndrome (AIS). These SNPs induce altered binding with cofactors and could therefore affect androgen action and circulating androgen levels (Quigley et al. 2004; Black et al. 2004; Li et al. 2005).

In healthy men, also an ***intraindividual variability*** in serum T levels exists. The release of T from the testes is episodic and occurs in response to a pulsatile LH stimulus, with peak levels between 7 am and 10 am (Mitamura et al. 1999). Different physiological conditions have been shown to temporarily alter androgen levels. Heavy resistance exercise training in men is known to induce an acute increase in serum T and FT levels, which return back to baseline within 30 min of recovery (Vingren et al. 2010). On the other hand, prolonged submaximal exercise was shown to result in a decline in serum T concentrations until 1 or 2 days following recovery. Spaceflights (Amann et al. 1992), severe injuries (Ferrando 2000), reduced caloric intake and chronic stress (Demura et al. 1989) are also reported to be associated with marked reductions in serum T levels.

5.4 Androgens and the male skeletal musculature

Next to their role in sexual development and function, secondary sex characteristics, and spermatogenesis, androgens are considered to be the main sex steroids regulating body composition. When boys enter puberty they develop more muscle mass (±35% gain in muscle mass) and strength, resulting from an increase in the CSA of muscle fibers. Next to GH and IGF1, androgens are particularly required to maintain muscle mass and strength in men. The relationship between androgens and muscle mass in conditions outside the physiological range is well-described. Boys with delayed puberty have lower T levels and lower lean mass when compared with age-matched controls (Han et al. 2006). A similar observation has been made in men with hypogonadism (Katznelson et al. 1996) or with suppressed serum T by administration of a GnRH-agonist (Mauras et al. 1998). Also the age-related loss in muscle mass and strength (sarcopenia) has been attributed in part to the decline of androgen levels with age (Kaufman and Vermeulen 2005; Orwoll et al. 2006). Androgen replacement therapy is well-known to be an effective therapy to improve muscle mass and function in hypogonadal (Bhasin et al. 1997), elderly (Allan et al.; Snyder et al. 1999; Storer et al. 2008) and normal men (Bhasin et al. 1996) as well as in those suffering from catabolic diseases such as cancer, human immunodeficiency virus (HIV)/AIDS, COPD and burn injuries (Hart et al. 2001; Gold

et al. 2006). The effect of androgens is dose dependent (Zitzmann and Nieschlag 2003a) and may be fiber type specific. However, it is unclear whether androgens predominantly affect fast- or slow-twitch muscles (Sinha-Hikim et al. 2002; Axell et al. 2006; Hulmi et al. 2008; Ophoff et al. 2009).

The effect of androgens on muscle strength is less clear. Reports of the effect of androgens on muscle force in both human and animal studies remain contradictory, with some studies demonstrating enhanced muscle strength following androgen administration (Jiang and Klueber 1989; Bhasin et al. 1996; Bhasin et al. 1997; Ferrando et al. 2002; Schroeder et al. 2003), whereas others failed to detect any significant effects on muscle strength despite gains in muscle mass (Tingus and Carlsen 1993; Snyder et al. 1999; Blackman et al. 2002; Wang et al. 2004).

5.5 Estrogens and the male skeletal musculature

E_2 is considered to be the main sex steroid involved in the development and maintenance of bone mass in both men and women (Lapauw et al. 2009). In addition, E_2 is important to initiate epiphyseal closure of long bones (Weise et al. 2001). As skeletal muscle myoblasts and mature fibers express functional estrogen receptors (ER), with two isoforms ERα and ERβ been described, a direct effect of E_2 on muscle cells may occur (Kahlert et al. 1997; Barros et al. 2006). Both animal and human studies suggest a role for E_2 in regulating glucose homeostasis (Heine et al. 2000; Barros et al. 2006), decreasing oxidative damage (Baltgalvis et al. 2010), stimulating muscle differentiation (Pedraza-Alva et al. 2009) and exerting anti-apoptotic effects (Vasconsuelo et al. 2008). Moreover, studies in rats demonstrated that E_2 is involved in the reduction of contraction-induced damage in skeletal muscle in both males and females (Bär et al. 1988; Koot et al. 1991; Tiidus 2003; Enns and Tiidus 2010), and hindlimb unloading experiments in ovariectomized rats have also shown that E_2 is involved in muscle recovery (Brown et al. 2001; McClung et al. 2006; Sitnick et al. 2006).

However, the possible effects of E_2 on the regulation of muscle mass and function in females are controversial. Hormone replacement therapy in female mice (Moran et al. 2007) and postmenopausal women (Phillips et al. 1993; Sipilä et al. 2013) have not always shown anabolic effects on muscle mass (Messier et al. 2011). Also a negative role of E_2 on the musculature in females has been suggested. Several studies observed a decrease in muscle mass and force after E_2 administration to ovariectomized rats (Ihemelandu 1981; Suzuki and Yamamuro 1985; Kobori and Yamamuro 1989; McCormick et al. 2004), while Brown et al. (2009) found an increase in muscle mass and function in ER KO female mice.

Research focusing on the association between muscle mass and serum E_2 levels in men are scarce. In elderly men, serum E_2 levels have been positively associated with lean and muscle mass,

independently from T levels (Vandenput et al. 2010; Auyeung et al. 2011), although not all studies could support this (van den Beld et al. 2000; Szulc et al. 2004b). Interestingly, intervention studies have shown that E_2 treatment resulted in increased lean mass and muscle mass in orchidectomized rodents (Vandenput et al. 2002; Svensson et al. 2010), indicating that further experiments examining the effects of E_2, following T aromatization, on skeletal muscle mass in men are of potential significance.

5.6 The (mis)use of anabolic steroids and its side effects

Because of its anabolic effects, androgens and its synthetic derivates are widely abused by athletes, especially weightlifters and bodybuilders, but also by recreational fitness sportsmen to enhance their physical appearance. However, this is not without risks. Androgens exert their effects systemically, hereby regulating many physiological processes including secondary sex characteristics, fat metabolism, bone metabolism, sexual and cognitive functions, cardiovascular functions and skin metabolism in a dose-dependent manner (Rhoden and Morgentaler 2004). Therefore, androgen therapy can induce important side effects.

Short- and long-term testosterone replacement therapy in men with androgen deficiency syndrome is generally safe but adverse effects such as an increase in hematocrit levels, acne and breast tenderness may occur. On the other hand, administration of androgens in eugonadal men results in supraphysiological testosteron blood levels which disrupt the normal production of hormones in the body. This often coincides with several side events depending on the dose, manner and frequency of administration. Acute adverse effects include acne, testicular atrophy, hypertension, jaundice, headaches, fluid retention, gastrointestinal irritation, diarrhea, stomach pains and oily skin, whereas chronic adverse effects include urogenital problems (impotence, reduced sperm production, shrinking of the testes, difficulty in urinating), acne, cardiovascular (hypertension, heart attack and stroke) and hepatic diseases, prostate cancer and neuropsychiatric effects. Moreover, the higher availability of T for the aromatase enzyme results in higher E_2 levels which can lead to breast pain and irreversible breast development (van Amsterdam et al. 2010; Fernández-Balsells et al. 2010).

Although most of these side effects only become irreversible when androgens are used in high dose over a prolonged time, they limit the use in clinical applications. Moreover, it is well-known that the use of anabolic steroids to enhance muscle mass in females will result in the development of more masculine characteristics and many of these side effects can become of major concern in the elderly (Spitzer et al. 2013). A first approach to encounter these side effects in the treatment of muscle atrophy is the development of a promising class of drugs, namely the selective androgen receptor

modulators (SARMs) which are tissue-selective AR-ligands with minimal androgenic action on other tissues (Thevis and Schanzer 2010). They bind to the AR with a high affinity and, unlike T, they are not substrates for aromatase and 5α-reductase. At the moment, several SARMs have successfully completed phase-II-clinical trials. Although none of these therapeutics have been clinically approved, they are already available on the black-market (Thevis et al. 2009). A second approach to achieve muscle selectivity is to identify muscle-specific signalling molecules downstream of the AR, which can give rise to novel therapeutic targets. Hence, understanding these signalling pathways and their cross-talk with other pathways will be important to reduce the potential side effects that can occur with SARM treatments.

"The undesirable effects of androgens limit their use in clinical applications. Therapeutic agents that could achieve anabolic effects on skeletal muscle without androgenic activities on other pheripheral tissues are of great clinical interest."

6 THE MOLECULAR REGULATION OF SKELETAL MUSCLE MASS BY ANDROGENS

6.1 Androgenic effects on satellite cells

As muscle satellite cells express a functional AR, a direct action of androgens may occur (Sinha-Hikim et al. 2004). Androgen-induced muscle hypertrophy has been reported to be associated with increased satellite cell number and increased myonuclear incorporation in young and older men (Kadi and Thornell 2000; Sinha-Hikim et al. 2003; Chen et al. 2005; Sinha-Hikim et al. 2006). Furthermore, androgens may act on the myogenic pathway by stimulating the differentiation of mesenchymal pluripotent progenitor cells to the generation of new satellite cells, and by inhibiting adipogenesis during muscle regeneration or hypertrophy (Jankowski et al. 2002; Singh et al. 2003). Although there is some evidence that androgens can activate satellite cell differentiation and proliferation, their contribution in increasing muscle mass is believed to be limited.

6.2 Androgenic effects on hypertrophy and atrophy signalling pathways

Studies in young and elderly men have shown that T administration increases skeletal muscle protein synthesis (Urban et al. 1995; Ferrando et al. 1998) and decreases muscle protein breakdown (Ferrando et al. 2002; Ferrando et al. 2003), at least for long-term androgen treatment. The effects of short-term treatment on the protein breakdown rate are less clear (Ferrando et al. 1998; Sheffield-Moore 2000).

The stimulation of the growth factors GH and IGF1 by T has been studied extensively. Several clinical studies have demonstrated that T therapy in healthy and elderly men is associated with increased serum IGF1 levels (Hobbs et al. 1993; Urban et al. 1995; Bhasin et al. 2001; Ferrando et al. 2002), whereas T administration to healthy rats was shown to increase intramuscular IGF1 mRNA expression (Yin et al. 2009). On the other hand, T deficiency in humans and androgen loss in rodents are associated with a reduction in both circulating (Grinspoon et al. 1996) and intramuscular IGF1 mRNA expression (Mauras et al. 1998). These results suggest that androgens can possibly affect the downstream signalling pathways of IGF1 i.e. Akt/mTOR and Akt/FoxO signaling pathways. In support of this hypothesis, an in vitro study using C2C12 cells demonstrated that T administration attenuates Atrogin-1 mRNA expression (Zhao et al. 2008b). Moreover, Xu et al. (2004) found increased levels of phosphorylated $p70^{S6K}$ in the LA muscle of castrated rats following DHT administration. Evidence for an androgenic regulation of myostatin signalling was provided by McMahon et al. (2003) who found lower myostatin protein levels in male mice compared with female mice. However, intervention studies have demonstrated either increased (Diel et al. 2007; Diel et al. 2008b; Diel et al. 2008a) or

decreased (Mendler et al. 2007) myostatin mRNA and protein levels in rodents or C2C12 cells treated with T or other anabolic steroids.

Hence, it is interesting to further investigate the in vivo effects of T on skeletal muscle atrophy and hypertrophy signalling pathways. During the course of this PhD-project, a number of other research groups similarly looked at the possible effects of androgens on signalling molecules regulating muscle mass, albeit with different methodological approaches compared to the current thesis (Pires-Oliveira et al. 2010; Svensson et al. 2010; Haren et al. 2011; Ibebunjo et al. 2011; White et al. 2013). The results of these studies, combined with the results of the present thesis, will be discussed in the general discussion.

"The muscle-specific molecular factors targeted by androgen therapy have not been completely elucidated but can give rise to novel therapeutic targets."

7 AIMS

The maintenance of skeletal muscle mass is a critical component for health. Androgens are well-known for their anabolic effects on muscle mass, but their clinical application in the treatment of muscle wasting is limited because of its severe side effects. Investigating the molecular mechanisms involved in the androgenic regulation of skeletal muscle hypertrophy and atrophy can lead to the development of effective therapeutic strategies to combat the devastating effects of muscle atrophy. In the past few years, considerable progress has been made in understanding the molecular events that regulate skeletal muscle mass.

However, from the introduction it became clear that inconsistency exists regarding the signalling molecules involved among different atrophy models. These differences in results have raised the idea that the signalling pathways involved in muscle wasting are unique to the atrophy model, and depend on the muscle type, and the time course following the atrophic stimulus. The molecular regulation of skeletal muscle mass by androgens has only recently been investigated and is still poorly understood. Current available knowledge has mostly been derived from gene-expression analyses which do not necessarily correlate to the protein expression. Therefore, a thorough understanding of how gene and protein expression of atrophy and hypertrophy inducing molecules is regulated by androgens in different fiber types and in different atrophy models remains important to extend our knowledge of how skeletal muscles adapt to anabolic stimuli. We have conducted a series of studies in order to gain more insight into the androgenic regulation of muscle mass and the signalling molecules and pathways involved.

In **study 1**, we investigated the relationship between androgens and muscle mass and function, as well as the influence of environmental and some genetic factors in a cohort of healthy young men. Next to age, physical activity, anthropometrics and circulating sex steroids, we tried to identify genetic variations in the AR that are associated with muscle mass and function. Next, the effects of T treatment on the signalling pathways regulating muscle protein degradation and synthesis in two different mice atrophy models were explored in study 2 and 3. **Study 2** focused on the regulation of muscle atrophy-inducing targets (Atrogin-1, MuRF1 and myostatin) in an "*androgen deprivation-induced muscle atrophy model",* with or without T or E_2 supplementation. In **study 3** atrophy and hypertrophy signalling molecules including IGF1/Akt/p70s6k, as well as myostatin, REDD1, atrogin-1 and MuRF1 were examined during "*disuse atrophy"* with or without T supplementation (Figure 10).

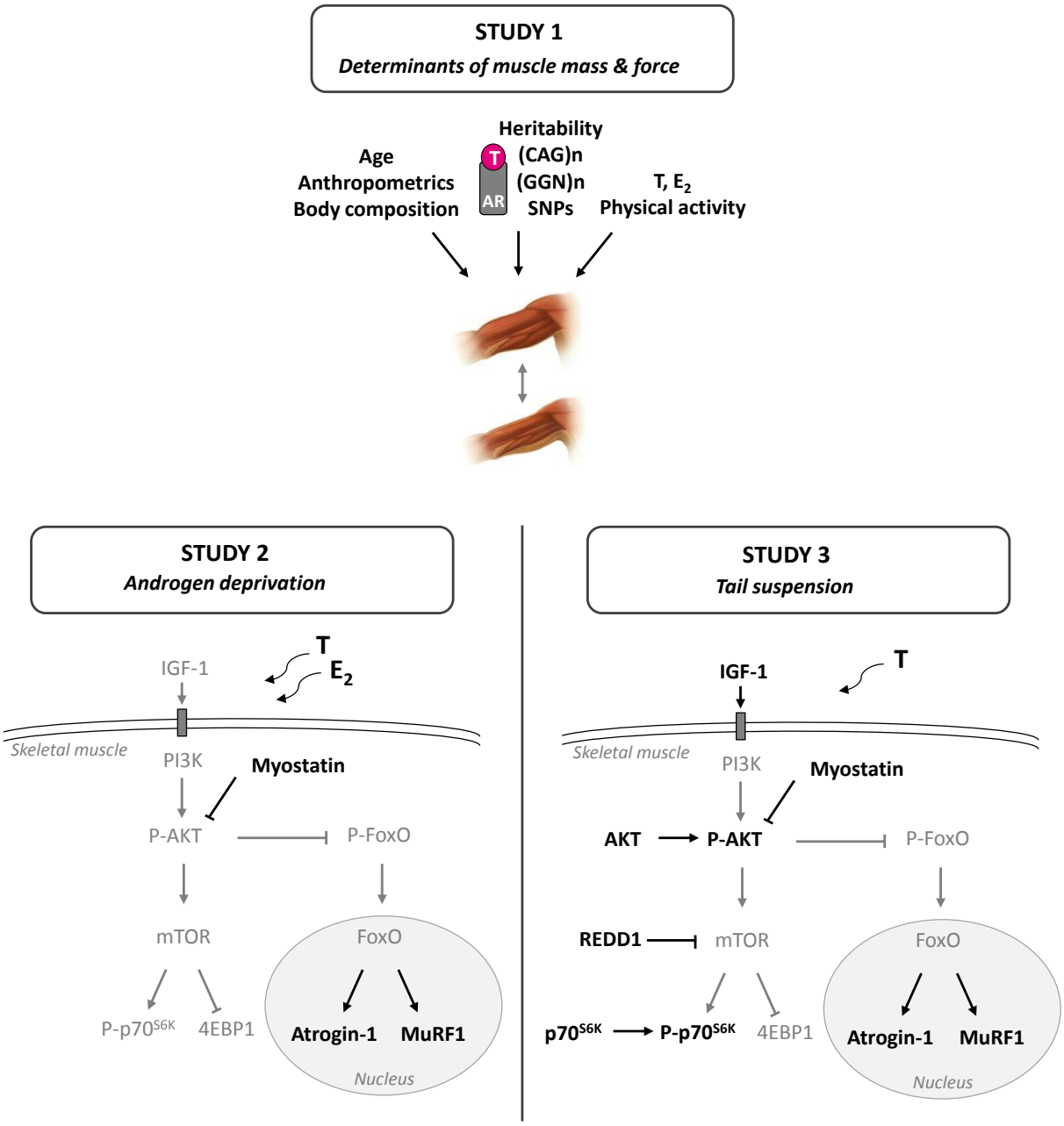

Figure 10. Schematic overview of the studies presented in this thesis. Study 1: Determinants of skeletal muscle mass and force in eugonadal men. Study 2: Effects of androgen deprivation with or without testosterone (T) or estradiol (E_2) treatment on skeletal muscle atrophy inducing targets. Study 3: Effects of tail suspension with or without testosterone (T) treatment on skeletal muscle atrophy and hypertrophy inducing targets. Targets shown in grey were not measured.

The following aims and underlying hypotheses were investigated in this thesis:

1. In study 1, we aimed to gain more insight into the interindividual variation in muscle mass in young healthy men. As polymorphisms in the AR gene causes differences in androgen sensitivity, we hypothesized that genetic variations in the AR contribute to the variation in muscle mass in young healthy men.

2. The anabolic effects of T administration during androgen deprivation-induced muscle atrophy and disuse atrophy were examined in study 2 and 3. We expected that T replacement would result in complete recovery of muscle mass following orchidectomy and that T supplementation during tail suspension would be able to ameliorate muscle mass. Also the potential of E_2 to induce anabolic effects was investigated following androgen deprivation (study 2).

3. Furthermore, we aimed to explore whether tail suspension was associated with changes in circulating T levels (study 3). Based on the literature, tail suspension was supposed to be associated with, although not invariably, reductions in serum T levels.

4. The aim of both study 2 and 3 was to investigate the regulation of atrophy-inducing targets during orchidectomy and tail suspension at 3 different time-points and in 3 different muscle types (extensor digitorum longus (EDL), soleus (SOL) and levator ani/bulbocavernosus muscles (LA/BC)). We hypothesized that changes in Atrogin-1, MuRF1, myostatin and REDD1 expression during muscle atrophy depend on muscle type and the time course following androgen loss and tail suspension.

5. The regulation of atrophy and hypertrophy inducing molecular factors by sex steroids was further elucidated in our 2 muscle atrophy models (study 2 and 3). An increase in IGF1/Akt/p70s6k levels and a decrease in REDD1, myostatin, Atrogin-1 and MurF1 gene and protein expression levels was expected following T treatment.

II. STUDY DESIGN

In this chapter, an overview of the study population and animal models is given. For more technical details on the methodologies, we refer to chapter 3 to 5.

1. SIBLOS POPULATION (STUDY 1)

Participants included in the analysis were part from a larger population recruited for the SIBLing-paired OSteo-study (SIBLOS study). This study is a population-based cross-sectional study, designed at the Departement of Endocrinology of Ghent University Hospital to investigate the determinants of bone mass and sex steroid levels in young brothers, focusing on general lifestyle, body composition and genetic background. Previous findings have been the subject of several publications (Crabbe et al. 2007; Bogaert et al. 2008; Lapauw et al. 2009; Bogaert et al. 2009; Taes et al. 2009b; Taes et al. 2009a; Vanbillemont et al. 2010; Taes et al. 2010b; Taes et al. 2010a; Roef et al. 2012; Vanbillemont et al. 2012). The study protocol was approved by the ethical committee of the Ghent University Hospital.

Participants of the SIBLOS study were recruited from the population registries of 3 semi-rural to suburban communities around Ghent, Belgium. A total of 12446 men between 25 and 45 years old were contacted by direct mailing, briefly describing the study purpose and asking if they had a brother within the same age range also willing to participate (maximal age difference between brothers was set at 12 yrs). The response rate was 30%. Finally, 768 young healthy men who fulfilled the primary inclusion criterion of having a brother within the same age range agreed to participate.

Exclusion criteria were defined as illnesses or medication use affecting body composition, hormone levels or bone metabolism. After exclusions, 677 men were included in study 1 (Figure 11). Two hundred and ninety six pairs of brothers (for a total of 592 men) were included in addition to 64 single participants, when their brother could not participate in the study, 19 men were included as third brother in a family and 2 as fourth brother. All participants gave their written informed consent and completed questionnaires about previous illness and medication use.

Relations between genetic variation in the AR gene (CAGn, GGNn and SNPs, determined by genotyping analysis), sex steroid levels (T and E_2, measured by liquid chromatography tandem mass spectrometry (LC-MS/MS)), body composition (lean and fat mass, measured by dual-energy x-ray absorptiometry (DXA)), muscle cross-sectional area (CSA) at radius and tibia (measured by peripheral quantitative computed tomography (pQCT)), muscle force (grip strength and isokinetic peak torque of biceps and quadriceps, measured by a dynamometer) and anthropometrics (body weight, height, armspan, sternum height, finger- and hand length) were studied using linear mixed-effect modelling.

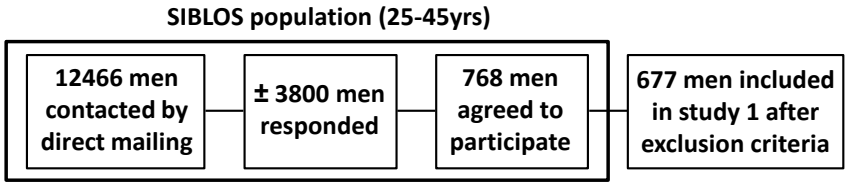

Figure 11. Overview of the SIBLOS population

2. EXPERIMENTAL ANIMAL MODELS

Mice used in study 2 and 3 were adult male C57BL/6JOla inbred mice (8w). All experimental protocols were approved by the Ethical Committee for Animal Research of Ghent University.

2.1 Androgen deprivation-induced skeletal muscle atrophy model (study 2)

Androgen deprivation in study 2 was induced by orchidectomy, i.e. surgical removal of the testicles under isoflurane inhalation. After making an incision at the tip of the scrotum, the testis and epididymis were pulled out and the vas deferens and blood vessels were ligated. Finally, the testes were cut and removed, and the incisions were sutured. A total of 100 mice were included in this study and were randomly assigned into 4 intervention groups: sham-operated (SHAM), orchidectomized with control vehicle (ORX+V) or orchidectomized and treated with testosterone (ORX+T) or β-estradiol (ORX+E_2) via subcutaneous silastic implants at the cervical region (Figure 12). Sham surgery was performed to omit the incidental effects caused by anesthesia or incisional trauma. Under isoflurane inhalation, an incision was made at the tip of the scrotum and the testes were pulled out and then replaced. Sham surgery was also performed at the cervical region to isolate the effects of the silastic tubes implantation.

Animals were killed 1 day, 7 days or 30 days after surgery. The twitch and tetanus contractile properties of the SOL and EDL muscles were measured in vitro by stimulation with capacitor discharges between platinum electrodes following 30 days of orchidectomy. Muscle biopsies from LA/BC, SOL and EDL were carefully dissected at the end of each intervention period, were weighted, and were used for gene and protein expression analysis by qPCR- and Western blot respectively.

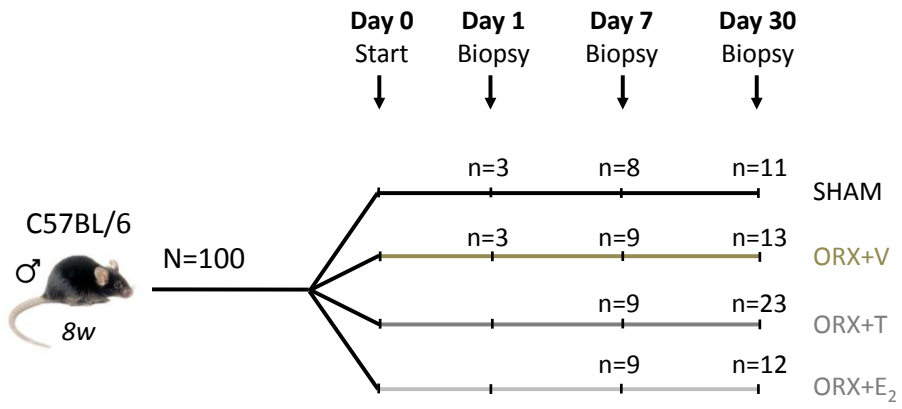

Figure 12. Summary of the experimental design of study 2.

2.2 Disuse-induced skeletal muscle atrophy model (study 3)

Muscle disuse in study 3 was imposed by tail suspension by a modification of the protocol described by Morey-Holton and Globus (2002). Tail suspension was performed by lifting the tail up so that the hindlimbs were unloaded. The mice were maintained in approximately 30° head-down tilt, so that the forelimbs remained loaded. The tail was suspended with adhesive tape on a metallic wire which was connected to a 360° free rotating hook. The hook was hung on a rail system above the cage (type III), which allowed free movement along the rail.

Animals were randomly assigned into 3 intervention groups: Sham-operated control group (SHAM, n=30), tail suspended (TS) with control vehicle treatment group (TS+V, n=30), and tail suspended with testosterone (T) (TS+T, n=30), administered via subcutaneous silastic implants at the cervical region (Figure 13). Sham surgery was performed at the cervical region to omit the effects of the silastic tubes implantation (section 2.2). Hindlimb unloading was imposed for 1, 5 and 14 days.

Blood was collected by cardiac puncture and analysed by LC/MSMS for the determination of serum T levels. The EDL and SOL muscles of both legs were carefully dissected, weighed, and used for qPCR and Western blotting analyses.

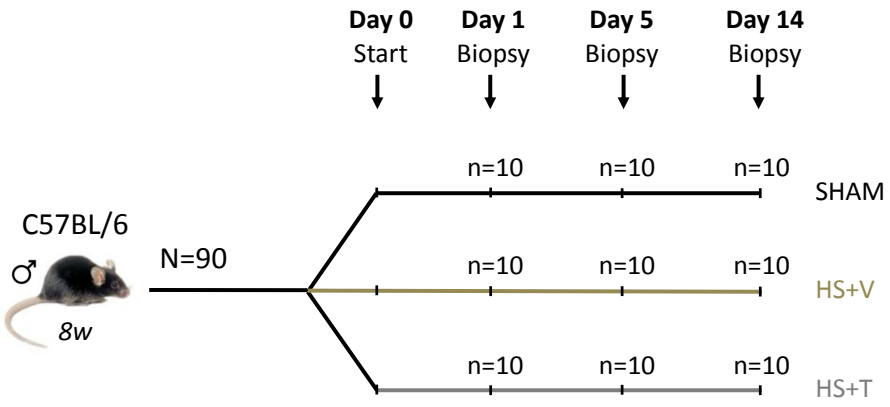

Figure 13. Summary of the experimental design of study 3.

III. STUDY 1: GENETIC VARIATIONS IN THE ANDROGEN RECEPTOR ARE ASSOCIATED WITH STEROID CONCENTRATIONS AND ANTHROPOMETRICS BUT NOT WITH MUSCLE MASS IN HEALTHY YOUNG MEN

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ABSTRACT

Objective: The relationship between serum testosterone (T) levels, muscle mass and muscle force in eugonadal men is incompletely understood. As polymorphisms in the androgen receptor (*AR*) gene causes differences in androgen sensitivity, no straightforward correlation can be observed between the interindividual variation in T levels and different phenotypes. Therefore, we aim to investigate the relationship between genetic variations in the *AR*, circulating androgens and muscle mass and function in young healthy male siblings.

Design: 677 men (25-45 years) were recruited in a cross-sectional, population-based sibling pair study.

Methods: Relations between genetic variation in the *AR* gene (CAGn, GGNn, SNPs), sex steroid levels (by LC-MS/MS), body composition (by DXA), muscle cross-sectional area (CSA) (by pQCT), muscle force (isokinetic peak torque, grip strength) and anthropometrics were studied using linear mixed-effect modelling.

Results: Muscle mass and force were highly heritable and related to age, physical activity, body composition and anthropometrics. Total T (TT) and free T (FT) levels were positively related to muscle CSA, whereas estradiol (E_2) and free E_2 (FE_2) concentrations were negatively associated with muscle force. Subjects with longer CAG repeat length had higher circulating TT, FT, and higher E_2 and FE_2 concentrations. Weak associations with TT and FT were found for the rs5965433 and rs5919392 SNP in the *AR*, whereas no association between GGN repeat polymorphism and T concentrations were found. Arm span and 2D:4D finger length ratio were inversely associated, whereas muscle mass and force were not associated with the number of CAG repeats.

Conclusions: Age, physical activity, body composition, sex steroid levels and anthropometrics are determinants of muscle mass and function in young men. Although the number of CAG repeats of the *AR* is related to sex steroid levels and anthropometrics, we have no evidence that these variations in the *AR* gene also affect muscle mass or function.

Keywords: sex steroids, muscle mass, muscle function, androgen receptor, androgens

INTRODUCTION

Skeletal muscle mass and function are highly heritable [1] and influenced by age, anthropometrics, sex steroid status and lifestyle-related factors [2–4]. The clinical relationship between androgens and muscle mass is well-described. Androgen deficiency (i.e. hypogonadism) leads to significant muscle loss and weakness [5], whereas testosterone (T) supplementation has dose-dependent anabolic effects [6,7]. Moreover, impaired steroid production or low androgen sensitivity could interfere with normal bone development and closure of the epiphyseal growth plates at the end of puberty.

However, the interrelationship between T levels, muscle mass and muscle force in eugonadal men is less clear [8]. Serum T levels are maintained at appropriate levels by the hypothalamic-pituitary-gonadal feedback loop. In healthy men, a large interindividual variation in serum T levels exists [9]. This between-subject variability in T levels has been related to age, BMI, environmental conditions such as smoking [10], and is considerably influenced by genetic factors [11,12]. The sensitivity to circulating T is determined in part by the transcriptional activity of the androgen receptor (*AR*). Polymorphisms in the *AR* gene have been described to alter this activity. We have previously shown that diminished androgen feedback, and consequently higher serum T concentrations, are associated with the CAG repeat length, and to a lesser extend with the GGN repeat length [9,13]. Furthermore, some single nucleotide polymorphisms (SNP) in the *AR* gene, resulting in an altered binding with cofactors, have been linked with the androgen insensitivity syndrome (AIS) [14–16] and could therefore affect androgen action and circulating androgen levels.

In order to gain more insight into the between subject variation in muscle mass in young healthy men, we investigated the relationship between androgens and muscle mass and function, as well as the influence of genetic components. We hypothesized that genetic variations in the *AR*, causing differences in androgen sensitivity, contribute to the variation in muscle mass in young healthy men.

MATERIALS AND METHODS

Ethics statement

The study protocol was conducted according to the Helsinki Declaration and was approved by the ethical committee of the Ghent University Hospital. All participants gave their written informed consent and questionnaires about previous illness and medication use were completed. Physical activity was scored using the questionnaire as proposed by Baecke *et al.* [17].

Study design and population

This population-based cross-sectional study is part of a larger study, from which inclusion criteria and study design were described previously [18]. Participants were recruited from the population registries of 3 semi-rural to suburban communities around Ghent, Belgium. Men (n = 12446), 25-45 years of age were contacted by direct mailing, briefly describing the study purpose and asking if they had a brother within the same age range also willing to participate (maximal age difference between brothers was set at 12 yrs). The overall response rate was 30.2%. Finally, a sample of 768 young healthy men who fulfilled the primary inclusion criterion of having a brother within the same age range agreed to participate. After exclusions, 677 men in total were included in the study. Two hundred ninety six pairs of brothers (for a total of 592 men) were included in addition to 64 men as single participants, when their brother could not participate in the study; 19 men were included as third brother in a family and 2 as fourth brother. Exclusion criteria were defined as illnesses or medication use affecting body composition, hormone levels or bone metabolism.

Body composition and muscle strength

Body weight and anthropometrics (arm span, hand and finger length) were measured in light indoor clothing without shoes. Sternum height was measured using a wall-mounted Harpenden stadiometer (Holtain, Crymych, UK). Lean and fat mass of the whole body were measured using dual-energy x-ray absorptiometry (DXA) with a Hologic QDR-4500A device (software version 11.2.1; Hologic, Bedford, MA, USA). Isokinetic peak torque of biceps and quadriceps muscles was assessed at the dominant limbs using an isokinetic dynamometer (Biodex, New York, NY, USA). Grip strength at the dominant hand was measured using an adjustable hand-held standard grip device (JAMAR hand dynamometer; Sammons & Preston, Bolingbrook, IL, USA). Their maximum performance was assumed to best reflect the current status and the history of their musculoskeletal adaptation.

Cross-sectional muscle area

A peripheral quantitative computed tomography (pQCT) device (XCT-2000, software version 5.4; Stratec Medizintechnik, Pforzheim, Germany) was used to scan the dominant leg (tibia) and forearm (radius). Muscle cross-sectional area (CSA) was estimated using a threshold below water equivalent linear attenuation set at 0.22/cm. This threshold eliminated skin and fat mass with lower linear attenuation in the cross-sectional slice. From the remaining area, bone area was subtracted, revealing the muscle at its maximum CSA.

Biochemical determinations

Venous blood samples were obtained between 08:00 and 10:00 AM after overnight fasting. All serum samples were stored at –80°C until batch analysis. Serum total testosterone (TT) and estradiol (E_2) levels were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) (AB Sciex 5500 triple-quadrupole mass spectrometer; AB Sciex, Toronto, Canada). Serum limit of quantification was <0.5pg/mL (1.9 pmol/L) for E_2 and 1.2 ng/dL (0.04 nmol/L) for T. The interassay coefficients of variation (CV) were 4.0% at 21 pg/mL (77 pmol/L) for E_2, and 8.3% at 36.7 ng/dL (1.3 nmol/L) and 3.1% at 307.8 ng/dL (10.7 nmol/L) for T [19]. Commercial radioimmunoassays were used to determine serum levels of sex hormone binding globulin (SHBG) (Orion Diagnostica, Espoo, Finland), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (ECLIA; Roche Diagnostics, Mannheim, Germany). Free testosterone (FT) and free estradiol (FE_2) concentrations were calculated from serum TT, E_2, SHBG and albumin concentrations using a previously validated equation derived from the mass action law [20, 21].

Genotyping of the androgen receptor

Genomic DNA was extracted from EDTA-treated blood using a commercial kit (Puregene kit; Gentra Systems, Minneapolis, MN, USA). The CAG and GGN repeats were determined as previously described [13].

Genotyping data for the AR gene for the Caucasian CEPH population was downloaded from the International Haplotype Mapping Project web site (http://www.hapmap.org) and the data was incorporated into the Haploview program [22]. The tagger function within Haploview was used to assign Tag SNPs. The tagging SNPs were chosen, by aggressive tagging (use 2- or 3-marker haplotypes), to capture the variations within the gene and the surrounding area with minor allele frequency (MAF) 0.01 and a minimum r^2 of 0.80 (for their location and the SNPs which they tag). For the SNP analyses, SNPlex [23] was carried out on fragmented gDNA at a final concentration of 25 ng/µl (total volume of 9 µl). Samples were run on an ABI 3730xl DNA Analyzer (Applied Biosystems,

Foster City, CA, USA) and data were analysed using Gene Mapper v. 3.7 software (Applied Biosystems). Genotype analysis was performed based on the SNPlex_Rules_3730 method following the factory default rules. Missing genotypes in the SNPlex analysis were obtained using TagMan Pre-Designed SNP Genotyping Assays® (Applied Biosystems) which were run on the StepOne System (Applied Biosystems). In total, 5 SNPs of the AR gene were genotyped.

Statistics

Descriptives are expressed as mean ± standard deviation or median [1^{st}-3^{rd} quartile] when criteria for normality were not fulfilled (Kolmogorov-Smirnov) and variables were log-transformed in subsequent linear models. Linear mixed-effects modelling with random intercepts and a simple residual correlation structure was used to study the effect of anthropometrics, sex steroid concentrations and genetic variations in the *AR* on muscle mass and function, with adjustment for the confounding effect of age, adult height and weight or fat mass and taking into account the interdependence of measurements between brothers. Parameters of fixed effects were estimated via restricted maximum likelihood estimation and reported as estimates of effect size (β) with their respective standard error. A sample size of 677 subjects allowed us a 81% power to detect a minimum effect size of 0.01 at a two-sided significance level of 5%. Validity of the models was assessed by exploring normality of distribution of the residuals. SNPs were considered as a categorical variable, whereas CAG and GGN lengths were analysed as continuous variables for assessing association, and as categorical variable (quartiles) with groups compared by one-way analysis of variance (ANOVA). Associations were considered significant at p-values less than 0.05. Statistical analyses were performed using S-Plus 7.0 (Insightful, Seattle, WA, USA). The polygenic program in SOLAR 2.0 (Southwest Foundation for Biomedical Research, San Antonio, TX, USA) was used to estimate upper-limit heritability (t^2), using a variance component model.

RESULTS

Study population and characteristics

Six hundred seventy seven subjects with a mean age of 34.5 ± 5.5 years are included in the study. Mean height is 1.79 ± 0.06 m and mean weight 81.4 ± 11.8 kg, with a body mass index of 25.3 ± 3.5 kg/m^2. Body composition and muscle function parameters are given in Table 1.

Table 1 General characteristics and hormone concentrations of all study participants (n = 677)

	Mean ± SD
Age (yr)	34.5 ± 5.5
Weight (kg)	81.4 ± 11.8
Height (m)	1.79 ± 0.06
BMI (kg/m^2)	25.3 ± 3.5
Testosterone (ng/dL) (nmol/L)	579 (20.1) [467.0-703.8]
Free testosterone (ng/dL) (nmol/L)	14.2 (0.49) [11.9-17.0]
Estradiol (pg/mL) (pmol/L)	21.2 (77.8) [16.7-25.7]
Free estradiol (pg/mL) (pmol/L)	0.4 (1.5) [0.3-0.5]
SHBG (nmol/L)	23 [18.4-29.7]
LH (U/L)	4.3 [3.1-5.5]
FSH (U/L)	3.8 [2.7-5.4]
Whole body lean mass (kg)	62.2 ± 6.6
Whole body fat mass (kg)	16.4 ± 6.4
Radius 66 % muscle area (cm^2)	45.2 ± 5.9
Tibia 66 % muscle area (cm^2)	82.6 ± 11.1
Grip strength (kg)	51.7 ± 8.0
Biceps force (Nm)	57.3 ± 10.5
Quadriceps force (Nm)	203 ± 42
Arm span (cm)	182.7 ± 7.3
Hand length (cm)	20.5 ± 1.0
Digit 2 finger length (cm)	7.4 ± 0.5
Digit 4 finger length (cm)	7.6 ± 0.5
Sternum height (cm)	61.5 ± 2.7

Non-Gaussian distribution: data presented as median [1st-3rd quartile]. Free testosterone and free estradiol serum concentrations were calculated using previously validated equations [20, 21].

As expected, the level of physical activity was associated with muscle mass. Biceps force was positively associated with the level of physical activity during work (β : 0.18 ± 0.03; p<0.0001) but not related to physical activity during sports (p=0.96), whereas quadriceps force was related to sports (β : 0.11 ± 0.04; p=0.004) and not to physical activity during work (p=0.52), independent from age, height and weight.

Age, weight and height in relation to muscle mass and force

Both fat (β : 0.2 ± 0.05 kg/y; p=0.0001) and lean mass (β : 0.1 ± 0.05 kg/y; p=0.03) increased with age, as well as muscle CSA at the radius (β : 21 mm^2/y ± 4; p<0.0001) and tibia (β : 32 mm^2/y ± 8; p=0.0001), which remained positive after additional adjustment for height, physical activity level and body fat (radius: p<0.0001 and tibia: p=0.004). With increasing age, lower limb muscle force indices slightly decreased after adjustment for height and weight (p=0.02). Biceps muscle force and maximal grip strength were unrelated to age.

Whole body lean mass was positively associated with height (β : 0.22 ± 0.02; p<0.0001) and weight (β : 0.78 ± 0.02; p<0.0001). Also a close relationship between muscle CSA and weight (β : 0.54 ± 0.03; p<0.0001 for radius, and β : 0.56 ± 0.03; p<0.0001 for tibia) was found. Moreover, maximal grip strength and muscle force indices at upper (biceps) and lower limb (quadriceps) were all positively related to height (all p<0.0001) and weight (all p<0.001).

Whole body lean mass exhibited a strong positive association with muscle CSA and muscle function (all p<0.0001), whereas whole body fat mass was inversely related to muscle CSA at radius (p<0.0001) and grip strength and muscle force of biceps (p<0.001).

The relationship of muscle CSA and muscle force (grip, biceps and quadriceps) with height and weight are represented in Figure 1.

Heritability of muscle mass and function

Table 2 illustrates the upper-limit heritabilities (t^2) of muscle mass and function parameters. All parameters are highly heritable (p<0.0001), with the highest t^2 observed for whole body lean mass.

Table 2 Upper-limit heritability estimates of selected muscle parameters (*)

	t^2
Whole body lean mass (kg)	0.86 ± 0.09
Whole body fat mass (kg)	0.73 ± 0.10
Radius 66 % muscle area (mm^2)	0.67 ± 0.10
Tibia 66 % muscle area (mm^2)	0.63 ± 0.10
Grip strength (kg)	0.56 ± 0.10
Biceps force (Nm)	0.76 ± 0.10
Quadriceps force (Nm)	0.67 ± 0.10

Muscle mass and force in relation to anthropometric measurements

Whole body lean mass and muscle CSA at the radius were positively associated with arm span (β : 0.29 ± 0.05; p<0.0001 and β : 0.31 ± 0.07; p<0.0001 respectively) as well as with finger (p=0.0001 to 0.04) and hand length (all p<0.0001) adjusted for height, weight and age. Fat mass was negatively associated with arm span (β : -0.23 ± 0.03; p<0.0001). Moreover, biceps flexion and hand grip force were related to arm span (β : 0.46 ± 0.06; p<0.0001 for biceps and β : 0.48 ± 0.07; p<0.0001 for grip), even more strongly than to hand length (β : 0.32 to 0.34 ± 0.05; p<0.0001 for biceps and β : 0.33 ± 0.05; p<0.0001 for grip) and finger length (β : 0.19 to 0.24 ± 0.04 ; p<0.0001 for biceps and β : 0.25 to 0.30 ± 0.04; p<0.0001 for grip). Muscle force and muscle CSA were unrelated to sternum height (data not shown). All associations remained positive after additional adjustment for fat or lean mass.

Sex steroids in relation to muscle mass and function

TT and FT concentrations were positively related to whole body lean mass (β : 0.07 ± 0.02; p=0.0002 and β : 0.08 ± 0.02; p<0.0001 respectively) and inversely to fat mass (β : -0.07 ± 0.02; p=0.0001 and β : -0.08 ± 0.02; p<0.0001 respectively), adjusted for age, weight and height. TT concentrations were positively related to muscle CSA at the tibia (β : 0.07 ± 0.04; p=0.04), and FT was positively associated with muscle CSA at the radius (β : 0.07 ± 0.04; p=0.03). E_2 and FE_2 concentrations were negatively associated with maximal grip strength (β : -0.08 ± 0.04; p=0.04 and β : -0.10 ± 0.04; p=0.007 respectively) and quadriceps force (β : -0.08 ± 0.04; p=0.02 and β : -0.11 ± 0.04; p=0.002 respectively), even after additional adjustment for T. No influence of TT or FT on muscle force was observed (data not shown). The 2D:4D finger length ratio and arm span were unrelated to circulating steroid concentrations (data not shown).

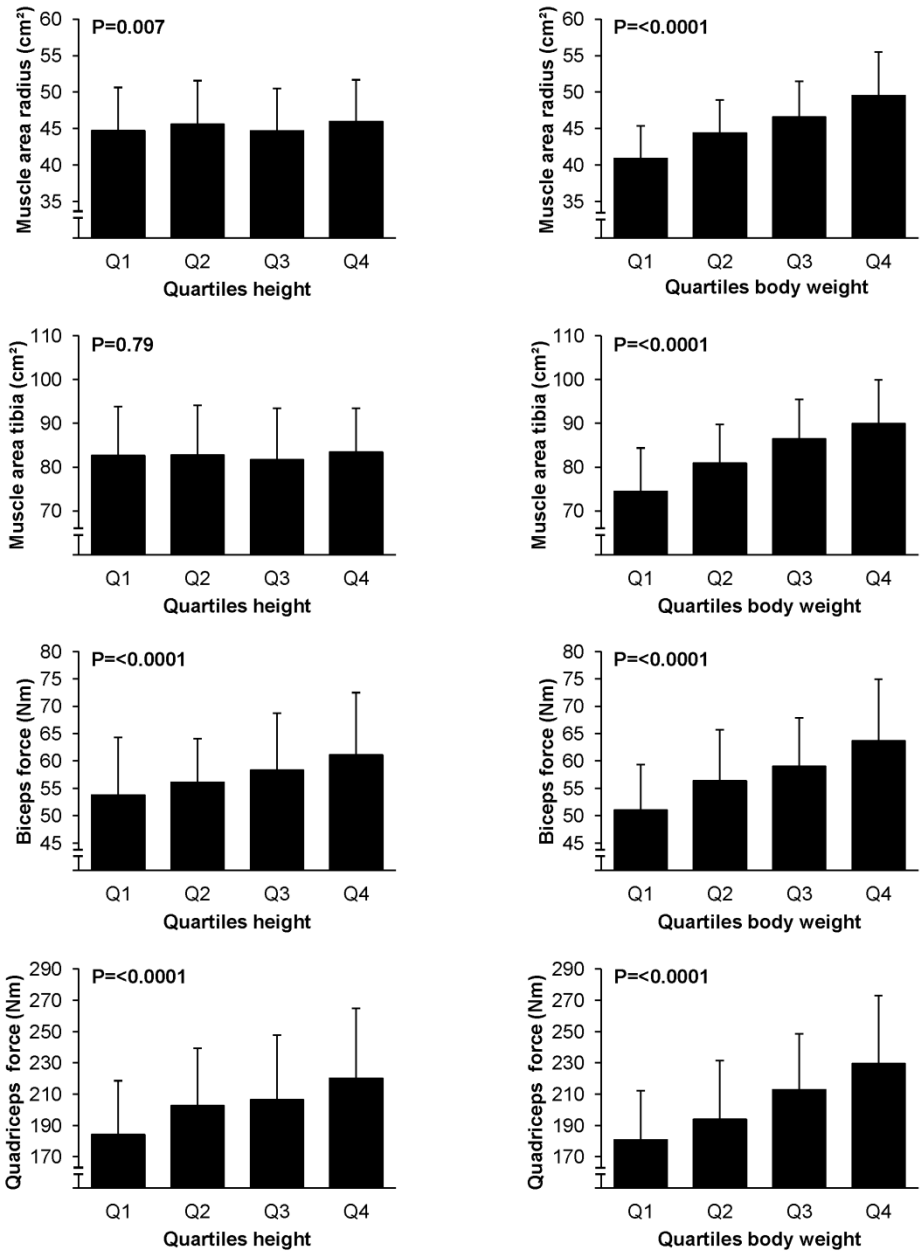

Figure 1 Muscle CSA and muscle force (grip, biceps and quadriceps) according to quartiles of height and weight. P-values result from ANOVA (overall difference between categories). Each bar represents the mean ± standard deviation (SD).

Genetic variation in AR in relation to circulating sex steroids, anthropometrics and muscle mass and function

The influence of genetic variation in the *AR* on circulating gonadal steroids, body composition and muscle function is shown in Table 3. The CAG repeat demonstrated a positive association with circulating TT and FT concentration, as well as with E_2 and FE_2 concentrations. Weak associations were found for the rs5965433 and rs5919392 polymorphisms in the *AR*. However, only the association between CAG repeat and TT and FT remained significant after Bonferroni correction. No

associations between GGN repeat polymorphism and TT or FT concentrations, as determined by LC-MS/MS, were found.

No consistent effects of the *AR* polymorphisms or CAG/GGN repeats were found on either body composition, muscle mass or muscle force (Table 3). Figure 2 illustrates the influence of the CAG repeat polymorphism on anthropometrics. Arm span was inversely associated with the number of CAG repeats (β : -0.09 ± 0.02; p=0.0001). Adult height (Figure 2), hand and digit 4 length (data not shown) were unrelated to CAG length, but digit 2 length at both left and right hand was inversely related to the CAG polymorphism (right β : -0.04 ± 0.01; p=0.0002 and left β : -0.04 ± 0.01; p=0.002 adjusted for age and height). From the 7 genetic variations analysed, only the CAG repeat length was found to be negatively related to the 2D:4D finger length ratio (right β : -0.05 ± 0.01; p=0.0006 and left β: -0.03 ± 0.01; p=0.01).

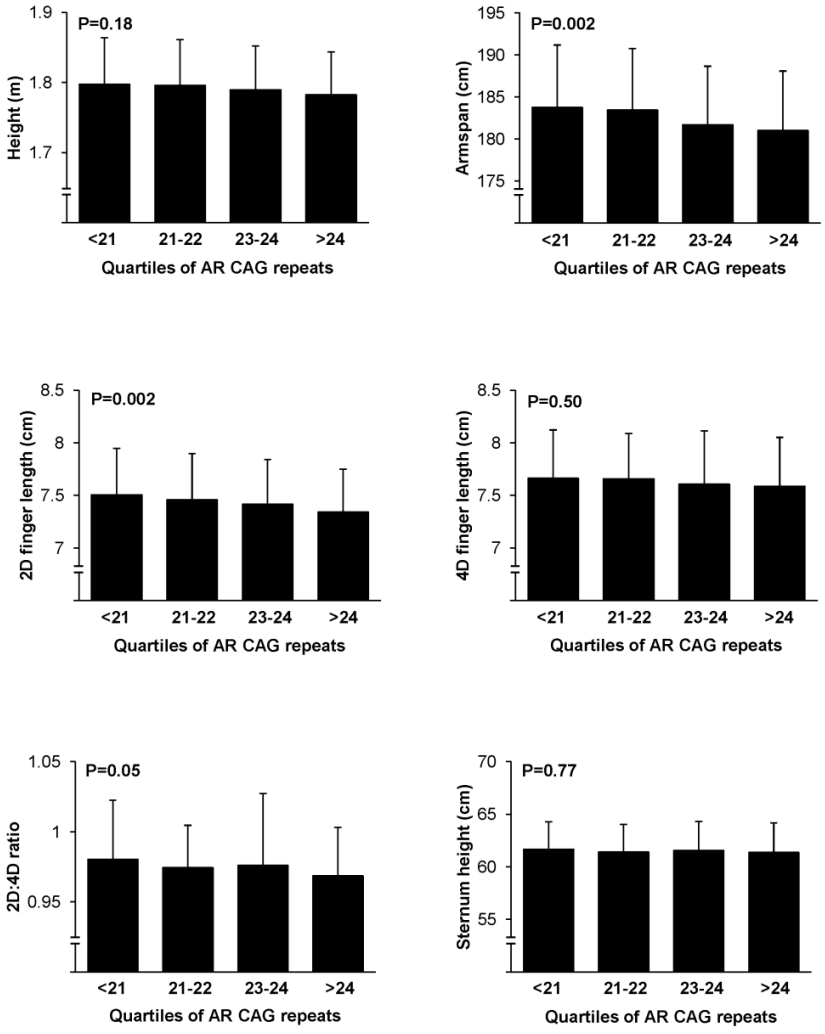

Figure 2 Anthropometrics according to quartiles of AR CAG repeat polymorphism. P-values result from ANOVA (overall difference between categories). Each bar represents the mean ± standard deviation (SD)

	CAG repeat	GGN repeat	rs17217069	rs5965433	rs5919392	rs6152	rs12011793
Testosterone (ng/dL)	**0.10 ± 0.04 (p=0.004)**	0.06 ± 0.04 (p=0.09)	0.44 ± 0.26 (p=0.10)	-0.28 ± 0.14 (p=0.05)	0.27 ± 0.16 (p=0.10)	0.16 ± 0.11 (p=0.12)	0.03 ± 0.14 (p=0.84)
Free testosterone (ng/dL)	**0.17 ± 0.04 (p<0.0001)**	0.06 ± 0.04 (p=0.08)	0.41 ± 0.28 (p=0.14)	**-0.30 ± 0.14 (p=0.04)**	**0.35 ± 0.17 (p=0.04)**	0.19 ± 0.11 (p=0.08)	0.12 ± 0.14 (p=0.40)
Estradiol (pg/mL)	**0.08 ± 0.04 (p=0.05)**	0.07 ± 0.04 (p=0.07)	0.32 ± 0.30 (p=0.029)	0.07 ± 0.15 (p=0.65)	0.16 ± 0.18 (p=0.39)	0.11 ± 0.12 (p=0.36)	0.25 ± 0.15 (p=0.09)
Free estradiol (pg/mL)	**0.10 ± 0.04 (p=0.014)**	0.07 ± 0.04 (p=0.07)	0.29 ± 0.29 (p=0.33)	0.06 ± 0.15 (p=0.68)	0.17 ± 0.18 (p=0.35)	0.10 ± 0.12 (p=0.37)	0.26 ± 0.15 (p=0.08)
SHBG (nmol/L)	-0.05 ± 0.04 (p=0.21)	0.02 ± 0.04 (p=0.52)	0.40 ± 0.27 (p=0.14)	-0.13 ± 0.14 (p=0.35)	0.02 ± 0.17 (p=0.89)	0.04 ± 0.11 (p=0.69)	-0.14 ± 0.14 (p=0.31)
LH (U/L)	0.06 ± 0.04 (p=0.14)	0.05 ± 0.04 (p=0.23)	0.22 ± 0.30 (p=0.45)	-0.12 ± 0.15 (p=0.42)	-0.05 ± 0.18 (p=0.80)	0.23 ± 0.12 (p=0.05)	0.22 ± 0.15 (p=0.14)
FSH (U/L)	-0.07 ± 0.04 (p=0.07)	0.06 ± 0.04 (p=0.15)	-0.34 ± 0.29 (p=0.24)	0.29 ± 0.15 (p=0.05)	-0.12 ± 0.18 (p=0.49)	-0.04 ± 0.11 (p=0.70)	0.10 ± 0.15 (p=0.51)
Whole body lean mass (kg)	-0.004 ± 0.017 (p=0.80)	0.02 ± 0.02 (p=0.20)	-0.17 ± 0.12 (p=0.17)	-0.08 ± 0.07 (p=0.24)	0.10 ± 0.08 (p=0.22)	0.09 ± 0.05 (p=0.08)	0.10 ± 0.06 (p=0.11)
Whole body fat mass (kg)	-0.005 ± 0.018 (p=0.80)	-0.02 ± 0.02 (p=0.30)	0.23 ± 0.13 (p=0.08)	0.06 ± 0.07 (p=0.40)	-0.08 ± 0.08 (p=0.34)	-0.10 ± 0.05 (p=0.05)	-0.12 ± 0.07 (p=0.07)
Radius 66 % muscle area (mm^2)	-0.04 ± 0.03 (p=0.17)	0.04 ± 0.03 (p=0.22)	-0.03 ± 0.24 (p=0.89)	-0.07 ± 0.12 (p=0.57)	0.33 ± 0.15 (p=0.02)	0.15 ± 0.09 (p=0.11)	0.17 ± 0.12 (p=0.15)
Tibia 66 % muscle area (mm^2)	0.005 ± 0.033 (p=0.90)	0.01 ± 0.03 (p=0.75)	-0.05 ± 0.24 (p=0.84)	-0.11 ± 0.12 (p=0.36)	-0.07 ± 0.15 (p=0.66)	0.08 ± 0.10 (p=0.39)	0.07 ± 0.12 (p=0.59)
Grip strength (kg)	-0.02 ± 0.04 (p=0.53)	-0.002 ± 0.036 (p=0.97)	-0.004 ± 0.271 (p=0.99)	-0.002 ± 0.140 (p=0.99)	-0.02 ± 0.17 (p=0.90)	0.09 ± 0.11 (p=0.41)	0.14 ± 0.14 (p=0.32)
Biceps force (Nm)	-0.05 ± 0.04 (p=0.21)	0.03 ± 0.03 (p=0.42)	-0.09 ± 0.27 (p=0.73)	-0.18 ± 0.13 (p=0.17)	0.29 ± 0.16 (p=0.07)	0.04 ± 0.10 (p=0.69)	0.04 ± 0.13 (p=0.77)
Quadriceps force (Nm)	-0.02 ± 0.04 (p=0.51)	0.03 ± 0.04 (p=0.38)	-0.16 ± 0.28 (p=0.56)	-0.07 ± 0.14 (p=0.59)	0.19 ± 0.16 (p=0.24)	-0.007 ± 0.104 (p=0.95)	0.02 ± 0.13 (p=0.87)

Table 3 Androgen receptor polymorphisms in relation to circulating gonadal steroids and muscle parameters.

Data are presented as standardized estimate ± SD (p-value). Results from mixed effects accounted for family structure and adjusted for age, height and weight.

DISCUSSION

In this cross-sectional study we investigated the interrelation between androgen sensitivity, heritability, circulating sex steroids, anthropometrics and muscle mass and function in a cohort of young men. We observed that the number of CAG repeats is associated with TT, FT, E_2 and FE_2 levels, and the 2D:4D finger length ratio and arm span. In contrast with the observed associations with circulating sex steroids, these genetic variations in the *AR* did not influence muscle mass or function in this cohort of young healthy men.

Our results are in agreement with twin studies reporting that muscle mass and strength are highly heritable [1]. Some of the remaining variance in muscle mass might be explained by antropometry, which is also under genetic control [2–4]. Height and weight were closely related to lean mass in our study. As taller subjects have longer bones, it is reasonable that they have longer muscles and thus higher muscle mass. Biceps force and hand grip force were also found to be related with anthropometric measurements, demonstrating that the strength of an individual is strongly determined by its body size.

Age has also an influence on skeletal muscle mass and function [3]. However, few studies have examined the relationship between age and lean mass in (young) adults [24,25]. In our study, we found a small but positive association between lean mass, muscle CSA and age. The lack of association between age and grip and biceps force, and the small inverse relationship with quadriceps force supports the results of Janssen *et al*. [25] which state that the muscle strength of the upper body is preserved better with increasing age than the muscle strength of the lower body.

The alterations in body composition with ageing are thought to be related to changes in sex steroid levels [26]. A loss of lean mass and an increase in fat mass are observed in elderly and hypogonadal men, whereas puberty in boys is associated with a remarkable gain in muscle mass [3,5]. However, the clinical relationship between androgens and muscle mass for variations within the normal range is less clear. In this cohort of eugonadal men, we demonstrated that whole body lean mass and muscle CSA are positively associated with both TT and FT. It is noteworthy that physical activity was also positively associated with serum T concentrations, indicating a higher impact of physical activity on muscle mass in men with higher serum T levels. However, and in agreement with Folland *et al.* [8], further analysis revealed that neither TT nor FT had any relation with muscle strength.

As mentioned earlier, between-subject differences in serum T levels within the physiological range are related in part to differences in androgen sensitivity and hypothalamus-pituitary feedback setpoint [9]. Genetic variations in the *AR* gene, in particulary CAG repeat polymorphisms, have been

associated with disorders linked to a reduced androgen activity [27]. We have previously shown that serum T levels are positively associated with the CAG and GGN repeat length in young, middle-aged and elderly men [9,13]. This is in contrast with the present study, in which we did not find any correlation between TT or FT and the GGN repeat length. It is noteworthy that the subjects of the current study are partly overlapping (358 unrelated men i.e. a single representative of the nuclear families out of 677 men) with the cohort of young men published by Crabbe *et al*. [9] and Bogaert *et al*. [13]. However, the serum concentrations of T have been re-determined by a highly precise LC-MS/MS method, as these were previously determined using less specific commercial immunoassay kits. Reports on associations between the GGN repeat and AR function are limited and inconsistent, with one study describing a positive association in a cohort of men with prostate cancer [28], whereas another study in young men could not find an association between the GGN repeat and serum T levels [29].

Based on studies reporting mutations in the *AR* gene related to AIS [14–16] we further screened for genetic polymorphisms in the *AR* that may affect the AR activity and thus circulating androgen levels. Interestingly, two SNPs (rs5965433 and rs5919392) were found to be significantly associated with FT, with the first also borderline significantly associated with TT. However, it is noteworthy that these associations did not remain significant after Bonferroni correction. Two recent genome-wide association studies [30,31] have identified several SNPs at different loci that were associated with serum T levels in middle-aged and elderly men. However, the *AR* gene was not described in these studies. Considering our relatively limited sample size, we suggest that analysis of our SNPs in those larger study populations may be required to confirm our findings.

Genetic variation in the *AR* gene influences circulating androgen levels, but may also affect body composition, muscularity or anthropometrics. Data on the association between CAG repeat length and muscle mass is limited and has been contradictory [8,32,33]. In our study, we could not find any relationship of CAG, GGN repeat length or the analysed SNPs in the *AR* with either body composition or measurements of muscularity. This might indicate that the relation of T with the muscle CSA is not related to genetic factors influencing androgen sensitivity, most likely because lower androgen sensitivity is compensated by elevated T levels.

Interestingly, we found that arm span and the 2D:4D finger length ratio were inversely associated with the number of CAG repeats, but not with the GGN repeat length or the analysed SNPs. The 2D:4D finger length ratio has been proposed as a marker of prenatal androgen action and of sensitivity to T, with a lower 2D:4D being associated with high androgen exposure [34,35]. Given the hypothesis that elevated T levels in men with lower androgen sensitivity do not necessary show

differences in androgen action, we can speculate that the negative effects on arm span and finger length might be mediated by the higher levels of FE_2 levels found in men with longer CAG repeat length, as suggested by Huhtamieni IT *et al.* [36]. As most E_2 produced in normal men is formed by aromatization of androgens [37], the higher T substrate availability in men with lower androgen sensitivity can explain the higher serum E_2 levels. E_2 is considered to be the main sex steroid involved in the development and maintenance of bone mass [18]. In addition, it is also important to initiate epiphyseal closure of long bones [38]. Therefore, we speculate that the presence of higher levels of E_2 in men with lower androgen sensitivity, but preserved estrogen action, resulted in earlier termination of longitudinal bone growth during puberty, an event wich is clearly observed in boys with aromatase excess syndrome or familiar hyperestrogenism [39,40].

To date, several studies have examined the possible relation of adult sex hormone concentrations [41,42] and *AR* CAG number [43–45] with 2D:4D, but results are controversial. To our knowledge, there is only one study that has examined the relationship between GGN repeat variation in the *AR* and 2D:4D ratios [46], but no reports on the relationship between SNPs in *AR* and 2D:4D ratios exist.

The higher serum E_2 levels found in men with a higher CAG repeat number might also play a direct role on muscle force since the negative association between E_2 and grip strength and biceps force, and between FE_2 and grip strength and biceps force in our study persisted after adjustment for T. Also Auyeung *et al.* [47] reported that E_2 levels, though positively related to muscle mass, were negatively related to muscle strength. However, it should be noted that the participants of the latter study were much older, with lower T levels.

Possible effects of E_2 on the regulation of muscle mass and function are still poorly understood. As skeletal muscle myoblasts and mature fibers express functional estrogen receptors (ER), a direct effect of E_2 in muscle cells may occur [48,49]. Although some studies have shown that E_2 is involved in muscle recovery [50,51] and has anabolic effects [52,53], a negative role of E_2 on the musculature has also been suggested by others. Several studies observed a decrease in muscle mass and force after E_2 administration of ovariectomized rats [54–57], and Brown M *et al.* [50] found an increase in muscle mass and function in *ER* knockout mice. However, the exact mechanism by which estrogens regulate muscle mass still has to be elucidated.

We recognize that our study has some limitations. First, our study may have been limited by the relatively small sample size, by which small but significant associations might have been missed, especially for the genetics analysis. Secondly, observations within brothers are not completely independent from each other. However, all analyses in this study were performed using linear

mixed-effects modelling with random intercepts to account for this interdependence. Furthermore, the cross-sectional design of this study does not allow us to draw conclusions on causality.

A major strength of this study is that we have used a highly precise LC-MS/MS method to determine T and E_2 serum concentrations. Most other studies used direct immunoassays, which are thought to have a reduced specificity at lower concentrations, especially those for serum E_2 [58,59], which could explain some of the conflicting results reported. Also, our cohort of healthy men in a well-defined age range may have strengthened our results.

In summary, in this study we showed that age, physical activity, body composition, sex steroid levels and anthropometrics are all determinants of muscle mass and function in young men. Although the number of CAG repeats were related to sex steriod levels and anthropometrics, we have no evidence that variations in the *AR* gene also contributes to the between subject variation in muscle mass or muscle function in young healthy men.

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Note added to manuscript in thesis:

Heritability estimates from sibling pairs do not only represent the genetic heritability, but also include a common environmental factor. These heritability estimates are therefore presented as upper-limit heritabilities or transmissibilities (t^2) rather than genetic heritabilities (h^2), as described in the publication.

Hormone levels are presented according to conventional units for laboratory testing and according SI units.

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IV. STUDY 2: ANDROGENIC AND ESTROGENIC REGULATION OF ATROGIN-1, MURF1 AND MYOSTATIN EXPRESSION IN DIFFERENT MUSCLE TYPES OF MALE MICE

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ABSTRACT

Purpose: The molecular factors targeted by androgens and estrogens on muscle mass are not fully understood. The current study aimed to explore gene and protein expression of Atrogin-1, MuRF1, and myostatin in an androgen deprivation-induced muscle atrophy model.

Methods: We examined the effects of orchidectomy either with or without testosterone (T) or estradiol (E_2) administration on Atrogin-1 gene expression, and MuRF1 and myostatin gene and protein expression. Measurements were made in soleus (SOL), extensor digitorum longus (EDL) and levator ani/bulbocavernosus (LA/BC) of male C57BL/6 mice.

Results: Thirty days of orchidectomy resulted in a reduction in weight gain and muscle mass. These effects were prevented by T. In LA/BC, Atrogin-1 and MuRF1 mRNA was increased throughout 30 days of orchidectomy, which was fully reversed by T and partially by E_2 administration. In EDL and SOL, a less pronounced upregulation of both genes was only detectable at the early stages of orchidectomy. Myostatin mRNA levels were downregulated in LA/BC and upregulated in EDL following orchidectomy. T, but not E_2, reversed these effects. No changes in protein levels of MuRF1 and myostatin were found in EDL at any time point following orchidectomy.

Conclusions: The atrophy in SOL and EDL in response to androgen deprivation, and its restoration by T, is accompanied by only minimal changes in atrogenes and myostatin gene expression. The marked differences in muscle atrophy and atrogene and myostatin mRNA between LA/BC and the locomotor muscles suggest that the murine LA/BC is not an optimal model to study orchidectomy-induced muscle atrophy.

Keywords: Atrogin-1, MuRF1, Myostatin, Skeletal muscle atrophy, Androgens

INTRODUCTION

Androgen deficiency refers to a significant decrease in circulating testosterone (T) levels and is associated with skeletal muscle loss and weakness (Mauras et al. 1998). T replacement therapy improves muscle mass and function in hypogonadal and elderly men (Bhasin et al. 1997, Snyder et al. 1999, Allan et al. 2008). Additionally, T and its synthetic derivatives stimulate anabolic effects in patients suffering from catabolic diseases, such as cancer, HIV/AIDS, COPD and burn injuries (Hart et al. 2001, Gold et al. 2006, Glass and Roubenoff 2010). Estrogen replacement therapy also has positive effects on skeletal muscle in post-menopausal women (Messier et al. 2011, Ahtiainen et al. 2012, Sipilä et al. 2013), while serum estradiol (E_2) levels in men are positively associated with lean- and muscle mass (Svensson et al. 2010, Vandenput et al. 2010, Auyeung et al. 2011). While androgen therapy has positive effects on muscle mass, it can have undesirable side effects such as increasing the risk of developing prostate cancer, cardiovascular disease, hepatotoxicity and infertility (Rhoden and Morgentaler 2004). Androgens and estrogens exert their effects by binding to their respective androgen (AR) (Lee and Chang 2003) and estrogen receptors (ER), with two ER isoforms in muscle (ERα or ESR1 and ERβ or ESR2) (Couse et al. 1997, Wiik et al. 2009). The molecular factors targeted by androgen and estrogen therapy that attenuate skeletal muscle loss and improve muscle function have not been completely elucidated. A better understanding of the muscle-specific molecules that are sensitive to androgen and estrogen therapy, and that regulate muscle atrophy, may identify therapeutic targets to improve clinical outcomes for patients suffering from diseases associated with skeletal muscle wasting (Glass and Roubenoff 2010, Sakuma and Yamaguchi 2010).

Skeletal muscle types have different levels of sensitivity to androgen deprivation and T administration (Axell et al. 2006, Hulmi et al. 2008, Ustunel et al. 2003). For example, the levator ani/bulbocavernosus muscle (LA/BC) is highly sensitive to androgen levels and is frequently investigated in androgen deprivation studies (Xu et al. 2004, Mendler et al. 2007, Pires-Oliveira et al. 2010, Serra et al. 2011). While both fast-twitch [extensor digitorum longus (EDL), tibialis anterior (TA) and plantaris] and slow-twitch [soleus (SOL)] muscles show androgen-dependent changes in muscle mass, the slow-twitch muscle appears to be more responsive to androgen deprivation and therapy (Axell et al. 2006). Therefore, when identifying muscle-specific molecules that may be sensitive to androgen deprivation and therapy, investigations should be completed in muscles with varying levels of androgen sensitivity, such as the LA/BC, EDL and SOL.

MAFbx/Atrogin-1 and MuRF1, muscle-specific ubiquitin ligases (Bodine et al. 2001, Gomes et al. 2001), are upregulated in numerous conditions of muscle atrophy (Foletta et al. 2011) and degrade proteins involved in muscle protein synthesis, regeneration and contraction (Lagirand-Cantaloube et

al. 2008, Li et al. 2004, Tintignac et al. 2005, Centner et al. 2001, Cohen et al. 2009, Fielitz et al. 2007). Myostatin, a member of the transforming growth factor-β (TGF-β) family, increases MAFbx/Atrogin-1 and MuRF1 mRNA levels (Lokireddy et al. 2011) and induces muscle atrophy; the latter a consequence of increased protein degradation and attenuated muscle protein synthesis (McPherron et al. 1997, Trendelenburg et al. 2009). The regulation of MAFbx/Atrogin-1, MuRF1 and myostatin in conditions of androgen deprivation and following androgen therapy remains equivocal, while little is known about the effects of E_2 administration. For example, orchidectomy causes an increase in MAFbx/Atrogin-1, MuRF1 and myostatin mRNA in triceps brachii (Ibebunjo et al. 2011), gastrocnemius (White et al. 2013) and LA muscles (Mendler et al. 2007, Pires-Oliveira et al. 2010); a response attenuated by the administration of T (Ibebunjo et al. 2011, Mendler et al. 2007, Pires-Oliveira et al. 2010) or nandrolone decanoate (White et al. 2013). However, others have observed no change in MAFbx/Atrogin-1 or MuRF1 mRNA levels in EDL muscle (Pires-Oliveira et al. 2010) or even a reduction in the mRNA levels of MAFbx/Atrogin-1 (Haren et al. 2011) and myostatin (Ibebunjo et al. 2011) in gastrocnemius and triceps brachii muscles of orchidectomized rats and mice. MAFbx/Atrogin-1 (Pires-Oliveira et al. 2010) and myostatin (Mendler et al. 2007) protein levels are upregulated in LA muscle following orchidectomy and return to baseline following T administration. No changes in MAFbx/Atrogin-1 protein in EDL muscle were reported (Pires-Oliveira et al. 2010) while MuRF1 protein levels were not measured. One week of E_2 administration in orchidectomized mice partially suppressed MAFbx/Atrogin-1 mRNA levels in gastrocnemius and LA muscle (Svensson et al. 2010). The effect of E_2 on MuRF1 and myostatin mRNA and protein levels has not been reported. Clearly further research is required to determine if MAFbx/Atrogin-1, MuRF1 and myostatin mRNA and protein levels are sensitive to androgen deprivation as well as T and E_2 therapy.

The aim of this study was to investigate the effect of androgen deprivation and T and E_2 administration on muscle mass and MAFbx/Atrogin-1, MuRF1, and myostatin mRNA and protein expression. All measurements were performed in muscles with varying androgen sensitivity, including the androgen-sensitive LA/BC, the slow-twitch SOL muscle and the fast-twitch EDL locomotor muscles, at three different time-points following orchidectomy.

MATERIALS AND METHODS

Animals and treatment

Adult male C57BL/6JOla inbred mice (8 weeks) were purchased from Harlan laboratories. Upon arrival, animals were allowed to acclimatize to their new surrounding for 2 weeks before surgery. All animals were given free access to water and food.

A total of 100 mice were included in this study and were randomly assigned into four intervention groups. Under isoflurane inhalation, 8-week-old mice were Sham-operated (Sham), orchidectomized (Orx+v) or were orchidectomized and treated with testosterone (Orx+T) or β-estradiol (Orx+E_2) (Sigma-Aldrich). Both hormones were administered using 0.5 cm subcutaneous silastic implants (Silclear tubing 1.57 × 2.41 mm, Degania, Israel) in the cervical region. A physiological rate of hormone release was obtained by diluting T and E_2 with cholesterol (respectively 1/2 and 1/16) as calculated by Vanderschueren et al. (2000), releasing 11.5 µg of T and 0.03 µg of E_2 daily. Empty silastic tubes were implanted in the 'Orx+v' group. Purity of the E_2 preparations was checked and no detectable contamination in the steroid preparations was found using LC-MS/MS (Applied Biosystems 5500). The efficacy of orchidectomy and dose of T treatment were verified by measurement of the seminal vesicles mass. To validate the dose of E_2 administration, the uterus weight of four mature female mice that were ovariectomized, and treated with the same dose of E_2 , was analyzed. Animals were killed at 7 days (n=8 for Sham, n=9 for Orx+v, Orx+T and Orx+E_2) or 30 days (n=11 for Sham, n=13 for Orx+v, n=23 for Orx+T and n=12 for Orx+E_2) after surgery to examine the short- and long-term effects of androgen deprivation and treatment. The ultrashort effects of androgen deprivation were determined at 24h after orchidectomy of six mice. Because data from a pilot experiment were found to be effective, they were included in the study. All experimental protocols were approved by the Ethical Committee for Animal Research of Ghent University.

Muscle excision and tissue biopsy

At the end of each intervention period, mice were weighed and anesthetized with an intra-abdominal injection containing a mixture of 80 % Ketamine (50 mg/ml, 100 mg/kg) and 20 % Xylazine (20 mg/ml, 10mg/kg). As described in detail below, after a 4-week intervention period, EDL and SOL of the right hindlimb were carefully dissected, and incubated for 15 min in Krebs-Henseleit solution, before contractility measurements were carried out. Animals were then euthanatized by cervical dislocation, and muscles of the left lower hindlimb, the LA/BC and seminal vesicles were removed and immediately frozen in liquid nitrogen. Biopsies were stored at -80 °C prior to weight determination, qPCR- and Western blot analysis.

Muscle contractile properties

After 30 days of Orx, twitch and tetanus contractile properties of the right hindlimb muscles were measured in vitro by stimulation with capacitor discharges between platinum electrodes (Radnoti), as previously described (Derave et al. 2005). In detail, after dissection, wires were attached to the tendons of EDL and SOL of the right hindlimb and were mounted on a heated (37 °C) incubation bath filled with Krebs-Henseleit-buffer (117 mM NaCl, 24.6 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 8 mM Mannitol and 2 mM Pyruvate). This solution was continuously oxygenated with a gaseous mixture of 95 % O_2 and 5 % CO_2. At the start of the experiment, muscles were set to their optimal length at which maximal isometric twitch forces are produced. Subsequently, the muscles were subjected to a series of five single twitch pulses of 500 µs duration with 1 Hz stimulation frequency and the average absolute force was calculated. A force-frequency curve was obtained by tetanic stimulation of the muscles at different frequencies (10, 20, 35, 50, 75, 100 Hz for SOL and 25, 40, 55, 70, 100, 125 Hz for EDL). A resting interval of 60 and 120s was allowed between each frequency for SOL and EDL, respectively. Because of the time-consuming technique of measuring in vitro muscle force, we have chosen to limit the measurements to the main intervention (orchidectomy) and only one time-point, at 30 days. Yet, we have measured muscle force of both SOL and EDL muscle.

RNA-isolation and qPCR

Total RNA was isolated from SOL, EDL and LA/BC using the TriPure Isolation Reagent (Roche Diagnostics, Vilvoorde, Belgium), followed by a purification with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). An on-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen). RNA was quantified by a Nanodrop 2000C spectrophotometer (Thermo Scientific), and RNA purity was assessed by calculating the A260/A280 ratio. Using a blend of oligo (dT) and random primers, 500 ng RNA was reverse transcribed with the iScript cDNA Synthesis kit (Biorad, Nazareth, Belgium), according to the manufacturer's instructions. qPCR was carried out on a Lightcycler 480 system (Roche) using an 8 µl reaction mix containing 3 µl template cDNA (1/10 dilution), 300 nM forward and reverse primers and 4 µl SYBR Green PCR Master Mix (Applied Biosystems, Halle, Belgium). Samples were analyzed according to the maximization method (Hellemans et al. 2007). The cycling conditions comprised a polymerase activation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15s, 60 °C for 60s. Primer sequences for MAFbx/Atrogin-1 and MuRF1 were adapted from the literature or selected from the RTprimerDB site (http://medgen.ugent.be/rtprimerdb/). Primers for myostatin, AR, estrogen receptor α (ESR1) and estrogen receptor β (ESR2) were designed using Primer Express software 3.0 (Applied Biosystems) (Table 1). Sequence specificity was confirmed using

NCBI Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To control the specificity of amplification, data melting curves were inspected, and PCR efficiency was calculated (between 90 and 110 % for all genes). Normalised gene expression values were calculated by dividing the relative gene expression values (calculated by the delta-Ct method) for each sample by the geometric mean of Ppia, Rplp0 and B2m as selected by GeNorm (Vandesompele et al. 2002).

Protein isolation and Western blots

Protein extraction was performed according to previously described procedures (Wallace et al. 2011). Total protein was extracted from ~ 11 mg of EDL using 1x RIPA buffer (15µl/mg sample) (Millipore, North Ryde, NSW, Australia) with the addition of protease inhibitor cocktail (Sigma, Castle Hill, NSW, Australia) and Halt Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific, Rockford, IL, USA). Following homogenization, the lysate was rotated at 4 °C for 60 min before being centrifuged at 13,000 rpm at 4 °C for 15 min. Total protein content of the supernatant was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Since the LA/BC complex was extremely atrophied after Orx, in particularly for the LA itself, there was not enough muscle left to carry out protein isolation. As no differences in protein expression were found in de EDL, we did not expect to find any differences in the SOL muscle.

Protein samples (30 µg) were denaturated in loading buffer and were separated by a 12 % SDS-PAGE gel in a buffer containing 25 mM Trisbase, 192 mM glycine and 0.1 % SDS, pH 8.8. Following separation, proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA) in a cold (4 °C) transfer buffer (25 mM Tris, 192 mM glycine, 10 % methanol, pH 8.3) during 2h, and membranes were then blocked for 1 h at room temperature in 5 % BSA or 5 % skim milk in PBS. Membranes were subsequently incubated overnight by gentle shaking at 4 °C with the following primary antibodies diluted 1:1,000 in 5 % BSA/PBS for myostatin (Millipore), and in 5 % skim milk/PBS for MuRF1 (ECM Biosciences, Versailles, KY, USA). After 4 x 5 min washing with PBS, the membranes were incubated for 1 h with a goat anti-rabbit IgG antibody labeled with an infrared-fluorescent 800-nm dye (Alexa Fluor 800; Invitrogen, Carlsbad, CA, USA) diluted 1:5,000 in PBS containing 50 % Odyssey blocking buffer (LI-COR Biosciences) and 0.01 % SDS. After washing, the specific proteins were visualized using the Odyssey Imaging System (LI-COR Biosciences) and individual protein band optical densities were quantified with the Odyssey software. Samples were alternately loaded and an internal control sample was loaded on each gel to account for run-to-run variation between the samples. To control

for protein loading, blots were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (G8795; Sigma-Aldrich, Sydney, Australia).

Statistical analysis

Statistical analyses were performed using SPSS statistical software (SPSS 19.0, Chicago, IL). Statistical differences between the Sham and the three intervention groups (Orx+v, Orx+T and Orx+E_2) at day 1, day 7 and day 30 were determined via a one-way analysis of variance (ANOVA) and a subsequent post hoc LSD test when a significant group effect was observed. An independent sample *T* test was used to compare the muscle twitch force between orchidectomized and control mice for the SOL and the EDL muscle. The effect of orchidectomy on absolute tetanic muscle force was assessed using a repeated measurements two-way ANOVA (frequency x intervention) for the SOL and EDL muscle. Normalized mRNA expression data were log-transformed to fulfill criteria for normality. Values are presented as means ± SD, and $P \leq 0.05$ was considered significant.

Table 1 Primers used in qPCR analysis

Gene symbol[a]	Source	Forward primer 5'-->3' Reverse primer 5'-->3'	GenBank accession number
Ppia	RTprimerDB	CAA-ATG-CTG-GAC-CAA-ACA-CAA-ACG GTT-CAT-GCC-TTC-TTT-CAC-CTT-CCC	NM_008907
Rplp0	RTprimerDB	GGA-CCC-GAG-AAG-ACC-TCC-TT GCA-CAT-CAC-TCA-GAA-TTT-CAA-TGG	NM_007475
B2m	RTprimerDB	CAT-GGC-TCG-CTC-GGT-GAC-C AAT-GTG-AGG-CGG-GTG-GAA-CTG	NM_009735
Fbxo32/MAFbx/ Atrogin-1	Pires-Oliveira et al. (2010)	GCA-GAG-AGT-CGG-CAA-GTC CAG-GTC-GGT-GAT-CGT-GAG	NM_026346
Myostatin	Primer express	TGC-TAT-AAG-ACA-ACT-TCT-GCC-AAG-A AAG-AGC-CAT-CAC-TGC-TGT-CAT-C	NM_010834
Trim63/MuRF1	Tang et al. (2010)	TGG-AAA-CGC-TAT-GGA-GAA-CC ATT-CGC-AGC-CTG-GAA-GAT-G	NM_001039048
Ar	Primer express	TCT-ACT-TTG-CAC-CTG-ACT-TGG-TTT ACT-CTT-GAG-ACA-GGT-GCC-TCA-TC	NM_013476.3
Esr1	Primer express	ACT-ACA-TAC-CCC-CGG-AAG-CA CAG-GGA-TTC-TCA-GAA-CCT-TTC-G	NM_007956.4
Esr2	Primer express	TGA-TGG-TCA-GAA-GTG-GGA-CAT-G AAG-CGC-AAC-GTG-GGT-AAG-G	NM_010157.3

[a]*Ppia* Peptidylprolyl isomerase A, *Rplp0* Ribosomal protein large P0, *B2m* Beta-2 microglobulin, *Fbxo32* F-box protein 32, *MAFbx* Muscle Atrophy F-box, *Trim63* Tripartite motif-containing 63, *MuRF1* Muscle RING Finger 1, *Myostatin* Myostatin, *Ar* Androgen receptor, *Esr1* Estrogen receptor α, *Esr2* Estrogen receptor β

RESULTS

Regulation of body weight and muscle mass

Compared to the Sham group, body weight gain was attenuated in Orx+v mice when measured 30-day post-operation. Administration of T for 30 days, but not E_2, prevented the Orx-induced attenuation in body weight gain (Fig. 1a). Androgen deprivation was successfully induced as confirmed by measuring seminal vesicle mass which was reduced by 68 and 89 % at 7- and 30-day post-operation, respectively. T, but not E_2, administration prevented seminal vesicle atrophy at both time points (Fig. 1b).

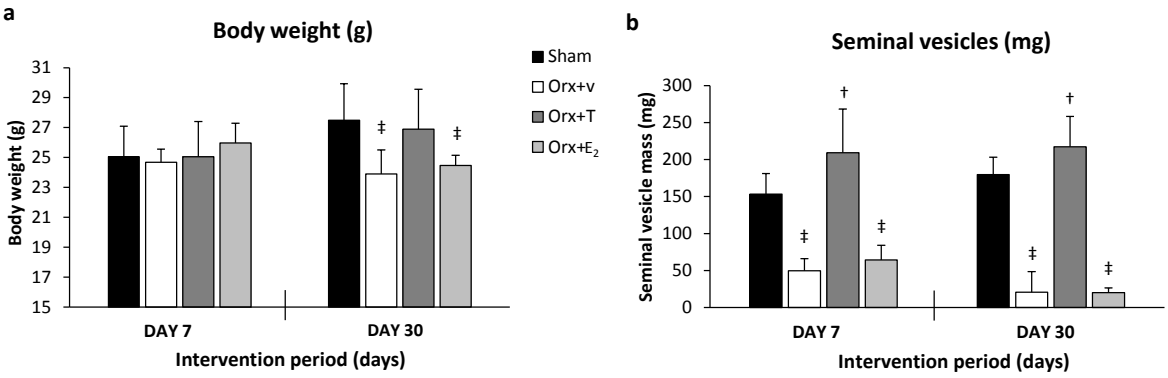

Figure 1. Effect of short- (7 days) and long-term (30 days) orchidectomy (Orx+v), orchidectomy and testosterone treatment (Orx+T), and orchidectomy and estradiol treatment (Orx+E_2) on body weight (**a**) and seminal vesicles (**b**). Each bar represents the mean ± standard deviation (SD). Differences between groups were assessed by one-way analysis of variance (ANOVA). * (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from Shams.

LA/BC muscle mass was reduced by 22 and 63 % when measured 7- and 30-day post-Orx, respectively; an effect blocked by T treatment at both time points. E_2 treatment for 7, but not 30 days, blocked the loss of LA/BC muscle mass (Fig. 2a). SOL and EDL muscle mass was also reduced by 13 and 14 % respectively in the Orx+v group, 30-day post-intervention; an effect attenuated by T, but not E_2 (Fig. 2b, c). No difference in body weight or SOL and EDL muscle weights was observed between treatment groups when measured 7-day post-intervention (data not shown).

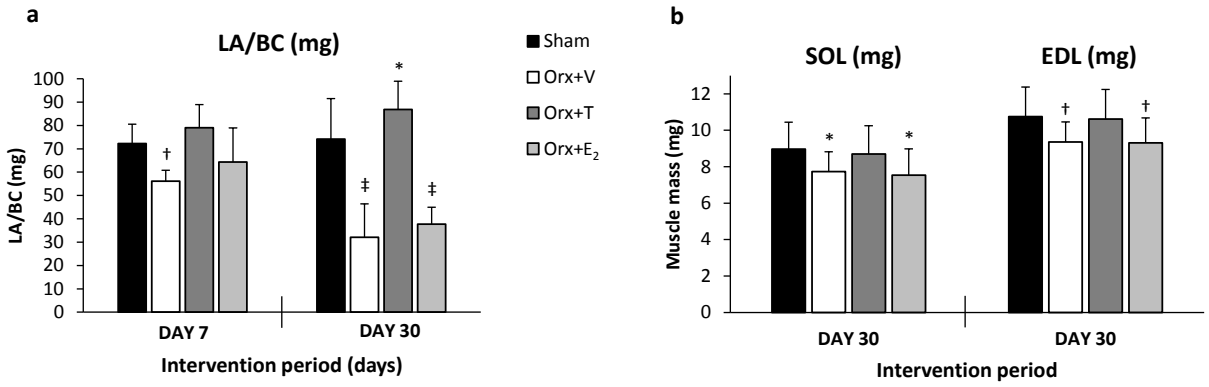

Figure 2. Effect of orchidectomy (Orx+v), orchidectomy and testosterone treatment (Orx+T), and orchidectomy and estradiol treatment (Orx+E_2) on LA/BC muscle mass, 7- and 30-day post-intervention (**a**), and SOL and EDL muscle mass, 30-day post-intervention (**b**). Differences between groups were assessed by one-way analysis of variance (ANOVA). *($P < 0.05$), †($P < 0.01$), ‡($P < 0.001$): significantly different from Shams.

It was of interest to determine if the different response to T and E_2 for the muscle types was associated with the expression levels of AR and estrogen (ESR1 and ESR2) receptors. AR mRNA expression was 300 % higher in the LA/BC muscle when compared with SOL ($P < 0.00001$) and EDL muscles ($P < 0.00001$) in Sham-operated mice (Fig. 3a). No difference in AR mRNA expression between EDL and SOL was found ($P = 0.40$). ESR1 mRNA levels were 110 and 550 % higher in EDL when compared with the SOL and LA/BC muscle, respectively (Fig. 3b). ESR1 expression was also 210 % higher in the SOL compared with the LA/BC muscle. ESR2 mRNA levels were 620 and 480 % higher in LA/BC muscle when compared with the SOL and EDL muscle, respectively; the latter two muscles having similar ESR2 expression levels (Fig. 3c).

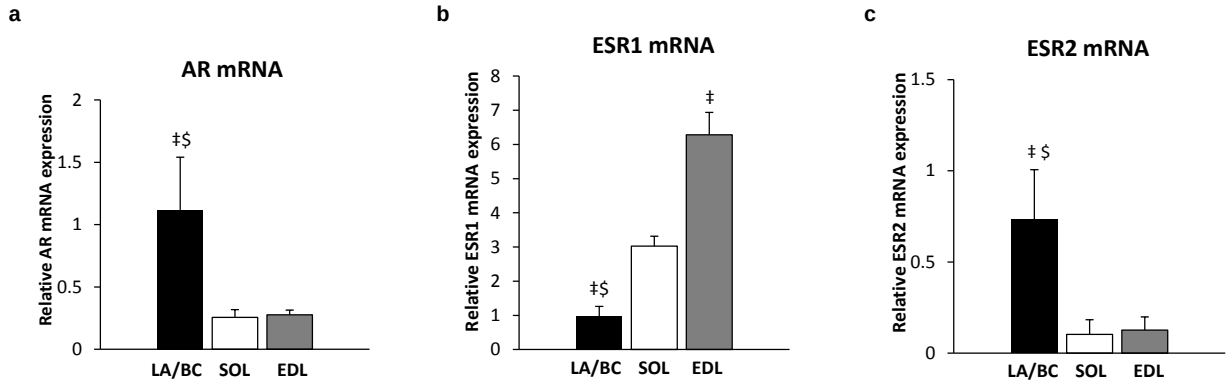

Figure 3. Relative mRNA expression levels of the androgen receptor (AR) (**a**), estrogen receptor α (ESR1) (**b**), and estrogen receptor β (ESR2) **(c)** in LA/BC, SOL and EDL muscle. Differences between groups were assessed by one-way analysis of variance (ANOVA). ‡($P < 0.001$): significantly different from SOL, $($P < 0.001$): significantly different from EDL

Influence of testosterone on skeletal muscle force production

A 37 % reduction (*P* = 0.02) in absolute twitch force and a decrease in contraction and relaxation speed in SOL muscle were observed 30-day post-Orx; no significant effect was observed for the EDL muscle (Fig. 4a). As presented in the force-frequency curve (Fig. 4b), a main effect (*P* = 0.042) for group indicated that absolute tetanic force was also reduced in the SOL muscle of the Orx+v mice when compared with Sham mice. When expressing the SOL muscle twitch and tetanic force relative to muscle mass, there was no difference between the Orx+v and Sham-treated mice (data not shown). Orx did not affect absolute or relative EDL muscle force production (data not shown).

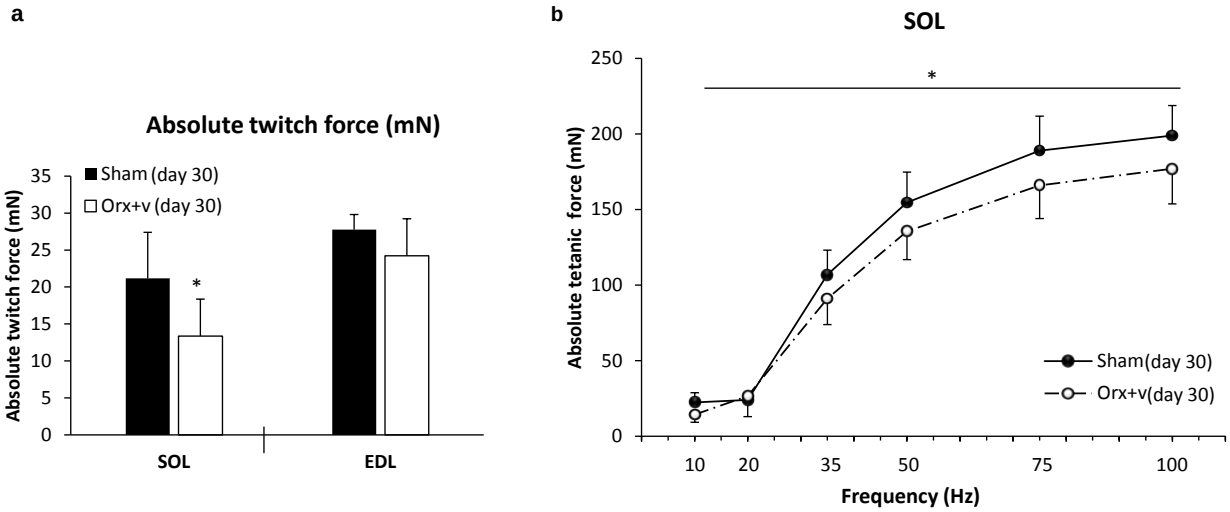

Figure 4. Absolute twitch force in SOL and EDL (**a**) and force-frequency relationship of SOL (**b**) muscle in Sham-operated (Sham) and orchidectomized (Orx+v) mice, 30-day post-intervention. Differences in absolute twitch force between groups were assessed by independent sample *T* tests and differences in absolute tetanic force between groups were assessed by two-way ANOVA repeated measurements (frequency x intervention). *(*P* < 0.05) significantly different from Shams.

LA/BC muscle

In the LA/BC muscle, MAFbx/Atrogin-1 mRNA increased by 300-900 % when measured 1, 7 and 30 days following Orx (Fig. 5a). A similar mRNA expression pattern was observed for MuRF1 (Fig. 5b). In contrast, myostatin mRNA levels were reduced by 86, 79 and 77 % when measured 1, 7 and 30 days after Orx (Fig. 5c). Administration of T blocked, while E_2 attenuated, the Orx-induced increase in MAFbx/Atrogin-1 and MuRF1 mRNA (Fig. 5a, b). Only T administration for 30 days was able to maintain myostatin levels when compared to the Sham group (Fig. 5c).

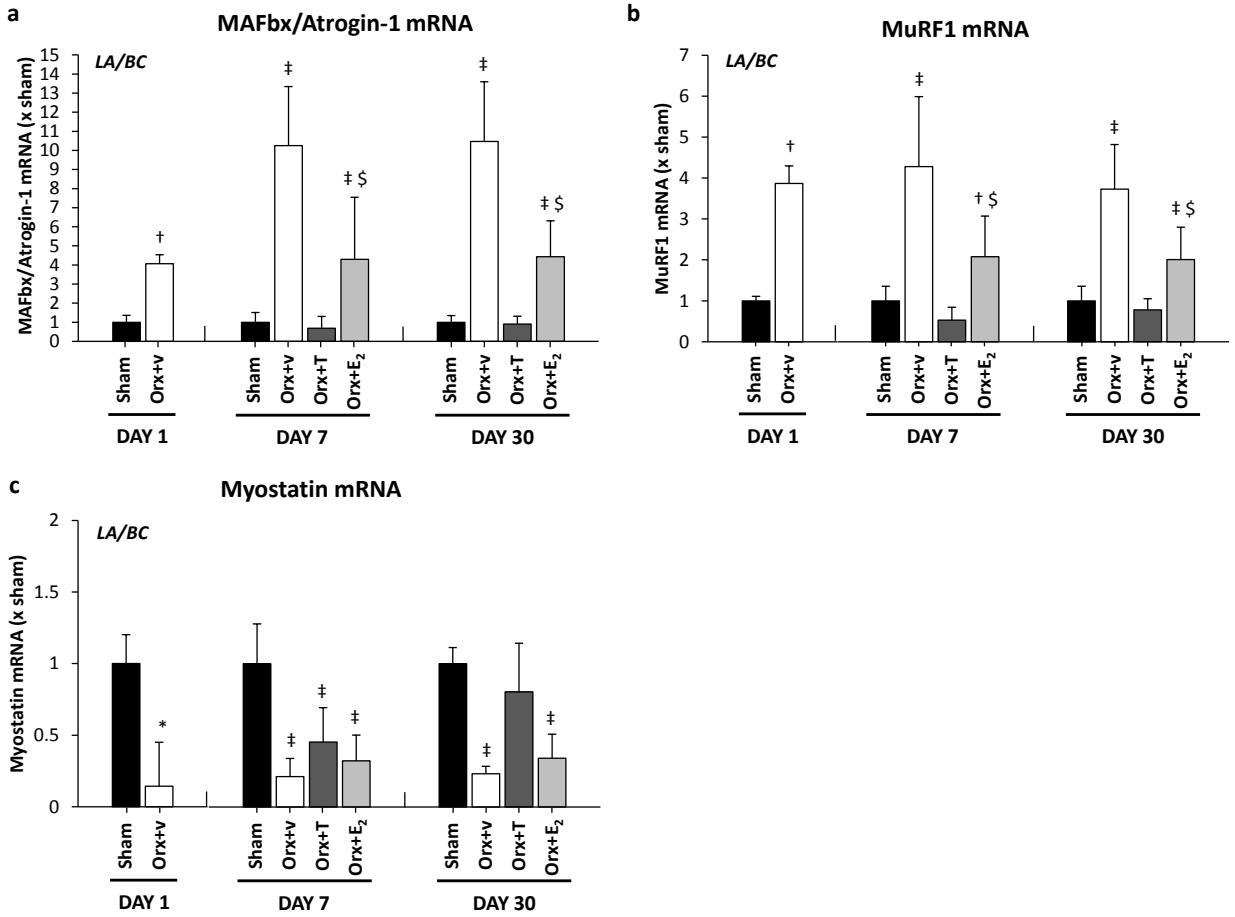

Figure 5. Effect of ultrashort (1 day), short- (7 days) and long-term (30 days) orchidectomy (Orx+v), orchidectomy and testosterone treatment (Orx+T), and orchidectomy and estradiol treatment (Orx+E_2) on MAFbx/Atrogin-1 mRNA expression (**a**), MuRF1 mRNA expression (**b**), and myostatin mRNA expression (**c**) in levator ani/bulbocavernosus (LA/BC) muscle. Data are shown as fold change from the control group for each gene. Differences between groups were assessed by one-way analysis of variance (ANOVA). *($P < 0.05$), †($P < 0.01$), ‡($P < 0.001$): significantly different from Shams. $($P \leq 0.001$): significantly different from Orx+v.

SOL muscle

In the SOL muscle, Orx did not alter MAFbx/Atrogin-1 mRNA levels; however, treatment with E_2 for 7 days increased MAFbx/Atrogin-1 mRNA by 60 % ($P = 0.02$) (Fig. 6a). MuRF1 mRNA was increased by 38 % ($P = 0.04$) 7 days, but not 30 days, post-Orx (Fig. 6b). At 30-day post-Orx, T and E_2 administration reduced MuRF1 mRNA in the Orx group (Fig. 6b). There was no effect on myostatin mRNA in any of the treatment groups (data not shown).

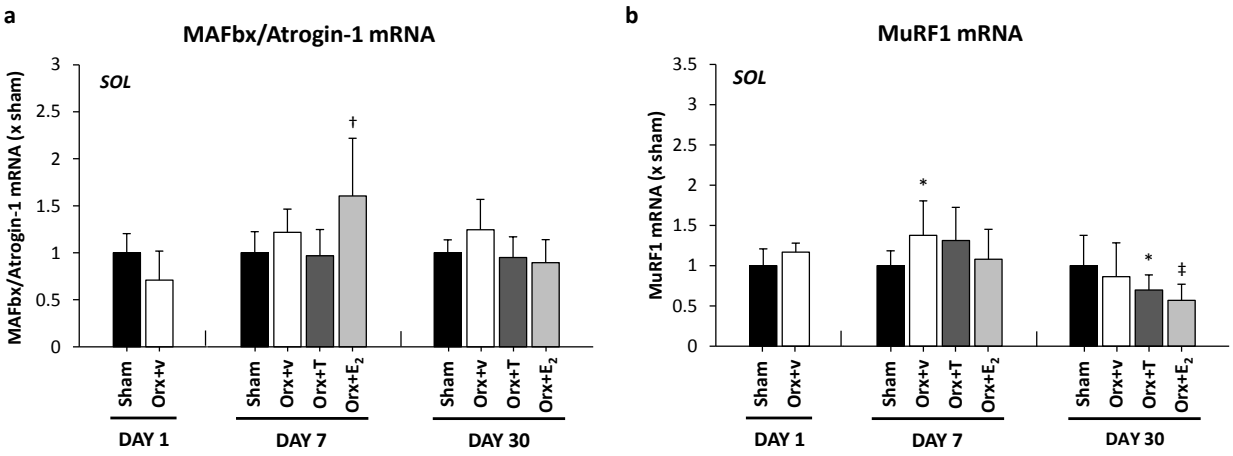

Figure 6. Effect of ultrashort (1 day), short- (7 days) and long-term (30 days) orchidectomy (Orx+v), orchidectomy and testosterone treatment (Orx+T), and orchidectomy and estradiol treatment (Orx+E_2) on MAFbx/Atrogin-1 mRNA expression (**a**), and MuRF1 mRNA expression (**b**) in soleus (SOL) muscle. Data are shown as fold change from the control group for each gene. Differences between groups were assessed by one-way analysis of variance (ANOVA). *($P < 0.05$), †($P < 0.01$), ‡($P < 0.001$): significantly different from Shams.

EDL muscle

In EDL muscle, there was 100 % increase in MAFbx/Atrogin-1 and MuRF1 mRNA 1 day following Orx when compared with the Sham group. This increase was not detectable when measured at 7 or 30 days following Orx (Fig. 7a, b). T and E_2 administration did not influence the MAFbx/Atrogin-1 and MuRF1 mRNA levels (Fig. 7a, b). Myostatin mRNA levels were upregulated by 50 % 7 days following Orx, an effect suppressed by T administration (Fig. 7c).

MuRF1 or myostatin protein levels were not altered in the EDL muscle in any of the intervention group studied (data not shown). Representative blots are shown in Figure 8.

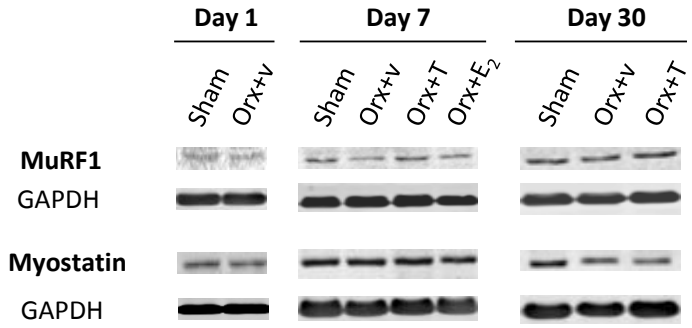

Figure 8. Protein blots of MuRF1 and myostatin measured in EDL muscle

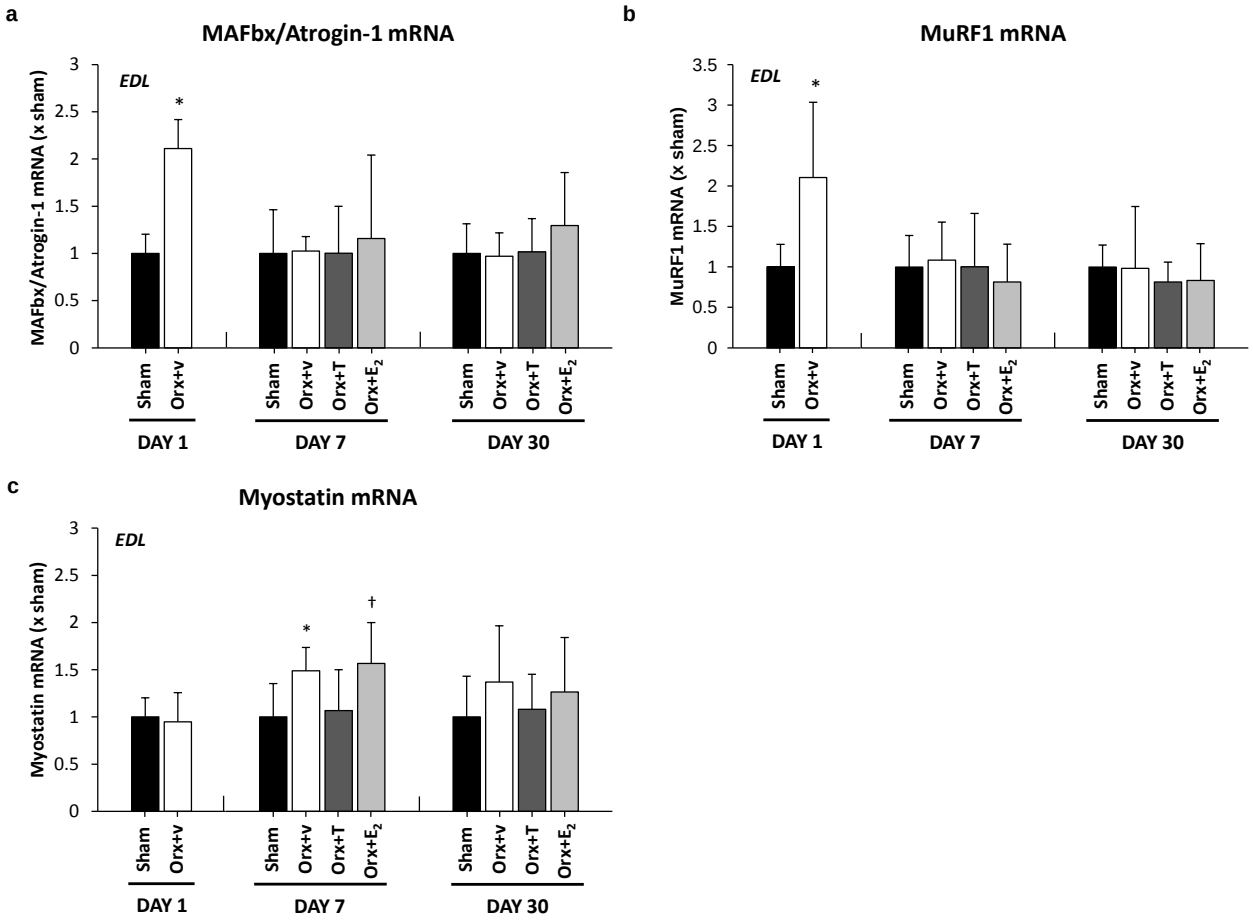

Figure 7. Effect of ultrashort (1 day), short- (7 days) and long-term (30 days) orchidectomy (Orx+v), orchidectomy and testosterone treatment (Orx+T), and orchidectomy and estradiol treatment (Orx+E_2) on MAFbx/Atrogin-1 mRNA expression (**a**), MuRF1 mRNA expression (**b**), and myostatin mRNA expression (**c**) in extensor digitorum longus (EDL) muscle. Data are shown as fold change from the control group for each gene. Differences between groups were assessed by one-way analysis of variance (ANOVA). *($P < 0.05$), †($P < 0.01$): significantly different from Shams.

DISCUSSION

Skeletal muscle atrophy occurs following androgen deprivation. While androgen and estrogen therapy can improve muscle mass, these therapies come with considerable side effects. Understanding the muscle atrophy-stimulating factors regulated by both androgen deprivation and androgen and estrogen therapy is important for the identification of potential muscle-specific therapeutic targets to improve the clinical outcomes of muscle wasting diseases. The present study used an orchidectomy (Orx)-induced skeletal muscle atrophy mouse model, with and without T and E_2 treatment, to establish the expression levels of key targets that stimulate skeletal muscle atrophy.

Orx resulted in an expected reduction in body weight as well as a rapid and continual decrease in LA/BC muscle weight, demonstrating the successful manipulation of our experiment model. However, the reduction in muscle mass of the locomotor SOL and EDL muscles was less severe. The strong and rapid androgenic response in muscle mass in the LA/BC may have been influenced by the higher mRNA levels of AR found in this muscle when compared to the SOL and EDL muscle. AR knock-out mice (ARKO) (MacLean et al. 2008) and myocyte-specific ARKO mice (Ophoff et al. 2009) demonstrated severe atrophy of the LA muscle compared to only a slight atrophy in the locomotor muscles such as the tibialis anterior, gastrocnemius, EDL and SOL muscle.

T administration is known to attenuate Orx-stimulated body weight loss and muscle atrophy in the LA/BC, SOL and EDL muscles (Axell et al. 2006, Mendler et al. 2007); a response observed in the present study. Interestingly, we observed that E_2 treatment in our Orx mice partially suppressed muscle loss in the LA/BC, but not in SOL and EDL muscles. Our observation that ESR2 mRNA levels are highly expressed in the LA/BC muscle, with very low expression levels in the SOL and EDL muscle, suggests that ERβ may be involved in the estrogen-mediated anabolic effects on LA/BC muscle mass. However, it is clear that more studies are needed before conclusive statements may be drawn.

In light of these changes in muscle mass, functional analysis of the SOL and EDL muscles demonstrated that Orx reduced absolute, but not relative, twitch force in the SOL muscle, supporting previous observations (Axell et al. 2006). This suggests that the reduction in muscle force in the SOL muscle following Orx is predominantly due to muscle wasting. Surprisingly, even though the EDL muscle had the same amount of muscle wasting as the SOL, Orx did not influence absolute or relative force production. The reason for this is unclear and may be caused by larger methodological variability of the *in vitro* contractility measures as compared to the determination of muscle mass.
Orx resulted in a rapid and persistent upregulation of MAFbx/Atrogin-1 and MuRF1 mRNA in the LA/BC during the 30-day period; an observation supporting others (Pires-Oliveira et al. 2010,

Svensson et al. 2010). However, we observed for the first time that this response was less pronounced and transient in the locomotor SOL and EDL muscles following Orx. Myostatin mRNA levels were decreased in the LA/BC muscle, whereas it was upregulated in the EDL following Orx. In contrast to the present study, a reduction in myostatin mRNA in the triceps brachii muscle of orchidectomized mice (Ibebunjo et al. 2011) and an increase in myostatin mRNA in the LA of orchidectomized rats (Mendler et al. 2007) have been observed. Why such discrepancy in androgenic modulation of myostatin mRNA levels among muscle types exist is not clear. The regulation of MAFbx/Atrogin-1 and MuRF1 mRNA levels by androgens in different locomotor muscles remains equivocal. Ibebunjo et al. (2011) and White et al. (2013) found an upregulation of MAFbx/Atrogin-1 and MuRF1 mRNA in triceps brachii and gastrocnemius of mice after 7- and 30-day Orx, an effect which was fully reversed by T (Ibebunjo et al. 2011) or nandrolone decanoate (White et al. 2013). In contrast, other studies did not observe altered MAFbx/Atrogin-1 and MuRF1 mRNA levels in the EDL muscle (Pires-Oliveira et al. 2010), while others observed a reduction in MAFbx/Atrogin-1 mRNA (Haren et al. 2011) in the gastrocnemius of orchidectomized rats. These differences are most likely due to the androgen-sensitivity levels of the different locomotor muscles as well as the different study designs. It is noteworthy that the mice in our study were still growing during the experimental period. Therefore, Orx-stimulated muscle atrophy coincided with a reduction in growth, which could have influenced the expression levels of the molecular targets measured.

Discordance between mRNA and protein levels of MuRF1 has already been described in other skeletal muscle atrophy models (Drummond et al. 2008) and highlights the need to measure both mRNA and protein levels when investigating MuRF1 in muscle atrophy conditions. Unlike the mRNA levels, we observed no change in MuRF1 or myostatin protein in the EDL muscle. In contrast, an increase in myostatin protein has been observed in the LA/BC muscle (Mendler et al. 2007). It is noteworthy that previous studies have not examined MuRF1 protein levels during androgen deprivation. However, we were unable to validate a specific antibody for MAFbx/Atrogin-1 protein. It must be noted that the lack of activity assays MAFbx/Atrogin-1 and MuRF1 remains a limitation in this field of research.

Androgen and estrogen therapy is able to attenuate the loss in muscle (Bhasin et al. 1997, Messier et al. 2011). In the present study, T administration blunted Orx-induced changes in MAFbx/Atrogin-1 and MuRF1 mRNA in LA/BC muscle only, with no effect on MAFbx/Atrogin-1 mRNA or MuRF1 mRNA in the SOL and mRNA and protein in EDL muscles. Therefore, we could not provide evidence that MAFbx/Atrogin-1 and MuRF1 are regulated by T during Orx-induced atrophy in locomotor SOL and EDL muscles. In the LA/BC muscle, E_2 administration also attenuated the increase in MAFbx/Atrogin-1

supporting observations by Svensson et al. (2010). However, we also observed that E_2 attenuated the increase in MuRF1 mRNA. Our findings indicate that the androgenic and estrogenic regulation of LA/BC muscle mass is likely influenced by transcriptional modulation of both MAFbx/Atrogin-1 and MuRF1.

CONCLUSIONS

Based on the different regulatory profile of MAFbx/Atrogin-1, MuRF1 and myostatin mRNA levels between the SOL and EDL locomotor muscles and the LA/BC muscle, we suggest that the murine LA/BC muscle is not an appropriate muscle to study skeletal muscle atrophy as it is not representative for the androgen sensitivity of other more functionally and clinically relevant skeletal muscle types. Furthermore, the lack of changes in MuRF1 and myostatin protein levels in EDL questions the involvement of these proteins in the regulation of the locomotor muscle mass by androgens. Finally, our data indicate that further experiments examining the effects of E_2 on skeletal muscle mass are of potential significance.

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V. STUDY 3: EFFECTS OF TAIL SUSPENSION ON SERUM TESTOSTERONE AND MOLECULAR TARGETS REGULATING MUSCLE MASS

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Under review in Muscle and Nerve

ABSTRACT

Introduction: The contribution of reduced testosterone levels on tail suspension (TS)-induced muscle atrophy remains equivocal. The molecular mechanism by which testosterone regulates muscle mass during TS has not been investigated.

Methods: Effects of TS on serum testosterone levels (by LC-MS/MS), muscle mass and expression of muscle atrophy and hypertrophy inducing targets were measured in soleus (SOL) and extensor digitorum longus (EDL) muscles following testosterone administration during 1, 5 and 14 days of TS in male mice.

Results: TS resulted in an increase, followed by a transient drop, in testosterone levels and a decrease in muscle mass. Testosterone supplementation did not affect muscle mass or IGF1, p-AKT, p-$p70^{S6K}$, REDD1, Atrogin-1 and MuRF1 protein expression during TS. Apparent differences in *Igf1*, *Mstn* and *MAFbx/Atrogin-1* gene expression between SOL and EDL following TS were found.

Discussion: The known anabolic effects of testosterone are not sufficient to ameliorate muscle mass in TS-induced muscle atrophy.

Keywords: Tail suspension, Testosterone, Disuse atrophy, Atrogin-1, AKT

INTRODUCTION

Tail suspension (TS) in rodents is a commonly used muscle atrophy model causing a rapid and extensive loss of skeletal muscle mass and function.[1,2] Previous studies have shown that TS is associated with,[3,4] although not invariably,[5,6] reductions in serum testosterone levels. In male rodents the inguinal canal remains open throughout life so the testes can periodically move into the abdomen when subjected to TS. Because of the increased testicular temperature in this new environment testosterone production can be affected.[7] However reduced caloric consumption and stress can also interfere with testosterone production.[8]

Testosterone deficiency (i.e. hypogonadism, castration) is associated with significant skeletal muscle loss and weakness,[9] suggesting that hormonal changes associated with TS can contribute to skeletal muscle atrophy. In support of this hypothesis, supplementation of nandrolone decanoate, a synthetic analog of testosterone, can attenuate, but not inhibit, TS-induced skeletal muscle atrophy in rats.[10-12]

Skeletal muscle mass is determined by the balance between protein synthesis and breakdown.[13] AKT, a serine/threonine kinase, plays a pivotal role in the regulation of skeletal muscle mass. In the canonical insulin-like growth factor 1 (IGF1)/AKT/mammalian target of rapamycin (mTOR) pathway, AKT activates mTOR, which subsequently phosphorylates p70-ribosomal S6 kinase ($p70^{S6K}$) and factor 4E-binding protein 1 (4E-BP1), resulting in protein synthesis.[14] Testosterone administration increases *Igf1* mRNA in rat muscle,[15] but the androgenic effect on AKT signaling remains equivocal.[16,17] In addition to protein synthesis regulation, AKT can inhibit protein degradation. AKT phosphorylates the forkhead family (FoxO) of transcription factors,[18] whereby they are sequestered to the cytoplasm and unable to transcribe the two muscle-specific ubiquitin protein ligases, *muscle atrophy F-box (MAFbx/Atrogin-1)* and muscle RING (really interesting new gene) finger-1 **(***MuRF1).*[19] Both genes are rapidly upregulated in rodent muscle during different atrophy models including disuse-induced[20,21] and castration-induced muscle atrophy,[17,22] although not all reports have confirmed the latter.[16,23] Myostatin (MSTN), a member of the transforming growth factor-β (TGF-β) family, attenuates muscle growth by inhibiting AKT signaling.[24] However, the regulation of MSTN during disuse[25-28] or androgen treatment[29,30] has been controversial. Regulated in Development and DNA Damage responses 1 (REDD1), is another negative regulator of muscle mass as it represses mTOR activity.[31] *Redd1* gene expression is increased in hindlimb muscles during casting,[32] and is inhibited by testosterone in dexamethasone treated rats.[33]

Determination of gonadal steroids in mice can be cumbersome as a circulating sex hormone binding globulin is absent in adult rodents, resulting in a high individual variation in serum testosterone

levels.[34] Moreover, only a limited amount of blood sample can be derived from those small animals. Radioimmunoassays are commonly used for the determination of serum testosterone levels,[3,5-7] although this technique has low sensitivity and specificity.[35] So far, no studies measuring testosterone levels in rodents following tail suspension-induced muscle atrophy have used the highly sensitive liquid chromatography tandem mass spectrometry method (LC-MS/MS).[36] Accurate measurement of testosterone levels by LC-MS/MS may therefore help in clarifying the potential change in testosterone levels associated with TS.

To date, the muscle-specific molecular factors targeted by androgen therapy that attenuate skeletal muscle mass loss during TS have not been studied. Understanding the molecular factors involved, may identify therapeutic targets to improve clinical outcomes for patients suffering disuse muscle atrophy.[37] Therefore, we aimed to investigate the effects of 1, 5 and 14 days of TS, without and with testosterone supplementation, on serum testosterone levels, skeletal muscle mass and gene and protein expression levels of the molecular factors that regulate muscle mass including IGF/AKT/p70^{S6K}, MSTN, REDD1, MAFbx/Atrogin-1 and MuRF1. As different muscle types respond differently to TS induced atrophy[38] and testosterone supplementation,[39] both slow-twitch soleus (SOL) and fast-twitch extensor digitorum longus (EDL) muscles were analysed.

MATERIALS AND METHODS

Animals

Ninety 8-week old male C57BL/6JOla inbred mice (Harlan laboratories) were included in this study. The mice were housed two animals per cage and were allowed to acclimatize to their new surrounding for 2 weeks before surgery. All mice were given free access to water (hydrogel) and standard chow on the ground, and were maintained under a 12/12h light/dark cycle. The welfare of the animals, body weight and damage to the TS-material was monitored daily. The experimental protocol was approved by the Ethical Committee for Animal Research of Ghent University, and the housing conditions were as specified by the Belgian Law of November 14, 1993, on the protection of laboratory animals.

Tail suspension

Animals were randomly assigned into 3 intervention groups: Sham-operated control group (SHAM, n=30), tail suspended (TS) with control vehicle (V) group (TS+V, n=30), and tail suspended with testosterone (T) treatment group (TS+T, n=30). Testosterone (Sigma-Aldrich) was administered using 1 cm subcutaneous silastic tubes (Silclear tubing 1.57 × 2.41 mm, Degania, Israel) implanted in the cervical region under isoflurane inhalation. A daily hormone release of 23 µg/day was obtained, as calculated by Vanderschueren et al.[40] The efficacy of androgen treatment was verified by measurement of the seminal vesicles mass and serum testosterone levels (see below). Empty silastic tubes were implanted in the 'TS+V' group. To allow recovery of the surgery, silastic tubes were implanted 2 days before the start of the TS protocol.

TS was performed by lifting the tail up so that the hindlimbs were unloaded.[1] The mice were maintained in approximately 30° head-down tilt, so that the forelimbs remained loaded. The tail was suspended with adhesive tape on a metallic wire which was connected to a 360° free rotating hook. The hook was hung on a rail system above the cage, which allowed free movement along the rail.

Hindlimb unloading was imposed for 1, 5 and 14 days. At the end of each intervention period, mice (n=10 per intervention group) were anesthetized with an intra-abdominal injection containing a mixture of 80% Ketamine (50 mg/ml, 100 mg/kg) and 20% Xylazine (20 mg/ml, 10 mg/kg). Special care was taken so that no weight bearing occurred before extraction of muscles. Mice were subsequently weighed and blood was collected by cardiac puncture. These samples were centrifuged at 2000 rpm for 10 min, and serum was collected and stored at -80 C prior to analysis of testosterone levels. Mice were then euthanatized by cervical dislocation. The EDL and SOL muscles of both legs and the seminal vesicles were carefully dissected, weighed, and immediately frozen in

liquid nitrogen for storage at -80°C. Muscles from the right leg were used for qPCR analysis and the contralateral muscles were used for immunoblotting.

LC-MS/MS for serum testosterone

Serum testosterone levels were determined using liquid chromatography tandem mass spectrometry (LC-MS/MS). One hundred µl of serum samples with added testosterone-d3 (internal standard) were extracted with diethylether. Ten µl was injected onto a High-Performance liquid chromatography system for 2D-LC operation (Shimadzu Scientific Instruments, Columbia, MD, USA) coupled to an AB Sciex Triple Quad mass spectrometer (AB SCIEX 5500 triple-quadrupole mass spectrometer, Toronto, Canada). The serum limit of quantification (LOQ) for testosterone was 1.2 ng/dL (0.04nmol/L), and was considered as the lowest measurable point where compounds could be detected with an intra-assay coefficient of variation (CV) of <=20% determined on a minimum of 5-6 measurements of a sample. The intra-assay CVs were 2.7% at 9 ng/dL (0.3 nmol/L), 1.7% at 318 ng/dL (11 nmol/L) and 1% at 634 ng/dL (22 nmol/L), and the inter-assay CVs were 8.3% at 36.7 ng/dL (1.3 nmol/L) and 3.1% at 307.8 ng/dL (10.7 nmol/L) respectively.[41] The bias, defined as the percentual difference between the measured concentration and the theoretical concentration, was 8.6% for 2 ng/mL, 4% for 20 ng/ml, -0.5% for 100 ng/mL and -1.3% for 2000 ng/ml.

RNA-isolation and qPCR

Total RNA was isolated using the TriPure Isolation Reagent (Roche Diagnostics, Vilvoorde, Belgium), followed by purification with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). An on-column DNase treatment was performed using the RNase-Free DNase Set (Qiagen). RNA was quantified by a Nanodrop 2000C spectrophotometer (Thermo Scientific), and RNA purity was assessed by calculating the A260/A280 ratio. Using a blend of oligo(dT) and random primers, 500 ng RNA was reverse transcribed with the iScript cDNA Synthesis kit (Biorad, Nazareth, Belgium), according to the manufacturer's instructions. qPCR was carried out on a Lightcycler 480 system (Roche) using an 8 µl reaction mix containing 3 µl template cDNA (1/10 dilution), 300 nM forward and reverse primers and 4 µl SYBR Green PCR Master Mix (Applied Biosystems, Halle, Belgium). The cycling conditions comprised a polymerase activation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 60°C for 60s. Primer sequences for *MAFbx/Atrogin-1*, *Murf1*, *Mstn*, *Igf1* and *Redd1* are shown in table 1. Sequence specificity was confirmed using NCBI Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To control the specificity of amplification, data melting curves were inspected and PCR efficiency was calculated (between 90 and 110% for all genes). Normalised gene expression values were calculated by dividing the relative gene expression values

(calculated by the delta-Ct method) for each sample by the geometric mean of peptidylprolyl isomerase A *(Ppia),* ribosomal protein large P0 (*Rplp0)* and beta-2 microglobulin *(B2m)* as selected by GeNorm.[42]

Protein isolation and Western blots

Protein isolation

Protein extraction was performed according to previously described procedures.[44] Total protein was extracted from approximately 9 mg of SOL using 1x RIPA buffer (15 µl/mg sample) (Millipore, North Ryde, NSW, Australia) with the addition of protease inhibitor cocktail (Sigma, Castle Hill, NSW, Australia) and Halt Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific, Rockford, IL, USA). Following homogenisation, the lysate was rotated at 4°C for 60 min before being centrifuged at 13,000 rpm at 4°C for 15 min. Total protein content of the supernatant was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

Western blots

Protein samples (30 µg) were denaturated in loading buffer and were separated on a 12% SDS-PAGE gel in a buffer containing 25 mM Trisbase, 192 mM glycine and 0.1% SDS, pH 8.8. Following separation, proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA) in a cold (4°C) transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3) for 2h then blocked for 1 hour at room temperature in 5% BSA or 5% skim milk in PBS. Membranes were subsequently incubated with the following primary antibodies diluted 1:1000 in 5% BSA/PBS for MSTN (Millipore, #AB3239), total $p70^{S6K}$ (Cell Signaling Technology, Inc., Beverly, MA, USA, #9202), total AKT (Cell Signaling, #9272), phospho-$p70^{S6K}$ (Cell Signaling, #9234), phospho-AKT (Cell Signaling, #9271), and 1:1000 in 5% skim milk/PBS for MuRF1 (ECM Biosciences, Versailles, KY, USA, #MP3401) overnight with gentle shaking at 4°C. After 4 x 5 minutes washing with PBS, the membranes were incubated for 1 h with a goat anti-rabbit IgG antibody labelled with an infrared-fluorescent 800-nm dye (Alexa Fluor 800; Invitrogen, Carlsbad, CA, USA) diluted 1:5,000 in PBS containing 50% Odyssey blocking buffer (LI-COR Biosciences) and 0.01% SDS. After washing, the specific proteins were visualized using the Odyssey Imaging System (LI-COR Biosciences) and individual protein band optical densities were quantified with the Odyssey software. An internal control sample was loaded on each gel to account for run-to-run variation between the samples. To control for protein loading, blots were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (G8795; Sigma-Aldrich, Sydney, Australia).

Statistical analysis

The statistical analyses were performed using SPSS statistical software (SPSS 19.0, Chicago, IL). A two-way between-groups analysis of variance (ANOVA) test was conducted to test the interaction of time and intervention. In case of a significant interaction effect, a subsequent one-way ANOVA was performed to test for differences between group means of SHAM, TS+V and TS+T for each of the time points measured. Differences between 1, 5 and 14 days were assessed by an one-way ANOVA on the difference scores of the TS+V and TS+T groups (i.e. the amount of change compared to SHAM). Differences in muscle mass and gene expression levels between SOL and EDL for the TS+V and TS+T groups (difference scores) were evaluated via one-way ANOVA for each time-point. A post-hoc LSD test was used when a significant group effect was observed. Normalized gene expression data and serum testosterone values were log-transformed to fulfil criteria for normality. Values are presented as means ± SD. P≤0.05 was considered signifcant.

Table 1 Primers used in qPCR analysis

Gene symbol*	**Source**	**Forward primer 5'-->3'** **Reverse primer 5'-->3'**
Ppia	RTprimerDB	CAA-ATG-CTG-GAC-CAA-ACA-CAA-ACG GTT-CAT-GCC-TTC-TTT-CAC-CTT-CCC
Rplp0	RTprimerDB	GGA-CCC-GAG-AAG-ACC-TCC-TT GCA-CAT-CAC-TCA-GAA-TTT-CAA-TGG
B2m	RTprimerDB	CAT-GGC-TCG-CTC-GGT-GAC-C AAT-GTG-AGG-CGG-GTG-GAA-CTG
MAFbx/Atrogin-1	Pires-Oliveira et al.[23]	GCA-GAG-AGT-CGG-CAA-GTC CAG-GTC-GGT-GAT-CGT-GAG
Mstn	Primer express	TGC-TAT-AAG-ACA-ACT-TCT-GCC-AAG-A AAG-AGC-CAT-CAC-TGC-TGT-CAT-C
Trim63/Murf1	Tang et al.[64]	TGG-AAA-CGC-TAT-GGA-GAA-CC ATT-CGC-AGC-CTG-GAA-GAT-G
Igf1	Primer express	TCA-ACA-AGC-CCA-CAG-GCT-ATG ACA-GCT-CCG-GAA-GCA-ACA-CT
Ddit4/Redd1	Primer express	GGT-GCC-CAC-CTT-TCA-GTT-GA CAG-AAC-TTA-ACA-GCC-CCT-GGA-T

* *Ppia*: peptidylprolyl isomerase A; *Rplp0*: ribosomal protein large P0; *B2m*: beta-2 microglobulin; *MAFbx/Atrogin-1*: Muscle Atrophy F-box; *Mstn*: myostatin; *Trim63*: tripartite motif-containing 63/*Murf1*: Muscle RING Finger 1; *Igf1*: insulin-like growth factor 1, *Ddit4*: DNA-damage-inducible transcript 4/*Redd1*: regulated in development and DNA damage responses 1.

RESULTS

Effect of TS on serum testosterone levels

Compared to the SHAM group, serum testosterone levels in the TS+V mice increased 2-fold at day 1, then dropped to very low values (8.8ng/dl or 0.3nmol/L) at day 5 of TS (Fig. 1A). The reduction in testosterone levels at day 5 of TS was accompanied with a reduction in the mass of testes and seminal vesicles by 16% and 22% respectively, when compared to SHAM (Fig. 1B,C). After 14 days of TS, circulating testosterone in the TS+V group subsequently raised towards SHAM levels (Fig. 1A). This occurred despite a lower testes and seminal vesicles mass measured at that time (Fig. 1B,C). In the TS+T group a 40-fold increase in serum testosterone levels and a 33% increase in seminal vesicles mass was observed as early as 1 day and was maintained after 5 and 14 days of TS when compared to SHAM (Fig. 1A,C).

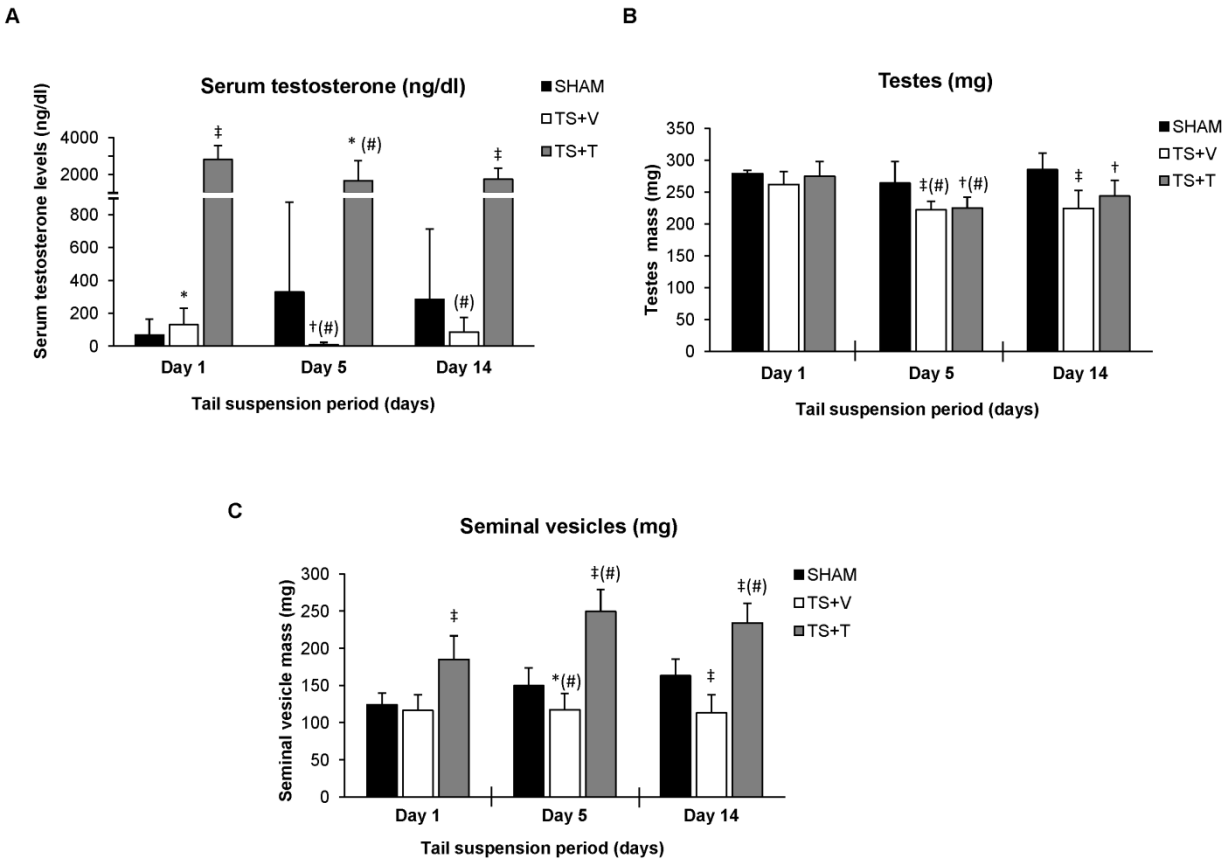

Figure 1. Effect of 1, 5 and 14 day of TS (TS+V), and testosterone treatment (TS+T) on serum testosterone levels (**A**), testes (**B**) and seminal vesicle (**C**) mass. Each bar represents the mean ± standard deviation (SD). Differences between intervention groups for each time point were assessed by one-way analysis of variance (ANOVA). * (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from SHAM. Differences between 1, 5 and 14 days of tail suspension for each intervention group were assessed by one-way analysis of variance (ANOVA). (#) (P<0.05): significantly different from previous time point.

Effect of TS and testosterone treatment on body weight and muscle mass

Body weight as well as SOL and EDL muscle mass were not significantly affected by testosterone treatment during the entire TS period (Fig. 2). Following 5 and 14 days of TS, body weight decreased by 24% and 19% in the TS+V and by 21% and 12% in the TS+T group, compared to the SHAM group. No significant differences were observed between the TS+V and TS+T treated mice (Fig. 2A). When compared to SHAM, SOL muscle mass decreased by 20% and 32% in the TS+V group after 5 and 14 days of TS respectively (Fig. 2B). EDL muscle mass decreased by 21% and 22% in the TS+V group after 5 and 14 days unloading respectively (Fig. 2C). The loss in both SOL and EDL muscle mass in the TS+V and TS+T groups became more pronounced from day 1 to day 5 of TS, but only the mass of the SOL muscle further atrophied between 5 and 14 days of TS. A significant difference between SOL and EDL muscle loss was found for the TS+T group, but not the TS+V group, at 14 days of TS (*P*=0.006).

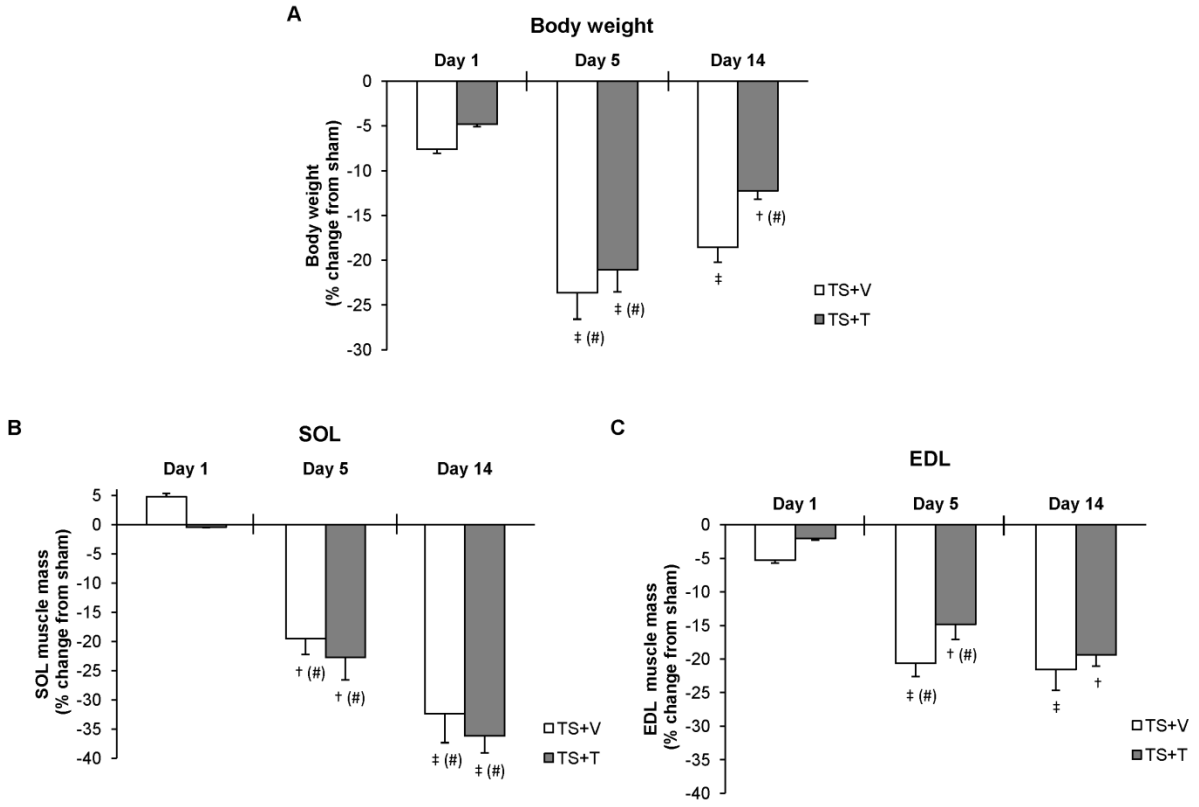

Figure 2. Effect of 1, 5 and 14 day of TS (TS+V), and testosterone treatment (TS+T) on body weight (**A**), SOL mass (**B**) and EDL mass (**C**). Data are shown as percent change from the control group. Each bar represents the mean ± standard deviation (SD). Differences between intervention groups for each time point were assessed by one-way analysis of variance (ANOVA). * (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from SHAM. Differences between 1, 5 and 14 days of tail suspension for each intervention group were assessed by one-way analysis of variance (ANOVA). (#) (P<0.05): significantly different from previous time point.

IGF1, AKT and p70^{S6K} gene and protein expression during TS and testosterone treatment

After 5 days of TS, *Igf1* mRNA levels in SOL muscle (Fig. 3A) were reduced by 50% in the TS+V group when compared to SHAM, and raised back to SHAM levels following 14 days of TS. In the EDL muscle (Fig. 3B), *Igf1* mRNA in the TS+V group was downregulated by 38% and 62% when measured at day 1 and day 5 respectively, but not at day 14 of TS when compared to SHAM. A significant difference (P<0.001) in *Igf1* mRNA expression was found between SOL and EDL in the TS+V group, as *Igf1* mRNA was already reduced at day 1 of TS in EDL but not in SOL. Although testosterone supplementation tended to prevent the reduction in *Igf1* mRNA in EDL at day 5 of unloading (p=0.09), none of the differences in *Igf1* mRNA levels between TS+V and TS+T groups were statistically significant in SOL or EDL at any of the time points.

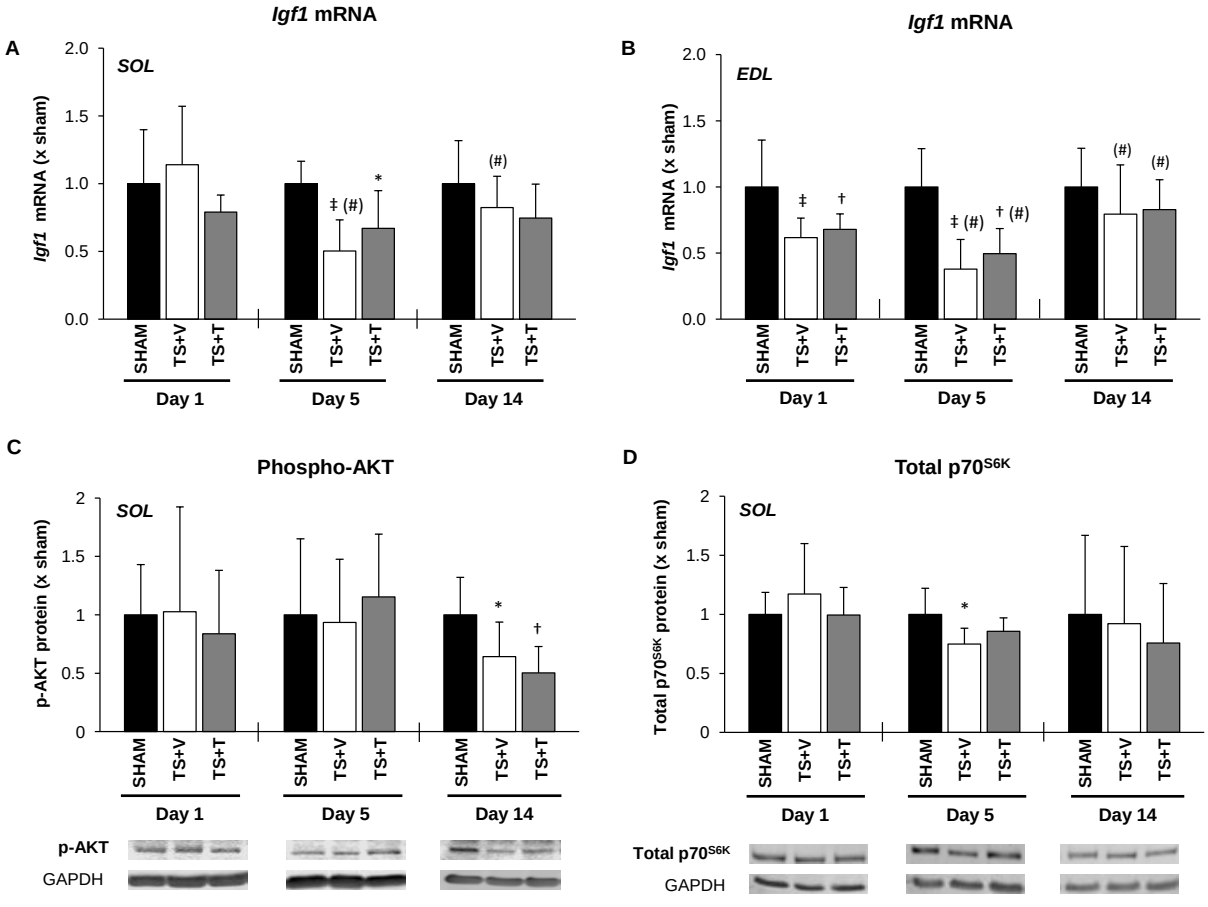

Figure 3. Effect of 1, 5 and 14 day of TS (TS+V), and testosterone treatment (TS+T) on *Igf1* mRNA expression in SOL muscle (**A**) and EDL muscle (**B**), and on phospho-AKT (**C**) and total p70^{S6K} (**D**) protein expression in SOL muscle . Data are shown as fold change from the control group for each muscle. Differences between intervention groups for each were assessed by one-way analysis of variance (ANOVA). * (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from SHAM. Differences between 1, 5 and 14 days of tail suspension for each intervention group were assessed by one-way analysis of variance (ANOVA). (#) (P<0.05): significantly different from previous time point.

In SOL, total AKT did not vary between groups over the 14 days of the study. However, when compared to SHAM, the phosphorylated levels of AKT were reduced by 36% and 50% in the SOL of TS+V and TS+T mice respectively, at the end of the TS period (Fig. 3C). Total $p70^{S6K}$ was slightly but significantly downregulated by 25% in the SOL muscle of TS+V mice on day 5 of TS when compared to SHAM (Fig. 3D). No significant changes in the phosphorylated levels of $p70^{S6K}$ among groups were found during the entire TS period.

MSTN and REDD1 gene and protein expression during TS and testosterone treatment

In SOL, *Mstn* mRNA levels were upregulated by 240% after 1 day of TS in the TS+V and TS+T mice when compared to SHAM (Fig. 4A).

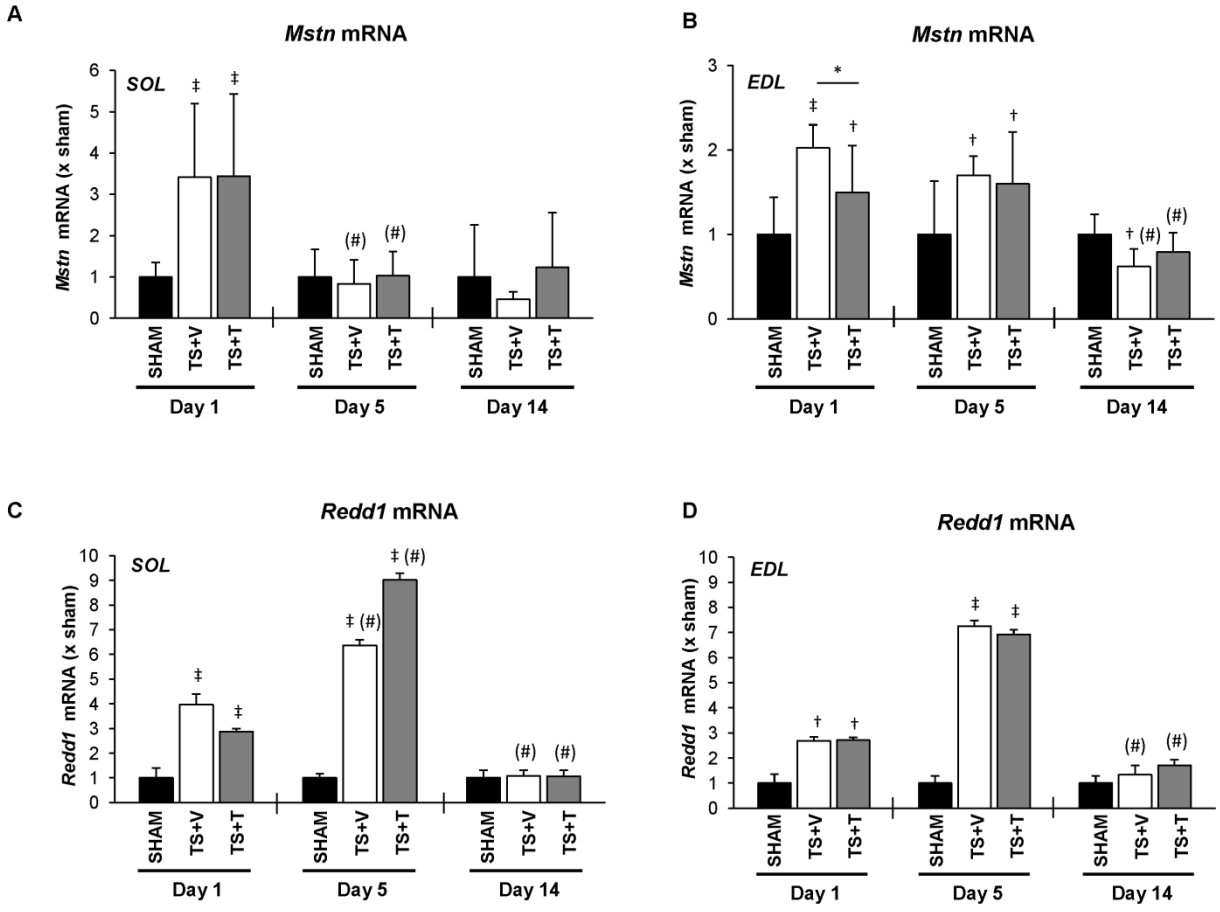

Figure 4. Effect of 1, 5 and 14 day of TS (TS+V), and testosterone treatment (TS+T) on *Mstn* mRNA expression in SOL muscle (**A**) and EDL muscle (**B**) and on REDD1 mRNA expression in SOL muscle (**C**) and EDL muscle (**D**). Data are shown as fold change from the control group for each muscle. Differences between intervention groups for each time point were assessed by one-way analysis of variance (ANOVA). * (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from SHAM. Differences between 1, 5 and 14 days of tail suspension for each intervention group were assessed by one-way analysis of variance (ANOVA). (#) (P<0.05): significantly different from previous time point.

In EDL, *Mstn* mRNA levels were increased in the TS+V group by 100% and 70% after 1 and 5 days respectively, but were decreased by 38% after 14 days of TS when compared to SHAM (Fig. 4B). In EDL, but not in SOL, testosterone supplementation during TS (TS+T) suppressed the increase in *Mstn* mRNA by 50% after 1 day of TS. These differences in *Mstn* mRNA expression response to TS between SOL and EDL were significant at day 5 for both TS+V (P=0.003) and TS+T groups (P=0.025). MSTN protein level in SOL was unchanged between all groups during the entire TS period.

In SOL, *Redd1* mRNA levels were increased by 300% and 540% in the TS+V group after 1 and 5 days of TS respectively, when compared to SHAM (Fig. 4C), and returned back to baseline levels after 14 days of TS. A similar expression pattern was observed in EDL muscle (Fig. 4D). No effect of testosterone treatment on *Redd1* mRNA levels was observed in either muscle during the entire TS period (Fig. 4C,D).

MAFbx/Atrogin-1 and MuRF1 gene and protein expression during TS and testosterone treatment

In the TS+V group, *MAFbx/Atrogin-1* mRNA levels in SOL and EDL muscles were increased by 180% and 90% respectively after 1 day of TS, and by 200% and 480% respectively after 5 days of TS when compared to SHAM (Fig. 5A,B). The increase in *MAFbx/Atrogin-1* mRNA expression in response to TS in EDL was significantly different from SOL at day 1 for the TS+V group (P<0.001) and the TS+T group (P=0.033) but not at day 5 or day 14 of TS. *MuRF1* mRNA expression in SOL and EDL muscles were also increased following 1 and 5 days of tail-suspension in both TS+V and TS+T groups, but no muscle type specific response was found (Fig. 5C,D). No significant differences in *MAFbx/Atrogin-1* and *Murf1* gene expression were found between the TS+V and TS+T treated mice. The changes in *Murf1* mRNA levels in SOL were not paralleled by changes in MuRF1 protein levels at any time point measured during TS.

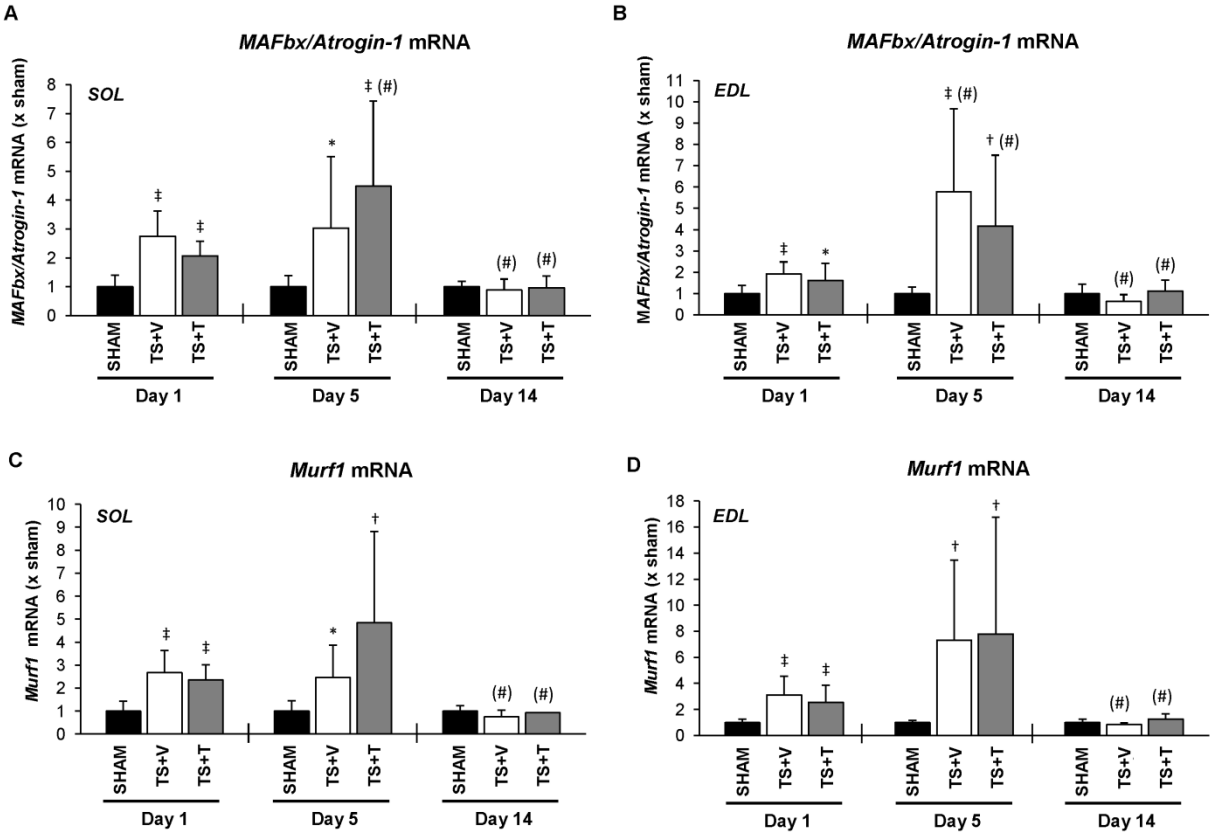

Figure 5. Effect of 1, 5 and 14 day of TS (TS+V), and testosterone treatment (TS+T) on *MAFbx/Atrogin-1* mRNA expression in SOL muscle (**A**) and EDL muscle (**B**), and on *Murf1* mRNA expression in SOL muscle (**C**) and EDL muscle (**D**) . Data are shown as fold change from the control group for each muscle. Differences between intervention groups for each time point were assessed by one-way analysis of variance (ANOVA). * (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from Shams. Differences between 1, 5 and 14 days of tail suspension for each intervention group were assessed by one-way analysis of variance (ANOVA). (#) (P<0.05): significantly different from previous time point.

DISCUSSION

The present study investigated testosterone supplementation in a tail suspension (TS)-induced skeletal muscle atrophy mouse model, with specific interest in key molecular targets that regulate muscle mass. By analysing both slow-twitch (SOL) and fast-twitch (EDL) muscles at different time-points following TS, our data demonstrated time- and muscle type dependent changes in gene expression levels of atrophy and hypertrophy inducing targets. The salient observation of our study is that TS in mice is associated with a transient increase, followed by a transient drop in serum testosterone levels. However, testosterone administration during TS did not restore muscle mass and only induced minimal changes in the mRNA and protein levels of the atrophy and hypertrophy molecular targets measured.

An important finding of this study is the transient changes in circulating testosterone levels measured following TS. Using a highly sensitive LC-MS/MS method we observed that circulating testosterone levels increased after 1 day of TS, rapidly and markedly decreased at day 5 of the TS period, but returned to baseline levels at the end of the 14 day intervention period. Acute stress is associated with a rapid but transient increase in circulating testosterone,[45,46] which may explain the increased testosterone levels observed after 1 day of TS in this study. On the other hand, chronic interference of stress-related hormones (e.g. corticosteroids) with the hypothalamic-pituitary-gonadal axis decreases sex steroid levels.[8,47] This may clarify the observed decrease in testosterone concentrations at day 5 of TS in our study. Next to stress, testicular retraction into the abdomen can be pointed out as the origin for the decline in testosterone production during TS in rodents.[7] However, this does not support the recovery of the testosterone levels at the end of the 14 day TS period which rather reflect a reduction in stress due to adaptation to the unloading protocol.

A rapid and extensive loss in muscle mass, with preferential atrophy of the slow-twitch muscles when compared to fast-twitch muscles, has been widely described in several disuse models including hindlimb suspension,[48] bed rest[49] and casting.[38] The greatest loss of muscle mass of both SOL and EDL muscle was observed between 1 and 5 days of TS in our study. This is also the time point at which the lowest testosterone levels in the non-treated TS group was observed. Although only SOL muscle further atrophied between 5 and 14 days of TS, we could not show statistically difference in percentage decreases in muscle loss between SOL and EDL.

Despite the major role testosterone plays in the regulation of skeletal muscle mass,[9] restoration of testosterone concentrations above physiological concentrations did not attenuate the TS-induced loss of muscle mass in the testosterone-treated group. Previous studies in androgen deficient models reported muscle atrophy only after a long-term castration period of 4 or 5 weeks.[17,50]

Therefore, the transient lower circulating testosterone levels during TS are unlikely to contribute to TS-induced muscle atrophy as the duration might have been too short to observe catabolic effects on muscle mass. Another possible explanation for the lack of androgenic effect could be a change in androgen receptor sensitivity. Previous studies in rodents have demonstrated that increased muscle activity resulted in increased AR expression in skeletal muscle and an enhancement of the sensitivity of androgen receptors. [51,52] It can therefore be speculated that TS might have resulted in androgen resistance at the level of the disused muscles.

An additional aim of the present study was to investigate the effects of testosterone on the gene and protein expression levels of some muscle hypertrophy and atrophy molecular targets following TS. TS resulted in a downregulation of *Igf1* mRNA in the SOL and EDL, an effect previously reported.[53,54] We additionally could show that this effect was more rapidly induced in the EDL muscle than in the SOL muscle, suggesting that IGF1 may contribute more to the initiation of the muscle loss in fast-twitch muscles compared to slow-twitch muscles during TS. Although we found a reduction in the downstream phophorylated levels of AKT after 14 days of TS, SOL muscle levels of phospho-p70^{S6K} remained unaltered. Similar findings have been observed in SOL and tibialis anterior muscle of mice and rats after 10 and 14 days of tail suspension,[54-56] however other studies could not show such changes[57] or reported a decrease in phosphorylation levels of both AKT and p70^{S6K}.[58] Nevertheless, although androgen treatment of normal and castrated mice is associated with an upregulation of targets of the IGF1/AKT/p70^{S6K} pathway,[17,22] we could not provide evidence for such anabolic effects in our TS model. The reduction in total p70^{S6K} levels after 5 days of TS remains unexplained. Hence, more studies are needed to clarify the inconsistency in muscle AKT/p70^{S6K} signaling during different periods of TS.

Apparent differences in *Mstn* mRNA expression between SOL and EDL muscle were found following TS. The upregulation of *Mstn* mRNA levels observed after 1 and 5 days of TS in EDL, but only after 1 day in SOL, suggest that muscle loss in fast-twitch muscles may depend more on Mstn than slow-twitch muscles. Moreover, we could show that *Mstn* mRNA expression was partially inhibited by testosterone treatment, only in EDL muscle. However, increased *Mstn* mRNA levels in SOL during TS were not associated with elevated MSTN protein levels; an observation previously reported in the literature.[30] Overall, the effect of muscle disuse on MSTN regulation remains unclear, and some studies have described increased levels,[25,26] whereas others failed to detect any changes in *Mstn* mRNA or protein content.[27,28]

Redd1, another negative regulator of muscle mass, seems to be rapidly and transiently induced by tail-suspension. Accordingly, Kelleher et al.[32] reported a 3.5-fold increase in *Redd1* mRNA content in

the SOL of rats subjected to 1, 2 and 3 days of unlilateral hindlimb casting, but did not investigate the effect of longer periods of disuse. Although there is evidence that *Redd1* is regulated by androgens,[17] our data indicate that testosterone does not influence *Redd1* gene expression during TS.

Similarly, studies in both humans and rodents demonstrated that the mRNA levels of the ubiquitin ligases *MAFbx/Atrogin-1* and *Murf1* are rapidly and transiently upregulated as early as 1 day following immobilization,[38,59,60] although muscle atrophy is only observed 2 days following immobilization.[54,61] Our results demonstrated a higher increase in *MAFbx/Atrogin-1* gene expression in SOL when compared to EDL after 1 day of TS, whereas no muscle type dependent differences in *MuRF1* gene expression were detected. This is in contrast to a study by Okamoto et al.[38] suggesting that MAFbx/Atrogin-1 and MuRF1 may contribute more to skeletal muscle atrophy in fast-twitch muscle fibers than in slow-twitch muscle fibers in cast immobilization-induced atrophy models.
We could not find any significant differences in the mRNA expression of these genes in the SOL and EDL of our testosterone treated mice when compared to non-treated mice. This suggests that testosterone does not regulate *MAFbx/Atrogin-1* and *Murf1* gene expression during TS. Moreover, the rapid and transient increase of *Murf1* mRNA levels observed in the SOL of TS+V and TS+T treated mice was not paralleled by changes in protein expression levels. The expression of MAFbx/Atrogin-1 and MuRF1 protein has been investigated during hindlbimb unloading,[62-64] and discrepancies between *Murf1* mRNA and MuRF1 protein levels have previously been described in paraplegic rat muscle and human bed rest models.[65,66]

In conclusion, our data indicate that decreased levels of circulating testosterone associated with TS do not contribute to TS-induced muscle atrophy in the slow-twitch SOL and the fast-twitch EDL muscle. Moreover, this study demonstrated apparent differences in *Igf1*, *Mstn* and *MAFbx/Atrogin-1* gene expression levels between SOL and EDL muscle following TS. These results suggest that the molecular regulation of disuse-induced muscle atrophy might be muscle type specific. Furthermore, testosterone treatment during TS does not restore alterations in the expression levels of catabolic and anabolic signaling molecules. Therefore, we suggest that the known anabolic effects of testosterone are not sufficient to maintain or ameliorate muscle mass in TS-induced muscle atrophy.

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VI. GENERAL DISCUSSION

1. OUTLINE

The prevention and therapy of skeletal muscle atrophy has been a topic of interest for many years and its applications are spreading from sport sciences to ageing and clinical medicine. Androgen treatment can be an effective therapeutic strategy, especially for patients incapable to exercise, but its application has been limited because of severe side effects. Enhancing our understanding of the androgenic regulation of skeletal muscle mass and the molecular factors modulated by androgens may help in the identification of novel muscle-specific therapeutic targets to combat the devastating effects of muscle wasting. In the first study of this thesis, we extensively investigated the determinants of muscle mass and function in healthy young men, as well as the influence of genetic variations in the AR. In addition, using two different muscle atrophy mice models, our results provided evidence for a complex mechanism by which T regulates muscle mass.

In the sections below, the determinants of muscle mass and its regulation by androgens in three different conditions (eugonadal, orchidectomy and tail suspension), and three different muscle types, are discussed with regard to the findings in this thesis. Second, the role of Atrogin-1 and MuRF1 during atrophy is questioned and other signalling molecules affected by androgens in skeletal muscle are summarized. Finally, the regulation of skeletal muscle mass by estrogens is discussed (Figure 14).

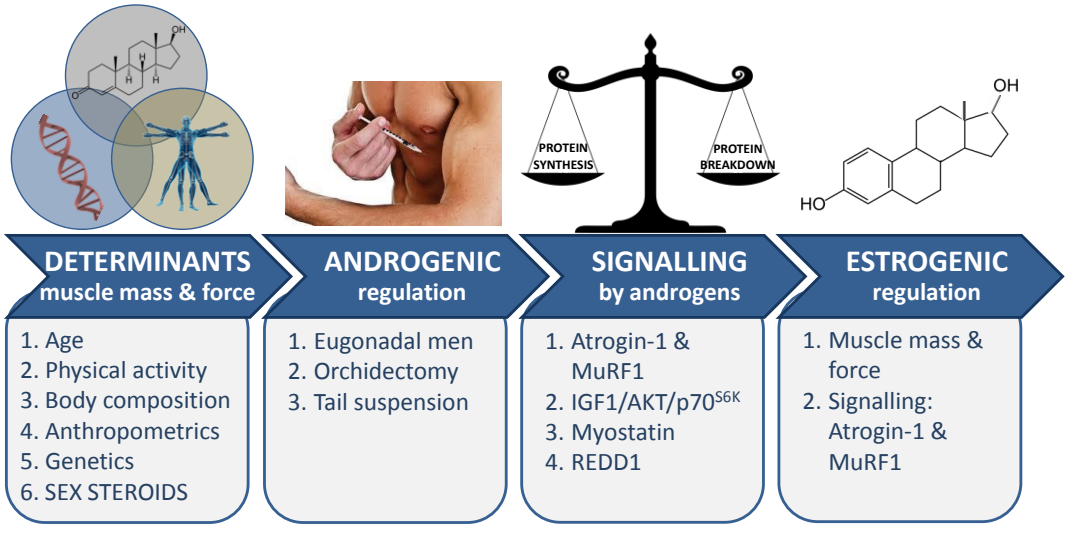

Figure 14. Overview of the topics discussed.

2. DETERMINANTS OF SKELETAL MUSCLE MASS IN HEALTHY YOUNG MEN AND THE INFLUENCE OF GENETIC FACTORS

2.1 Determinants of skeletal muscle mass in healthy young men

In study 1, we aimed at identifying environmental and genetic determinants of skeletal muscle mass and strength in young men. Our results confirmed that skeletal muscle mass and strength are highly heritable (Arden and Spector 1997). It is noteworthy that the heritability estimates are presented as upper-limit heritabilities or transmissibilities (t^2) rather than genetic heritabilities (h^2). In contrast to classical twin studies, genetic and environmental factors contributing to the total phenotypic variance between sibling pairs can not be distinguished (Huygens et al. 2004). The heritability estimates found in study 1 therefore not only represent the genetic heritability, but also include a common environmental factor by which the heritabilities might be overestimated. However, Huygens et al. (2004) argued that the transmissibility estimates for muscle mass phenotypes might be close to the true genetic heritability, since twin studies have shown that the common environmental component for muscle mass phenotype is small or negligible (Thomis et al. 1998).
Our results are also in line with previous findings that skeletal muscle mass and strength are influenced by age (Baumgartner et al. 1999), anthropometrics, body composition (Gallagher and Heymsfield 1998), physical activity (Geirsdottir et al. 2012) and sex steroid levels. However, we expanded our analyses with an extensive characterization of body composition (weight, fat mass), anthropometrics (height, armspan, hand length, finger length) and several measurements of muscle mass (lean mass, muscle CSA at tibia and radius) and muscle force (biceps, quadriceps and grip) by which we provided a more detailed overview of the determinants of muscle mass and force in healthy young men.

First, in our cohort of healthy men in a narrow age range (25-45y) (study 1), we found a small but positive association between age and lean mass and muscle CSA at the radius and tibia, whereas a small inverse relationship between age and quadriceps force was observed. Muscle mass generally peaks in the 20s or 30s and then declines (Baumgartner et al. 1999; Janssen et al. 2000). Our results suggest that this decline is preceded by a loss in muscle force of the lower body. Second, body weight was positively associated with all indices of muscle mass and force, whereas a negative relation between whole body fat mass and muscle CSA and force was found. Fat mass is known to have adverse effects on muscle function (Abdul-Ghani et al. 2008), and it is noteworthy that the negative association between fat mass and muscle CSA and force was only found in the upper body of our healthy men, probably because of the known male pattern of fat distribution around the trunk and upper body. Third, we demonstrated that taller men and subjects with longer armspan,

hand- and finger length have higher muscle mass and force. Finally, a relationship between serum T levels and muscle CSA was shown in our cohort of eugonadal men.

It is somewhat surprising that age and TT/FT were associated with muscle CSA, but not with the muscular strength measures. A possible explanation for this inconsistency might be that biceps force and quadriceps force are not the best representive measurements for muscle CSA at forearm and lower leg, respectively. Additional analysis indeed showed a rather small relationship between muscle CSA at the tibia and quadriceps force (β : 0,12 ± 0.04; p=0.0013); however, strong relationships were found between muscle CSA at the radius and biceps force (β : 0.49 ± 0.03; p<0.0001) and between muscle CSA at the radius and grip strength (β : 0.40 ± 0.03; p<0.0001). Methodological issues such as subject familiarization, limb position and subject motivation can lower the accuracy of the muscle force test protocol and could be another reason for the observed discrepancy.

2.2 The relationship between serum testosterone levels and muscle mass in eugonadal men is not influenced by genetic variations in the AR gene

As mentioned in the introduction, an interindividual variation in serum T levels exist in healthy young men. This between-subject variability can be attributed, in part, to polymorphisms in the AR gene. We confirmed previous findings (Crabbe et al. 2007; Bogaert et al. 2009) that the CAG repeat length of the AR gene is positively associated with serum T levels in healthy young men. As these polymorphisms likely affect androgen sensitivity, it is expected that a diminished negative feedback control of the hypothalamic-pituitary-gonadal axis results in subsequently higher T levels (Crabbe et al. 2007). Because of its influence on AR activity, the CAG repeat length has been associated with a series of androgen-related clinical effects such as prostate cancer risk, spermatogenesis, bone density, hair growth and cardiovascular risk factors (Zitzmann and Nieschlag 2003b). Next to this polymorphism, we demonstrated an association between serum T levels and two SNPs of the AR gene (rs5965433 and rs5919392), which have previously been linked with AIS. However, we could not find any relationship between the CAG repeat length or the analysed SNPs in the AR and either body composition or measurements of muscularity in our cohort of healty men. Lower androgen sensitivity of the AR, due to longer CAG repeats, likely causes a modulated setpoint in the negative feedback regulation of the hypothalamic-pituitary-gonadal axis. This means that the lower AR sensitivity might be compensated by higher levels of T (Crabbe et al. 2007), without changing impact on its target tissues. Therefore, other factors responsible for the remaining variation in T levels within the physiological range might contribute to the association of T with muscle CSA, especially because the variation in T explained by the CAG repeat is rather limited. Thus, part of the

interindividual variation in T levels in healthy men can be attributed to differences in androgen sensitivity, but no straightforward correlation between this variation in T levels and different phenotypes can be observed.

Interestingly, we found that anthropometric measurements i.e. arm span and the 2D:4D finger length ratio, were inversely associated with the number of CAG repeats in the AR gene. The 2D:4D finger length ratio has been proposed as a marker of prenatal androgen action and of sensitivity to T, with a lower 2D:4D being associated with high androgen exposure (McIntyre 2006). Given the hypothesis that elevated T levels in men with lower androgen sensitivity are not necessary associated with differences in androgen action, we speculate that the negative effects on arm span and finger length might be mediated by the higher levels of FE_2 levels found in men with longer CAG repeat length (Huhtaniemi et al. 2009). As most E_2 produced in normal men is formed by aromatization of androgens (MacDonald et al. 1979), the higher T substrate availability in men with lower androgen sensitivity can explain the higher serum E_2 levels. E_2 is considered to be the main sex steroid involved in the development and maintenance of bone mass (Lapauw et al. 2009). In addition, it is also important to initiate epiphyseal closure of long bones (Weise et al. 2001). Therefore, we hypothesize that the presence of higher levels of E_2 in men with lower androgen sensitivity, but preserved estrogen action, result in earlier termination of longitudinal bone growth during puberty, an event wich is clearly observed in boys with aromatase excess syndrome or familiar hyperestrogenism (Martin 2003).

"Age, physical activity, body composition, sex steroid levels and anthropometrics are determinants of muscle mass and function in young men. Although the number of CAG repeats of the AR are related to sex steroid levels and anthropometrics, we have no evidence that these variations in the AR gene also affect muscle mass or function."

3. ANDROGENIC REGULATION OF SKELETAL MUSCLE MASS

3.1 Testosterone and its effect on muscle mass and strength in different conditions

The anabolic effects of androgens are mainly observed when serum levels are inadequate as seen in elderly and hypogonadal men (Katznelson et al. 1996; Kaufman and Vermeulen 2005). Indeed, in study 2, we showed that orchidectomy resulted in tremendous atrophy of the LA/BC (-63%) and a significant loss in locomotor muscle weight (-13%), whereas T replacement completely negated the orchidectomy-induced muscle atrophy. These findings were strengthened by the observation that absolute, but not relative, twitch and tetanic force were reduced after androgen deprivation, indicating that the altered muscle force is predominantly due to changes in muscle mass. It is noteworthy that the anabolic effects of androgens on muscle mass in animal models are not straightforward. Orchidectomy or androgen treatment of male mice or rats have not always been shown to result in changes in muscle mass (Antonio et al. 1999; Brown et al. 2001; Pires-Oliveira et al. 2010; Haren et al. 2011) and function (Tingus and Carlsen 1993; Ophoff et al. 2009), which can be due to a lower AR expression found in rodent skeletal muscle when compared to humans.

In our cohort of eugonadal men (study 1), we could not show any association between T and muscle strength, despite a positive relation between T and muscle CSA. Inconsistency in the literature can possibly be attributed to different methodologies used to measure muscle strength, or may result from the muscle-specific and dose-dependent actions of androgens on muscle strength (Friedl et al. 1991; Storer et al. 2003; Schroeder et al. 2003; Sinha-Hikim et al. 2006).

An interesting finding from study 3 is the marked decrease in circulating testosterone levels following 5 days of tail suspension. Other studies investigating hindlimb suspension (Deaver et al. 1992; Ortiz et al. 1999; Wimalawansa and Wimalawansa 1999; Kamiya et al. 2003), but also studies using other disuse models like spaceflights (Philpott et al. 1985; Grindeland et al. 1990; Amann et al. 1992), severe injury (Ferrando 2000), head-down bed rest (Nichiporuk et al. 1998) and SCI (Clark et al. 2008) provided evidence for reduced serum T levels during muscle disuse. It is therefore likely that these hormonal changes contribute to the skeletal muscle atrophy. Therapeutic use of androgens in this setting could thus be beneficial. Several studies addressed this issue in a variety of muscle disuse atrophy models, but inconsistencies in the results have been reported (Table 1).

Table 1. Overview of studies investigating the effects of androgen supplementation in different muscle disuse atrophy models

STUDY	STRAIN	ANDROGEN (administration)	DOSE (frequency)	DURATION	MUSCLE	AMELIORATION OF MUSCLE ATROPHY?
Tail suspension						
De Naeyer et al. (study 3)	mice	T (silastic tubes)	1mg/kg (daily)	1, 5, 14d	SOL/EDL	**NO**
Tsika et al. (1987)	rats	ND (pellets)	2mg/kg (daily)	6w	SOL	**NO**
					PLT	**YES**
Wimalawansa et al. (1999)	rats	T (inj)	6mg/kg (once)	12d	QUADR	**YES**
		ND (inj)	6mg/kg (once)	12d	QUADR	**YES**
Bricout et al. (1999)	rats	T heptylate (inj)	10 mg/kg (weekly)	3w	SOL/PLT /EDL	**NO**
Joumaa et al. (2002)	rats	ND (inj)	15mg/kg (weekly)	6w pre-treatment + 3w TS	EDL	**NO**
					SOL	**YES**
Casting						
Witzmann et al. (1988)	rats	ND (inj)	7mg/kg (weekly)	5w	SOL	**NO**
Taylor et al. (1999)	rabbits	ND (IM inj)	15mg/kg (weekly)	8w	TA/EDL	**YES**
Denervation						
Zhao et al. (2008)	rats	ND + T (pumps)	0.75mg/kg (weekly) + 2.8mg/kg (daily)	3, 7, 14 d	GC	**NO**
				28d denervation + 3, 7, 28d treatment	GC	**YES**
Spinal cord injury						
Gregory et al. (2003)	rats	T (capsule)	replacement therapy	11w	SOL/GC/ TA/VL	**YES**
Ung et al. (2010)	mice	TP (inj)	5mg/kg (daily)	8w	EDL/SOL	**NO**
Amyotrophic lateral sclerosis						
Yoo et al. (2012)	mice	DHT (silastic implant)	NA	120d	TA/GC	**YES**
Head-down bed rest						
Zachwieja et al. (1999)	human	T enanthate (inj)	200mg (daily)	28d	lean mass	**YES**

T:testosterone, ND:Nandrolone decanoate, DHT:dihydrotestosterone, TP:testosterone propionate, SOL:soleus muscle, EDL:extersor digitorum longus muscle, TA:tibialis anterior muscle, VL:vastus lateralis muscle, PLT:plantaris muscle, QUADR:quadriceps muscle, GC:gastrocnemius muscle, d:days, w:weeks, inj:injection, IM:intra-muscular, NA: not available.

Androgen supplementation during tail suspension, casting, denervation and SCI in rodents has been shown to either improve muscle mass or to have no effects on the disuse atrophy (Table 1). In our tail suspended mice (study 3), T treatment showed no efficacy in manipulating skeletal muscle mass, even though the dose of T was double the amount as that used in our orchidectomized mice (study 2). There are several possible explanations for the conflicting results reported in the literature. First, it is possible that the duration of transient lower circulating testosterone levels during tail suspension in study 3 was too short to induce atrophy by androgen deprivation, and despite the high

dose of T administered (5- to 40-fold the physiological amount) it might have been not sufficient to prevent or ameliorate the tremendous muscle atrophy induced by disuse. It is often suggested that anabolic androgenic steroids increase muscle mass only in combination with muscle activity or with muscle loading. This would explain the anabolic effects found during casting and bed rest, since weight bearing contractile activity (isometric contraction in case of casting) is still possible under these conditions (Taylor et al. 1999). However, studies providing evidence for this statement are lacking. Moreover, this hypothesis is not supported by the beneficial effects of androgen treatment found following denervation (Zhao et al. 2008a), SCI (Gregory et al. 2003) or ALS (Yoo and Ko 2012). The efficiency of androgen treatment might also depend on the anabolic agent used. Nandrolone, DHT, and synthetic derivates of T are known to be biologically more potent androgens than T, due to a greater affinity for the AR or because aromatization into E_2 is impossible (Evans 2004). In addition, also other study variables such as species differences and the investigated muscle type may contribute to the discrepancies among studies.

It is noteworthy that no studies have used the highly sensitive LC-MS/MS method for measuring T levels in rodents following disuse-induced muscle atrophy. The clinical use of LC-MS/MS instruments for the measurement of serum sex steroid levels has grown during the last decade, and it has become the method of choice for detecting anabolic steroid usage in athletes (Thevis et al. 2008; Pozo et al. 2008). However, radioimmunoassays are still widely used, despite their lack of sensitivity and specificity. This leads to unreliable measurements, especially in the lower range concentrations (Taieb et al. 2003; Wang et al. 2004). These limitations are particularly problematic when measuring E_2 levels in men or T levels in rodent serum. In rodents, a circulating sex hormone binding globulin is absent, resulting in a high individual variation in serum testosterone levels (Sullivan et al. 1991). Another advantage of LC-MS/MS is that only a small amount of sample is needed for the analysis, which is especially interesting in the determination of sex steroid levels in rodents. Thus, by using the LC-MS/MS method we were able to accurately measure T levels in our tail suspended mice (study 3), and E_2 levels in our cohort of healthy men (study 1).

"Testosterone therapy is effective in restoring muscle mass and absolute muscle strength during androgen deprivation, but its effects during muscle disuse atrophy remains equivocal."

3.2 Muscle atrophy is determined by the atrophic stimulus and is muscle type-dependent

As mentioned in the introduction, there is evidence that the extent of muscle loss depends on the muscle fiber type distribution and on the atrophic conditions (Degens and Alway 2006). Indeed, the rapid and extensive loss in skeletal muscle mass found following tail suspension in study 3 (-22 to -32 %), is in contrast with the slow and limited muscle loss induced by androgen deprivation as observed in study 2 (-13%). Other muscle disuse atrophy models, e.g. casting and bed rest, have confirmed the preferential loss of the SOL muscle when compared to the EDL muscle following our tail suspension experiment (Gardetto et al. 1989; Okamoto et al. 2011; Miokovic et al. 2012). A possible explanation for this fiber type specific atrophic response during disuse might be the functional role of the muscles. The SOL is a constantly active tonic muscle, important in maintaining body posture, whereas the EDL is mainly active during dynamic movements. As a consequence, there is a difference in the extension of change in activity level between the muscles during disuse, with the SOL muscle undergoing a relatively larger reduction in activity than the EDL (Degens and Alway 2006).

Another remarkable difference in atrophy response was found between the LA/BC and the locomotor muscles following androgen deprivation (study 2). The perineal LA and BC skeletal muscles are highly androgen responsive and depend on androgens for their normal maintenance and function (MacLean et al. 2008). It is well established that the LA muscle displays rapid and extensive muscle loss in response to androgen deprivation which is likely due to high AR protein expression and/or high androgen sensitivity (Monks et al. 2004; Johansen et al. 2007; Ophoff et al. 2009; MacLean et al. 2010). Indeed, in study 1 we showed that the mRNA level of the AR is 4-fold higher in the LA/BC when compared with SOL and EDL muscles, supporting previous observations (Monks et al. 2004; Johansen et al. 2007). These observations indicate that the diverse response of different muscle types to androgens possibly reflects differences in AR expression between muscles. However, despite this well-kown muscle type dependent difference in androgenic-anabolic responsiveness, the LA muscle is still widely used in preclinical studies.

Whether muscles with differences in their twitch properties respond differently to T could not be deduced from study 2, as SOL and EDL lost an equal amount of muscle mass following androgen deprivation. Reports in the literature are not univocal, with some describing predominant changes in type I fibers, others in type II fibers and some reports finding no differential changes in CSA between fiber types following androgen withdrawal or androgen administration in humans and rodents (Table 2).

Table 2. Overview of studies investigating the effects of androgen withdrawal and androgen treatment in different muscle types**.**

STUDY	STRAIN	INTERVENTION	MUSCLE TYPE MOSTLY AFFECTED
		Androgen withdrawal	
Axell et al. (2006)	mice	orx	Type II (TA, biceps branchii, EDL vs SOL)
Ophoff et al. (2009)	mice	mARKO	Type II (EDL vs SOL, GC, QUADR)
Rowe et al. (1968)	mice	orx	Type II (TA, biceps branchii, EDL vs SOL)
Brown et al. (2001)	rats	orx	No effect (SOL, EDL,PLT, GC, PER)
De Naeyer et al. (2014) (study 2)	mice	orx	No differences (between EDL and SOL)
Sinha-Hikim et al. (2002)	human	GnRH agonist	No differences (within VL)
		Androgen treatment	
Axell et al. (2006)	mice	orx+T	Type I (SOL vs TA, biceps branchii, EDL)
Eriksson et al. (2005)	human	power lifting + T and anabolic steroids	Type I (within VL)
Kadi et al. (1999)	human	power lifting + anabolic steroids	Type I (within trapezius)
Sinha-Hikim et al. (2006)	Human	GnRH agonist + T enathate	Type I (within VL)
Ustunel et al. (2003)	rats	T	Type I (within GC)
Tsika et al. (1987)	rats	ND	Type II (PLT vs SOL)
Sinha-Hikim et al. (2002)	human	GnRH agonist+ T	No differences (within VL)
Frese et al. (2011)	rats	orx+DMT/NOR/TP	No differences (within GC)
Bricout et al. (1999)	rats	T	No effect (SOL, PLT, EDL)

T:testosterone, ND:Nandrolone decanoate, TP:testosterone propionate, SOL:soleus muscle, EDL:extersor digitorum longus muscle, TA:tibialis anterior muscle, VL:vastus lateralis muscle, PLT:plantaris muscle, QUADR:quadriceps muscle, GC:gastrocnemius muscle, PER: peroneus longus muscle, orx: oerchidectomy, mARKO:myocyte-specific androgen receptor knockout,GnRH: gonadotropin-releasing hormone , DMT: desoxymethyltestosterone NOR: norandrostenedione.

However, when taking a closer look to these reports, it seems that the slow-twitch fibers may be particularly responsive to androgen administration whereas the fast-twitch fibers are probably more sensitive to androgen withdrawal (Axell et al. 2006). Atlhough we did not find any differences in AR expression levels between SOL and EDL muscle (study 2), Hulmi et al. (2008) demonstrated a more intensive AR-specific immunofluorescence staining at the cell borders of type I muscle fibers compared to type II fibers in the vastus lateralis (VL) muscle of men, indicating that type I muscle fibers might be more responsive to the effects of T. On the other hand, myocyte-specific ARKO mice, with AR deletions only in mature postproliferative myofibers, showed a decrease in EDL weight but not in SOL (Ophoff et al. 2009), suggesting that type II muscles may depend more on androgens for

their growth than type I muscles. It is reasonable to assume that the androgen response may also depend on various factors, such as contractile activity (Hennig and Lømo 1985; Inoue et al. 1994), metabolic activity (Pette and Spamer 1986), and the dose (Storer et al. 2008) or type of androgen administered (Frese et al. 2011).

> *"Slow-twitch muscles are particularly responsive to muscle disuse and androgen administration whereas the fast-twitch muscles are likely more sensitive to androgen withdrawal. The extent of muscle loss highly depends on the atrophic stimuli."*

4. THE MOLECULAR REGULATION OF SKELETAL MUSCLE MASS DURING ANDROGEN DEPRIVATION AND TAIL SUSPENSION, AND THE INFLUENCE OF TESTOSTERONE TREATMENT

The main purpose of study 2 and study 3 was to investigate the regulation of muscle atrophy and hypertrophy inducing targets following orchidectomy and following tail suspension with or without T supplementation (Figure 15).

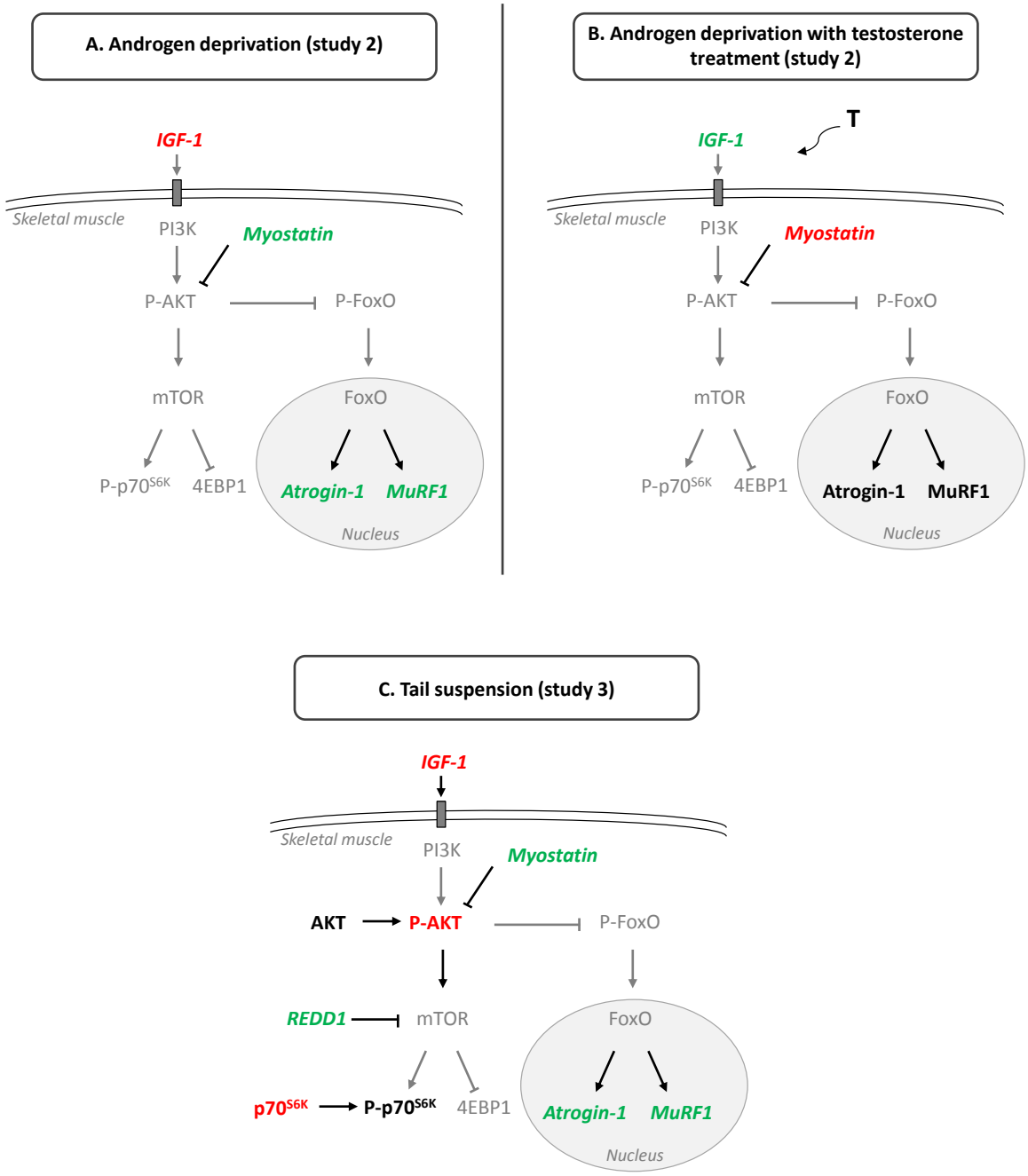

Figure 15. Effects of androgen deprivation (**A**), testosterone treatment following androgen deprivation (**B**) and tail suspension (**C**) on skeletal muscle atrophy and hypertrophy inducing targets. Genes (italic) or proteins in green were positively regulated, and genes or proteins in red were negatively regulated. The other genes or proteins shown were not measured (in grey) or were not regulated (in black) by the intervention.

4.1 Atrogene signalling

4.1.1 Atrogene signalling is time- and muscle type-dependent

Because Atrogin-1 and MuRF1 gene expression levels were, after their discovery, quickly believed to serve as a convenient marker of enhanced muscle protein breakdown (Sacheck et al. 2007), we hypothesized that T treatment would ameliorate muscle atrophy by suppressing atrogene expression. However, to date, inconsistencies in the involvement of the atrogenes in the atrophy process exist. A number of reports show no change in the mRNA levels of Atrogin-1 and MuRF1 during different catabolic conditions (Fareed et al. 2006; Léger et al. 2006a; Léger et al. 2008; Sakuma et al. 2009). Also, currently, little is known about the androgenic regulation of the ubiquitin ligases. Some very recent studies confirmed an upregulation of both Atrogin-1 and MuRF1 after orchidectomy and a downregulation of these genes after T supplementation (Ibebunjo et al. 2011; White et al. 2013), whereas others have reported no change (MacLean et al. 2008; Pires-Oliveira et al. 2010) or even a reduction (Svensson et al. 2010; Haren et al. 2011) in the mRNA levels of Atrogin-1 and MuRF1 in orchidectomized and ARKO mice.

With gene expression levels in both study 2 and 3 measured at multiple time-points, we provides evidence for a transient increase in Atrogin-1 and MuRF1 gene expression found in the SOL and the EDL muscles following androgen deprivation (Figure 16) and tail suspension (Figure 5 from study 3) respectively, even before muscle weight loss was observed.

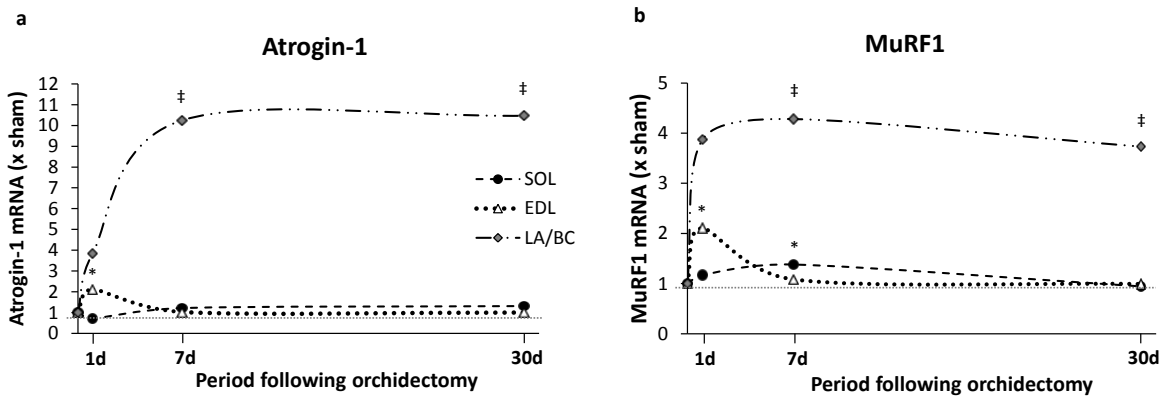

Figure 16. Effects of 1, 7 and 30 days of orchidectomy on MAFbx/Atrogin-1 (**a**) and MuRF1 (**b**) mRNA expression in SOL, EDL and LA/BC muscle. Data are shown as fold change from the control group for each muscle. Differences between Sham and Orx mice were assessed by independent sample T-tests. * (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from Shams. Interconnecting lines are drawn to indicate time effects (no other time-points measurements were made).

This important finding may clarify the contradictory results reported in the literature. Since most studies have measured gene expression levels at only one time-point, it is likely that transient changes could were missed. However, this only seems to be the case for the locomotor muscles, as results from study 2 demonstrated a rapid and sustained increase in Atrogin-1 and MuRF1 gene expression in the LA/BC muscle following androgen deprivation (Figure 16). The remarkably distinct response of Atrogin-1 and MuRF1 to androgen deprivation between the LA/BC and the locomotor muscles clearly point to a muscle type dependent regulation of gene expression. Moreover, our results suggest small differences in atrogene expression between slow- and fast-twitch muscles, as no upregulation of Atrogin-1 mRNA in SOL was found following orchidectomy, despite a significant upregulation of MuRF1 and a loss of muscle mass. In addition, as atrogene expression levels and kinetics differ between androgen deprivation- and tail suspension induced muscle loss, the changes in gene expression levels may also depend on the atrophic stimuli.

As mentioned in the introduction, the androgenic regulation of Atrogin-1 and MuRF1 gene expression can occur through direct action of the AR on the ARE of the atrogene (Lee and Chang 2003), or via indirect action through cross-talking with upstream signalling molecules like FoxO3 (Weissberger and Ho 1993; Hobbs et al. 1993; Baron et al. 2004). Studies in castrated mice demonstrated that Atrogin-1 and MuRF1 genes are upregulated with a concomitant decrease in phosphorylated and thus inactive levels of FoxO (Ibebunjo et al. 2011; White et al. 2013). However, a study in human C2C12 cells (Zhao et al. 2008b) provided evidence that the regulation of Atrogin-1 by testosterone is independent of FoxO3. In fact, human atrophy models including ALS (Léger et al. 2006b), COPD (Doucet et al. 2007) and ageing (Léger et al. 2008) have not yet supported a role for the FoxO transcription factors in the transcriptional regulation of Atrogin-1 and MuRF1.

"Atrogene signalling is time- and muscle type-dependent and is determined by the atrophic stimuli. Because Atrogin-1 and MuRF1 mRNA seems to be rapidly and transiently upregulated and precedes the loss of muscle mass, we and others (Murton, Foletta, Gomes MD 2001) believe that they might be involved in the initiation of the atrophy programme in the locomotor muscles."

4.1.2 Atrogin-1 and MuRF1: Lost in translation?

Although androgen deprivation is associated with transiently increased atrogene expression levels, results of our Western blot analyses and the lack of effects of T treatment in the locomotor muscles indicate that the regulation of the atrogenes by androgens is not as straightforward as previously

assumed. First, T treatment was able to completely inhibit the increase in Atrogin-1 and MuRF1 gene expression in the LA/BC, but did not alter atrogene expression levels following orchidectomy in the locomotor muscles (study 2), nor following tail suspension (study 3). However, the latter may be explained by the fact that also no differences in muscle mass were detected following T treatment in our tail-suspended mice. Second, the transient increase in MuRF1 mRNA levels in EDL following orchidectomy was not paralleled by changes in their protein expression, while Atrogin-1 protein expression was not measured due to technical problems in validating a specific antibody. Only one study has investigated the protein expression level of Atrogin-1 following androgen deprivation in rats (Pires-Oliveira et al. 2010). The authors found no changes in Atrogin-1 gene and protein expression which is reasonable since no muscle atrophy was detected in the latter study. In fact, very few studies have measured changes in protein levels of atrogin-1 and MuRF1 under atrophic conditions (Doucet et al. 2007; Léger et al. 2009; Nedergaard et al. 2012), and no data on MuRF1 protein levels during androgen deprivation and/or treatment were found in the literature. From both studies 2 and 3, it became clear that changes in MuRF1 gene expressions levels do not reflect changes in MuRF1 protein expression. Discordance between mRNA and protein levels of MuRF1 have already been described in other skeletal muscle atrophy models (Drummond et al. 2008). Lack of associations between gene and protein expression levels is commonly described (Chen et al. 2002; Lichtinghagen et al. 2002) and can be explained by different factors. Various molecular processes, such as microRNAs, post-transcriptional splicing, translational regulation and modifications can influence the quantity of proteins and thus results into true biological differences between mRNA and protein levels. In addition, E3 ligases are known to have a short half-life time due to auto-ubiquitination (Chen et al. 2012), making the proteins unstable (Cardozo and Pagano 2004; Bdolah et al. 2007). It is therefore reasonable to assume that the increase in mRNA expression allows rapid protein turnover, without affecting or mildly affecting protein levels. Also, the power to detect significant changes in protein levels could have been limited due to the lower sensitivity of the Western blotting technique compared to qPCR. Thus, finding no differences in protein expression levels does not rule out that MuRF1 plays a role in muscle atrophy.

It is noteworthy that growing doubt exists about the role of both E3 ligases in muscle atrophy. Both E3 ligases target different proteins that are mainly involved in protein synthesis instead of protein degradation (Attaix and Baracos 2010; Baehr et al. 2011; Murton 2011). MuRF1 was shown to interact with myofibrillar proteins such as myosin heavy chain (MHC) (Clarke et al. 2007), troponin I (Kedar et al. 2004), myosin binding protein C and myosin light chain (Cohen et al. 2009) and more recently actin (Polge et al. 2011), suggesting its role in muscle breakdown. On the other hand, the only identified substrates for Atrogin-1 are MyoD, a key transcription factor (Tintignac et al. 2005;

Lagirand-Cantaloube et al. 2009), and eIF3F, an eukaryotic initiation factor of protein synthesis (Lagirand-Cantaloube et al. 2008; Csibi et al. 2010). Both proteins play key roles in the control of muscle differentiation and protein synthesis, questioning the role of Atrogin-1 in muscle proteolysis (Attaix and Baracos 2010).

> *"Although both Atrogin-1 and MuRF1 are strongly regulated by T in the LA/BC muscle, the molecular regulation of these atrogenes by sex steroids in the locomotor muscles remains unclear, suggesting that both atrogenes are not the major mediators of androgen deprivation-induced muscle atrophy as initially believed."*

4.2 IGF1 is involved in the maintainance of muscle mass

Studies in transgenic mice overexpressing Akt in skeletal muscle have demonstrated that Akt plays a pivotal role in the regulation of muscle mass by promoting muscle hypertrophy, via mTOR, and by simultaneously blocking muscle atrophy, via FoxO (Bodine et al. 2001b; Lai et al. 2004). Akt is known to be activated by IGF1, one of the most well-characterized muscle growth-promoting factors.

The stimulation of IGF1 by androgens is well documented (Hobbs et al. 1993; Urban et al. 1995; Grinspoon et al. 1996; Mauras et al. 1998; Reisz-Porszasz et al. 2003; Yin et al. 2009). Testosterone administration to our orchidectomized mice in study 2 resulted in increased intramuscular IGF1 mRNA expression in SOL, whereas orchidectomy was associated with a reduction in IGF1 mRNA expression in the EDL muscle (Figure 17, unpublished data from study 1).

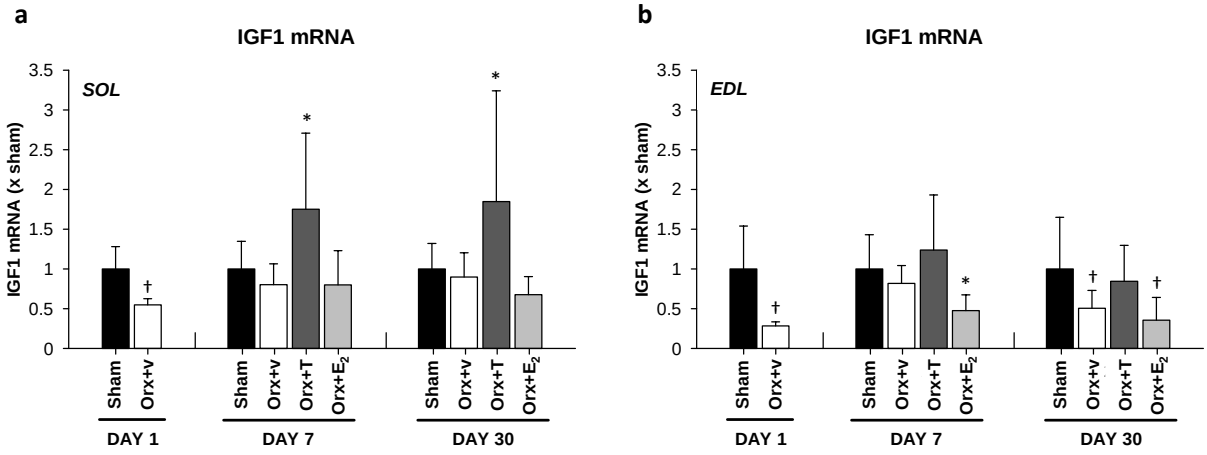

Figure 17. Effects of ultrashort (1 day), short- (7 days) and long-term (30 days) orchidectomy (Orx+v), orchidectomy and testosterone treatment (Orx+T), and orchidectomy and estradiol treatment (Orx+E_2) on IGF1 mRNA expression in SOL (**a**) and EDL muscle (**b**). Data are shown as fold change from the control group for each muscle. Differences between groups were assessed by one-way analysis of variance (ANOVA).
* (P<0.05), † (P<0.01), ‡ (P<0.001): significantly different from Shams (Unpublished data from study 2).

However, results from study 3 could not provide evidence for such an androgenic regulation during disuse atrophy, as no effects of T treatment on the IGF1-Akt-p70^{S6K} (phospho-) levels were found in our tail-suspended mice. More intriguing, very recent research has suggested that the IGF1 axis is not essential to mediate the anabolic effects of androgens, as T was found to increase muscle mass in GH-deficient rats, with low IGF1 serum levels (Serra et al. 2011). However, these controversial results do not exclude that locally produced IGF1, as measured in study 2 (Figure 17) is important in mediating the androgen action in muscle. This hypothesis is supported by a study of Ohlosson et al. (2009) demonstrating normal muscle development in liver-specific IGF1 KO mice in which muscle IGF1 production is still possible. The presence of two AREs in the upstream promoter region of the IGF1 gene (Wu et al. 2007) suggest that, next to the indirect crosstalk between androgens and circulating IGF1 by direct binding of the AR to the regulatory subunit of PI3K (Baron et al. 2004), T can locally influence IGF1 production by transcriptional activation.

Tail suspension in study 3 resulted in a downregulation of IGF1 mRNA during 1 and 5 days of tail suspension, and a subsequent reduction in the phosphorylated levels of Akt in the SOL and EDL after 14 days of tail suspension (Figure 15a). It is well established that IGF1 mRNA levels decrease during disuse (Adams et al. 2000; Litvinova and Shenkman 2007), and that muscle hypertrophy, induced by increased mechanical loading, is associated with elevated IGF1 mRNA and protein expression (Adams and Haddad 1996). However, the importance of IGF1 in regulating muscle mass was questioned by the experiments of Spangenburg et al. (2008), which revealed that loading induced muscle hypertrophy via Akt and p70^{S6K} can occur independently of a functional IGF1 receptor in transgenic mice. Also, Criswell et al. (1998) found that overexpression of IGF1 in skeletal muscle does not prevent unloading-induced muscle atrophy. So, although experiments in IGF1 KO and IGF1 receptor KO mice demonstrated a pivotal role of IGF1 in promoting myofiber hypertrophy during development and postnatal growth (Liu et al. 1993), its role as a major regulator of adult muscle mass during acute muscle unloading and reloading has been a subject of debate (Spangenburg et al. 2010). However, despite this controversy, these results do not rule out that IGF1 might be beneficial for optimizing long-term adaptive remodelling during altering mechanical loading via enhanced protein synthesis or through increased satellite cell activation.

From the literature it became clear that the effect of either tail suspension or androgens on muscle Akt signalling is uncertain. Tail suspension and androgen loss have demonstrated either reduced (Childs et al. 2003; Sugiura et al. 2005; Haddad et al. 2006; Ibebunjo et al. 2011; White et al. 2013), increased (Haren et al. 2011) or unchanged (Krawiec et al. 2005; Hourdé et al. 2009; Maki et al. 2012) phosphorylation of Akt and its downstream target p70^{S6K}. Such conflicting results could be

explained by the different disuse atrophy models used, the organism studied and the fact that the protein expression levels were measured at different time points during the atrophy process. However, it is interesting to note that Akt activity indirectly depends on the dietary state, as it contributes to the regulation of glycogen synthesis in the skeletal muscle (Cross et al. 1995; Hajduch et al. 2001). Upon food intake, insulin binds to its receptor, which activates insulin receptor substrate (IRS) and subsequently PI3K, Akt and 5'- adenosine monophosphate-activated protein kinase (AMPK), leading to the translocation of glucose transporter 4 (GLUT4) to the cell membrane. Once docked on the plasma membrane, GLUT4 is able to transport glucose into the cell (Tsakiridis et al. 1995; Zhou et al. 1999). As tail suspension is associated with increased stress, and as T is known to increase dietary intake (Ferreira et al. 2012), these research models are unavoidably associated with alterations in insulin secretion, hereby inducing changes in phospho-Akt levels.

"A diminished local production of IGF1 appears to be involved in the atrophy process in both androgen deprivation and muscle disuse models. However, activation of Akt and subsequently $p70^{S6K}$ during tail suspension remains equivocal."

4.3 Myostatin expression is under androgenic control but differs among muscle type

Next to atrogene signalling, it is likely that other molecules are responsible for the loss in skeletal muscle mass during T deprivation. As removal of androgens results in a decrease in anabolic stimuli, it is likely that signalling molecules involved in hypertrophy signalling are modulated. Therefore, we investigated the gene and protein expression levels of myostatin, an endogenous inhibitor of muscle growth, following orchidectomy (study 2) and tail suspension (study 3) (Figure 15). Our findings that androgen deprivation induced an upregulation of myostatin gene expression and that T administration following orchidectomy and tail suspension was able to inhibit this increase in myostatin mRNA in the EDL muscle let us believe that myostatin is indeed under control of androgens.

There are some indications in the literature for the involvement of androgens in the regulation of myostatin. An apparent difference in myostatin protein expression exists between males en females, with the higher muscle mass in males being associated with a decreased abundance of myostatin protein when compared to females (McMahon et al. 2003). Moreover, muscle-specific overexpression of myostatin in mice resulted in lowered muscle mass, which was more pronounced in males than in females (Reisz-Porszasz et al. 2003). The androgenic regulation of myostatin could be reasonable as the research group of Bhasin (Ma et al. 2001) has identified ARE in the myostatin promoter suggesting an AR-dependent transcriptional modulation of myostatin expression.

However, this may only be true in fast-twitch muscles as no upregulation in myostatin gene expression was found in SOL following orchidectomy (study 2), nor following T treatment during tail suspension (study 3). More intriguingly, in the LA/BC, a striking opposite direction of change in myostatin gene expression was found in comparison with the EDL. Muscle myostatin mRNA was paradoxically decreased following orchidectomy and increased following T treatment. This surprising finding was also reported by Ibebunjo et al. (2011) in mice triceps brachii muscles, and more recently by Dubois et al. (2014) in the LA and GC muscle of castrated mice. Comparably, Diel et al. (2007, 2008a, 2008b) demonstrated an increase in myostatin mRNA expression in the rat GC muscle and C2C12 cells after treatment with T or other anabolic androgenic steroids. A possible, yet not conclusive, explanation could be that the decreased expression of myostatin counteracts the fast and progressive atrophy of the LA/BC in order to prevent total muscle loss. Conversely, increased myostatin induced by T may prevent excessive hypertrophy. This may also explain the downregulation of myostatin in EDL following 14 days of tail suspension (study 3). However, conclusions are difficult to make, as no differences in muscle mass were found by T administration in the latter study.

An effect of androgen action could not be deduced from our Western blot data, as no significant differences in myostatin protein expression in SOL and EDL were found in study 2 and 3, respectively. Differences between myostatin mRNA and protein levels were also observed by Mendler et al. (2000) and McMahon et al. (2003) in regenerating rat muscles and male mice respectively. Possible reasons for this discrepancy are described above but other modifications such as cleavage of promyostatin into active myostatin (Lee and McPherron 2001), might also be responsible. Actually, only one study by Mendler et al. (2007) investigated the myostatin protein levels in the LA, but not the locomotor muscles, in an androgen-deprivation rat model. They showed a significant increase of the myostatin protein after orchidectomy and a downregulation of the myostatin protein levels back to baseline by T treatment of the orchidectomized rats.

> *"Myostatin gene expression is clearly under androgenic control, but the marked differences in the modulation of myostatin gene expression by T among muscle types questions its role in the regulation of muscle mass."*

4.4 REDD1 is involved in muscle disuse atrophy

Similar to the ubiquitin ligases, REDD1 showed a similar transient expression pattern following tail suspension in both SOL and EDL. The gene expression levels of this mTOR inhibitor quickly increased

(3- to 4-fold) and were further upregulated (7- to 9-fold) at day 5 of tail suspension, but did not differ from controls 14 days after the start of tail suspension. One research group recently investigated the gene expression levels of REDD1 during 3 days of unilateral hindlimb casting of rats (Kelleher et al. 2013), and found a similar increase in REDD1 mRNA content in the SOL. In fact, this rapid and substantial increase of REDD1 gene expression was also observed in the first days following starvation (McGhee et al. 2009) and dexamethasone treatment (Wang et al. 2006). It is noteworthy that long-term effects were not addressed in these latter studies, by which they were unable to observe the late drop in gene expression level.

Interestingly, Harvey et al. (2008) suggested that REDD1, like Atrogin-1 and MuRF1, is a transcriptional target of FoxO. We can therefore hypothesize that the rapid and transient increase in REDD1 mRNA, as well as Atrogin-1 and MuRF1 mRNA, might be induced by an acute activation of FoxO following the atrophic stimulus.

5. ESTROGENIC REGULATION OF SKELETAL MUSCLE MASS

5.1 Estradiol and its effects on muscle mass and strength

While the anabolic effects of T on muscle mass are quite established, a role for estrogens in regulating muscle mass remains unclear. E_2 treatment in our orchidectomized mice (study 2) partially suppressed LA/BC but not SOL and EDL muscle loss. As ERβ mRNA levels were higher in the LA/BC muscle compared with the SOL and EDL muscles, in which only a very low expression was observed (study 2), we might speculate that the estrogenic effect found on LA/BC muscle mass, but not in the locomotor muscles, is ERβ-mediated. Studies in ERs KO mice indicated that both ERα and ERβ receptors may have distinct actions on skeletal muscle, although their exact physiological responses remain unclear (Barros and Gustafsson 2011). Using ERs KO female rats, Velders et al. (2012) provided evidence for the involvement of ERβ signalling in the regulation of locomotor skeletal muscle growth by stimulating anabolic pathways. Our results may also clarify why Goyal et al. (2007) could not show any effects of ERα KO on the LA mass in male mice.

Most of our knowledge regarding E_2-responsiveness of male muscles has been derived from studies performed in elderly men, with studies finding a positive association (Vandenput et al. 2010; Auyeung et al. 2011), and studies finding no association (van den Beld et al. 2000; Szulc et al. 2004a) between E_2 and lean- and muscle mass. In our cohort of eugonadal men (study 1) we could not find an association between E_2 and the CSA of the locomotor muscles, and most intriguingly, the negative association between E_2 and grip strength and biceps force found in study 1 suggest a negative role of E_2 on muscle strength of the upper body of healthy men.

5.2 The molecular regulation of skeletal muscle mass by estradiol

The mechanism by which skeletal muscle functions are regulated by estrogens has received considerably little attention. Over the last 5 years, studies have tried to investigate the intracellular signalling molecules involved in estrogen-regulated muscle processes such as cell survival (Ronda et al. 2007; Boland et al. 2008; Vasconsuelo et al. 2008; Ronda et al. 2010) and myogenic differentiation (Galluzzo et al. 2009), as well as transcripts involved in muscle lipid metabolism (Maher et al. 2010; Rogers et al. 2010), fiber type distribution (Rogers et al. 2010) and energy metabolism (Riedl et al. 2010). There are only a limited number of studies that have examined the estrogenic effects on muscle atrophy and hypertrophy signalling pathways. The research group of Kovanen (Pollanen et al. 2010; Ahtiainen et al. 2012) showed that estrogen replacement therapy exerts anti-catabolic effects on ageing skeletal muscle by increasing gene expression levels of IGF1, mTOR and FoxO3. Interestingly, similar to Svensson et al. (2010) we demonstrated that E_2 is able to

suppress the increased gene expression levels of both MAFbx/Atrogin-1 and MuRF1 in the LA/BC muscle of orchidectomized mice, suggesting that E_2 is also involved in the modulation of atrophy inducing targets. However, this is in strong contrast with the results of Rogers et al. (2010), who found that 12 weeks of ovariectomy was associated with decreased gene expression of both ubiquitin ligases in the quadriceps muscle of female mice. While we could not show any effects of E_2 supplementation on myostatin gene and protein levels in our orchidectomized mice (study 2), results about the regulation of myostatin by estrogens in postmenopausal women (Dieli-Conwright et al. 2012) and ovariectomized rats (Tsai et al. 2007) have been controversial.

"E_2-treatment is able to attenuate LA/BC muscle atrophy during androgen-deprivation, presumable by suppressing Atrogin-1 and MuRF1 gene expression. This effect might be mediated via ERβ activation. However, it is clear that more functional studies are needed before conclusive statements may be drawn."

6. LIMITATIONS AND PERSPECTIVES

This thesis contributed to the better understanding of the molecular regulation of skeletal muscle mass by androgens; however, our findings also resulted in several new research questions and still a lot of ambiguities remain.

Although we could demonstrate important changes in the mRNA expression of various signalling molecules in two different atrophy models, their 'role' in the atrophy process was not examined. Western blot and qPCR are widely accepted techniques for determining protein and mRNA expression levels, respectively. However, they do not provide information on the specific function of the protein or gene examined, as such that results should strictly be interpreted according to the expression levels only. Activity assays, KO, knock-down and overexpression experiments in transgenic mice are required to assess the contribution of each molecule in the muscle atrophy and hypertrophy pathways regulated by T. Furthermore, it is important to identify novel transcriptional regulators and novel substrates for Atrogin-1 and MuRF1, to better understand their regulation and exact function. This is possible using protein-protein interaction approaches such as yeast two-hybrid screening, co-immunoprecipitation assays and affinity purification assays.

In addition, most of our understanding of the molecular targets regulating muscle mass has been derived from animal and in vitro models. However, there is growing evidence that considerable differences exist between rodents and humans. For example, in contrast to observations in rodents, human studies using clinical models of muscle atrophy have not demonstrated a predominant role of the FoxO transcription factors in the regulation of Atrogin-1 and MuRF1 so far. Also discordance in the alterations in gene expression levels of Atrogin-1 and MuRF1 during limb immobilisation (Gustafsson et al. 2010), running (Louis et al. 2007; Harber et al. 2009), fasting (Gomes et al. 2001; Larsen et al. 2006) and ageing (Clavel et al. 2006; Foletta et al. 2011) exist between rodents and humans. Regarding these species differences, it is necessary to verify whether the signalling molecules investigated in this thesis are also physiologically relevant to human muscle wasting, in order to validate their potential as therapeutic targets.

Furthermore, numerous other key factors of the ubiquitin-proteasome pathway have not been measured in the present thesis e.g. FoxO, mTOR, 4EBP1, GSK3, eIF2B, but could lead to a better understanding of the signalling cascades activated by T. FoxO, an upstream regulator of Atrogin-1 and MuRF1, and mTOR, a downstream target of IGF1-PI3K-Akt signalling, are key regulators whose role during androgen deprivation and tail suspension should be addressed. Moreover, mTOR is implicated in the control of autophagy, hereby providing a link between the ubiquitin-proteasome pathway and the autophagy-lysosomal pathway. Therefore, measurement of key targets of the

autophagy-lysosomal pathway in our androgen deprivation model would be of potential significance. Indeed, Serra et al. (2013) have recently provided evidence for changes in both proteasomal and lyosomal activity in the LA of castrated mice.

Moreover, study 1 may have been limited by the relatively small sample size, by which small but significant associations might have been missed, especially for the genetic analyses. The conduction of genome wide association studies may provide additional relevant SNPs associated with serum T levels and muscle mass.

Finally, by using the LC-MS/MS method we were able to show transient changes in circulating testosterone levels following tail suspension in mice (study 3) and to provide reliable serum E_2 levels in men (study 1). The use of this state of the art technique for the measurement of T and E_2 should be encouraged in future research in both rodent and human studies to overcome the lack of both specificity and sensitivity of the still widely used radioimmunoassays.

7. GENERAL CONCLUSIONS

With decades of research highlighting the anabolic effect of T on skeletal muscle growth, the present thesis aimed at exploring the androgenic and estrogenic regulation of known muscle atrophy- and hypertrophy-inducing proteins, in order to identify novel muscle-specific therapeutic targets to improve the clinical outcomes of muscle wasting diseases.

With this work we have shown that the androgenic regulation of skeletal muscle and its underlying mechanism are not straightforward. The following conclusions are drawn from the present thesis:

1) **Genetic variations in the androgen receptor are not associated with muscle mass or function.**
   Heritability, age, physical activity, body composition and anthropometrics in eugonadal men contribute to the interindividual variations in muscle mass. Although variations in serum T levels are associated with the number of CAG repeats and 2 SNPs (rs5965433 and rs5919392) in the AR, these genetic variations do not seem to influence muscle mass or function.

2) **Testosterone treatment is not effective in ameliorating SOL and EDL muscle mass during tail suspension in mice.**
   T therapy is effective in restoring muscle mass and absolute muscle strength during androgen deprivation, but does not ameliorate muscle mass during tail suspenion, despite transient lower serum T levels measured in this disuse atrophy model. The mechanisms underlying this lack of anabolic effects remain unclear.

3) **Atrogin-1, MuRF1 and REDD1 are rapidly activated before muscle atrophy is observed.**
   By measuring gene expression levels at different time points, Atrogin-1, MuRF1 and REDD1 mRNA levels were shown to be rapidly and transiently activated upon androgen deprivation and tail suspension, suggesting that they may contribute to the initiation of the atrophy program.

4) **The murine LA/BC muscle is not an optimal model to study muscle atrophy.**
   Based on the important kinetic differences in Atrogin-1, MuRF1 and myostatin expression between the LA/BC and EDL and SOL muscle, we suggest that the murine LA/BC is not an optimal model to study muscle atrophy since it is not representative for the androgen sensitivity of other more functionally and clinically relevant skeletal muscle types.

5) **Myostatin and IGF1 are under androgenic control but the regulation of Atrogin-1 and MuRF1 by testosterone in the locomotor muscles is unclear.**

   Locally produced myostatin and IGF1 are mediators of muscle mass and are regulated by T. However, T treatment during androgen deprivation or tail suspension was not able to alter Atrogin-1 and MuRF1 gene or protein expression levels in the locomotor muscles, questioning their regulation by sex steroids.

6) **Estradiol is effective in regulating LA/BC muscle mass but not the locomotor muscle mass.**

   E_2-treatment during androgen deprivation clearly showed anabolic effect on the LA/BC, but not on the locomotor muscles. Evidence for its anabolic effects is provided by its effect on Atrogin-1 and MuRF1 gene expression, which is likely mediated via ERβ activation.

Using two different muscle atrophy models, the present work has contributed to the better understanding of the androgenic regulation of skeletal muscle mass. However, it is clear that the application of therapeutic agents to inhibit skeletal muscle atrophy is still in its infancy. As more evidence exists that the regulation of muscle atrophy depends upon the atrophic stimuli, it will be important to develop specific therapies for each muscle wasting condition. Moreover, since muscle loss results from an interplay between multiple signalling pathways, it is reasonable to assume that the use of multiple medications, whether or not combined with exercise or protein intake, will be necessary for an effective treatment of muscle atrophy.

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De Naeyer H, Everaert I, De Spaey A, Kaufman JM, Taes Y, Derave W. Androgenic and estrogenic regulation of skeletal muscle mass and atrophy signaling in male mice. 15th European Congress of Endocrinology. Copenhagen, April 2013 (poster presentation).

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