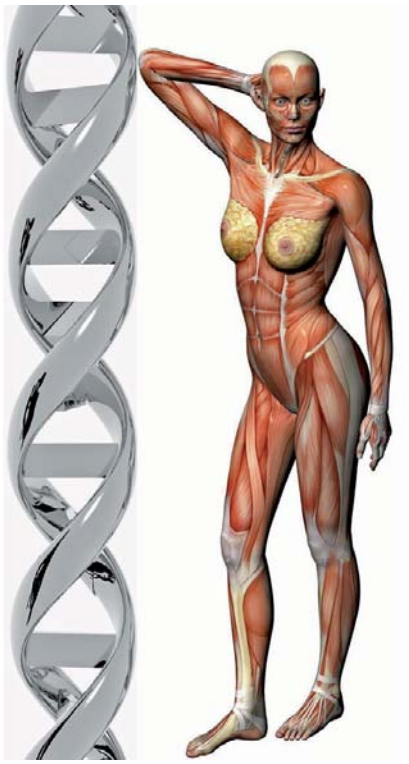
STUDIES IN SPORT, PHYSICAL EDUCATION AND HEALTH
169

Eija Pöllänen

Regulation of Gene Expression and Steroidogenesis in Skeletal Muscle of Postmenopausal Women

With Emphasis on the Effects of Hormone Replacement and Power Training



JYVÄSKYLÄN YLIOPISTO

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“Manuscripts don’t burn.”

- Mikhail Bulgakov -

ABSTRACT

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Regulation of gene expression and steroidogenesis in skeletal muscle of postmenopausal women - With Emphasis on the Effects of Hormone Replacement and Power Training
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The purpose of this study was to assess whether the use of estrogen-containing hormone replacement therapy (HRT) or plyometric power training (PT) has an effect on skeletal muscle properties by affecting global and/or IGF-1-related gene expression. In addition, the systemic and paracrine roles of steroid hormones in the regulation of muscle properties in post- and premenopausal women were investigated. Two different datasets were utilized: the Ex/HRT study, which was a year-long randomized placebo-controlled intervention with control (CO), HRT and PT groups and the Post/Premenop. study, which was a cross-sectional, case-control study. This thesis is largely based on the muscle samples obtained in the two studies in order to determine differences in gene expression and in latter case also in muscle hormone concentration. Samples were available from nine CO, ten HRT and eight PT women who were 50 to 57 years old and early-stage postmenopausal at the onset of the Ex/HRT study and from 13 postmenopausal 61 to 67 years old non-HRT users and 13 premenopausal 29 to 38 years old women not using contraceptives during participation in the Post/Premenop. study. According to the microarray studies, a wide range of changes occurs in muscle gene expression during the first postmenopausal years. In particular gene expression related to cellular catabolic processes, energy metabolism and muscle remodeling was affected. However, both HRT and PT seemed to balance or slow down some of these changes, and can therefore be considered as counteractive treatments. Furthermore, PT induced transcriptional changes in gene expression related to cytoplasm, actin binding and insulin signaling, while HRT affected gene expression related to protein post-translational modifications, proteolysis, cell proliferation and mitochondrial functions. PT and HRT had parallel effects on gene expression related to carbohydrate metabolism and calcium signaling. In addition, HRT was found to affect the gene expression of several genes related to the IGF-1 signaling pathway. The analysis of steroidogenesis in the post- and premenopausal women revealed that despite the extensive loss of steroid hormones in circulation, the skeletal muscle tissue of postmenopausal women has higher concentrations of estrogen and testosterone than the same tissue in premenopausal women. However, the postmenopausal women's muscle properties, including size, strength, power and quality, were poorer than those in the premenopausal women. Taken together, the results imply that postmenopausal HRT and PT induce beneficial changes in muscle gene expression, which are manifested as improved or better maintained muscle properties compared to controls. Furthermore, low circulating concentrations of steroid hormones were associated with decrements in skeletal muscle quality which the local steroidogenesis within the postmenopausal women's muscle was not able to counteract.

Keywords: skeletal muscle, hormone replacement therapy, plyometric power training, microarray study, gene expression, sex steroid hormone, aging, menopause

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Jyväskylä, May 2011
Eija Pöllänen

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which will be referred to in the text by their roman numbers. Additionally, some unpublished data are included in the thesis.

I. Pöllänen E, Ronkainen PHA, Suominen H, Takala T, Koskinen S, Puolakka J, Sipilä S, Kovanen V. 2007. Muscular transcriptome in postmenopausal women with or without hormone replacement. Rejuvenation Research 10(4): 485-500**.**

II. Pöllänen E*, Ronkainen PHA*, Horttanainen M, Takala T, Puolakka J, Suominen H, Sipilä S, Kovanen V. 2010. Effects of combined hormone replacement therapy or its effective agents on the IGF-1 pathway in skeletal muscle. Growth Hormone & IGF Research. 20(5): 372-379 *Equal contribution.

III. Pöllänen E, Fey V, Törmäkangas T, Ronkainen PHA, Taaffe D, Takala T, Koskinen S, Cheng S, Puolakka J, Kujala UM, Suominen H, Sipilä S, Kovanen V. 2010. Power training and postmenopausal hormone therapy affect transcriptional control of specific co-regulated gene clusters in skeletal muscle. AGE (Dordr). 32(3): 347-363.

IV. Pöllänen E, Sipilä S, Alén M, Ronkainen PHA, Ankarberg-Lindgren C, Puolakka J, Suominen H, Hämäläinen E, Turpeinen U, Konttinen YT, Kovanen V. 2011. Differential influence of peripheral and systemic sex steroids on skeletal muscle quality in pre- and postmenopausal women. Aging Cell. Published online March 9. DOI: 10.1111/j.1474-92726.2011.00701.x

ABBREVIATIONS

ANCOVA	analysis of co-variance
ANOVA	analysis of variance
ATP	adenosine triphosphate
BMI	body mass index
Ca^{2+}	calcium
cDNA	complementary DNA
CO	control
CSA	cross-sectional area
CT	computed tomography
CV	coefficient of variance
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
E_1	estrone
E_2	17β-estradiol
Ex/HRT study	Exercise and hormone replacement therapy study
FC	fold change
$fCSA_{QF}$	intramuscular fat area within *quadriceps femoris*
FDR	false discovery rate
GEO	Gene Expression Omnibus
GlcNAc	O-linked N-acetylglucoseamine
GO	gene ontology
HRT	hormone replacement therapy
HU	Hounsfield units
KE	knee extension strength
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBM	lean body mass
LC-MS/MS	liquid chromatography-tandem mass spectrometry
$mCSA_{QF}$	muscle CSA of *quadriceps femoris*
mRNA	messenger ribonucleic acid
Post/Premenop. study	Study on post- and premenopausal women
PT	power training
QF	*quadriceps femoris*
qPCR	quantitative polymerase chain reaction
RCT	randomized controlled trial
RNA	ribonucleic acid
RQ	relative quantity
SD	standard deviation
T	testosterone
$tCSA_{QF}$	total CSA of the *m. quadriceps femoris*

ABBREVIATIONS USED FOR GENES AND PROTEINS

18S	RNA, 18S ribosomal; RN18S1
4E-BP1	eukaryotic translation initiation factor 4E binding protein
actin	actin, alpha 1, skeletal muscle; ACTA1
ACVR2B	activin A receptor, type IIB; myostatin receptor
AKT	serine/threonine-protein kinase B; PKB, v-akt murine thymoma viral oncogene
AKT1	PKB, v-akt murine thymoma viral oncogene homolog 1
AKT2	PKBβ, v-akt murine thymoma viral oncogene homolog 2
AKT3	PKBγ, v-akt murine thymoma viral oncogene homolog 3
ALDOA	aldolase A, fructose-bisphosphate
AMPK	AMP-activated protein kinase
AR	androgen receptor
aromatase	aromatase cytochrome P450; CYP19A1
ATP5H	mitochondrial ATP synthase
atrogin-1	muscle atrophy F-box gene, MAFbx; FBXO32
β-Actin	beta actin; ACTB
CALM1	calmodulin 1
CALM2	calmodulin 2
CaMK2A	calcium/calmodulin-dependent protein kinase II alpha
CaMK2B	calcium/calmodulin-dependent protein kinase II beta
CASP9	caspase 9, apoptosis-related cysteine peptidase
CHAD	chondroadherin
COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2
DCTN1	dynactin 1
DDX52	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52
DVL1	dishevelled, dsh homolog 1
eNOS	nitric oxide synthase (endothelial cell); NOS3
ERK2	extracellular signal regulated kinase 2; MAPK1
ESR1	estrogen receptor 1; ESRα
ESR2	estrogen receptor 2; ESRβ
FASN	fatty acid synthase
FBXO11	F-box protein 11
FOXO	forkhead family of transcription factors
FOXO1	Forkhead box O1
FOXO3	Forkhead box O3
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GRB10	growth factor receptor-bound 10
GH	growth hormone
GLUT4	glucose transporter, solute carrier family 2 member 4; SLC2A4
GPER	G-protein coupled estrogen receptor; GPR30
GSK3	glycogen synthase kinase 3

HSD17B1	17β-hydroxysteroid dehydrogenase 1
HSD17B3	17β-hydroxysteroid dehydrogenase 3
HSD17B5	17β-hydroxysteroid dehydrogenase 5
HSD17Bs	17β-hydroxysteroid dehydrogenases
HSD3B1	3β-hydroxysteroid dehydrogenase 1
HSD3B2	3β-hydroxysteroid dehydrogenase 2
HSD3Bs	3β-hydroxysteroid dehydrogenases
HSPE1	heat schock 10 kDa protein 1 (chaperonin 10)
IDH3A	isocitrate dehydrogenase 3 (NAD+) alpha
IGF-1	insulin-like growth factor 1 (somatomedin C)
IGF-1Ea	insulin-like growth factor 1, splice variant Ea
IGF-1Eb	insulin-like growth factor 1, splice variant Eb
IGF-1Ec	insulin-like growth factor 1, splice variant Ec; MGF
IGF-2	insulin-like growth factor 2 (somatomedin A)
IL4I1	interleukin 4 induced 1
ING2	inhibitor of growth family, member 2;ING1L
LH	luteinizing hormone
MAPK	mitogen activated protein kinase
MEF2D	myocyte enhancer factor 2D
Megalin	low density lipoprotein receptor-related protein 2, LRP2; GP330
MGEA5	meningioma expressed antigen 5 (hyaluronidase); NCOAT; OGA
MGF	mechano growth factor; IGF-1Ec
MKNK2	MAP kinase interacting serine/threonine kinase 2
MRPL27	mitochondrial ribosomal protein L27
MRPL33	mitochondrial ribosomal protein L33
MRPS12	mitochondrial ribosomal protein S12
MRPS36	mitochondrial ribosomal protein S36
MTFP1	mitochondrial fission process 1; MTP18
mTOR	mechanistic target of rapamycin (serine/threonine kinase); FRAP1
mTORC1	mTOR complex including raptor-protein
mTORC2	mTOR complex including rictor-protein
MuRF-1	muscle-specific RING finger protein 1; TRIM68
MYF5	myogenic factor 5
MYF6	myogenic factor 6 (herculin); MRF4
MYH8	myosin, heavy chain 8, skeletal muscle, perinatal
MyHC I	myosin, heavy chain I slow isoform; MYH7
MyHC IIa	myosin, heavy chain 2a, skeletal muscle, adult; MYH2
MyHC IIx	myosin, heavy chain 2x; MYH1
MyHC	myosin, heavy chain
MYOD1	myogenic differentiation 1; MYF3
myogenin	myogenin (myogenic factor 4); MYOG; MYF4
Myostatin	MSTN; growth differentiation factor 8, GDF8
nebulin	actin-binding protein, component of sarcomere; NEB
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase
p38	MAPK14

PECI	peroxisomal D3,D2-enoyl-CoA isomerase
PHKG1	phosphorylase kinase, gamma 1
PI3K	phosphoinositide-3-kinase
PKM2	pyruvate kinase, muscle
PRKAR2A	cAMP-dependent protein kinase, regulatory subunit II, alpha
PTPMT1	protein tyrosine phosphatase, mitochondrial 1
RAB31	RAB31, member RAS oncogene family
Raf	Ras-associated serine/threonine MAP-3-kinase
Ras	protein superfamily of small GTPases
RNASEH2C	ribonuclease H2, subunit C
S6K1	ribosomal protein S6 kinase; p70-S6K
SHBG	sex hormone-binding globulin
SRD5A1	5α-reductase 1
SRD5A2	5α-reductases 2
SRD5As	5α-reductases ; steroid-5-alpha-reductase
STARS	striated muscle activator of Rho-dependent signaling; ABRA
STK11	serine/threonine kinase 11
STS	steroid sulfatase; ES
SULT1A1	sulfotransferase family, 1A1
titin	component of sarcomere; connectin; TTN
TNFα	tumor necrosis factor; TNF
TPI1	triosephosphate isomerase 1
tropomyosin	regulatory protein of striated muscle actin filament
troponin	regulatory protein of striated muscle actin filament
TST	thiosulfate sulfurtransferase (rhodanese)
UBE2E3	ubiquitin-conjugating enzyme E2E 3
UBE2G2	ubiquitin-conjugating enzyme E2G 2
USP1	ubiquitin specific peptidase 1
USP15	ubiquitin specific peptidase 15
USP2	ubiquitin specific peptidase 2
USP50	ubiquitin specific peptidase 50
VEGF	vascular endothelial growth factor

CONTENTS

1 INTRODUCTION

Aging is associated with a gradual reduction in skeletal muscle mass, strength and quality which in turn lead to a reduction in functional capacity. Major biological reasons for these decrements include reduction in diameter and number of muscle cells, impairments in neural functions as well as unfavorable changes in muscle composition due to infiltration of fat and collagenous extracellular matrix (Mohan & Radha 1980, Borkan et al. 1983, Lexell et al. 1988, Wokke et al. 1990, Delmonico et al. 2009). With increasing life expectancy, it is predicted that the number of people with decrements in muscle function will increase significantly in the near future.

Aging-related muscle wasting and weakness has during the past few decades come to be considered an important contributory factor to the quality of life of older persons. The term sarcopenia (greek "sarx" for flesh; "penia" for loss), was first used in the late 1980s to describe age-related decrease of muscle mass and has been further developed ever since to better describe aging-related changes in musculature (Rosenberg 1989, Rosenberg 1997). Several studies have examined the prevalence of sarcopenia, as indicated by height-adjusted muscle mass two standard deviations lower than that found in younger populations and have reported prevalences ranging from 4.1% to 35.1% at age 60 and over and 31% to 60% at age 80 and over (Baumgartner et al. 1998, Iannuzzi-Sucich et al. 2002, Tanko et al. 2002a, Lau et al. 2005, Kim et al. 2009, Kirchengast & Huber 2009). Recently, the European Working Group on Sarcopenia in Older People recommended a more comprehensive definition of sarcopenia, including both low muscle mass and impaired muscle functions, for diagnosis of sarcopenia (Cruz-Jentoft et al. 2010). To date no studies have been published in which this broader definition has been applied to estimate prevalence or consequences of sarcopenia.

Throughout the adult life, women are weaker and have less muscle mass than men. Furthermore, deterioration in muscle performance has been observed as early as during perimenopause (Calmels et al. 1995), rendering women more vulnerable to sarcopenia than age-matched men. Compared to the slow, gradual reduction in circulating steroid hormones in men, the decrement in steroid hormones, especially estrogens, in women during their fifties occurs rapidly.

Concomitant with such changes in the hormonal milieu, accelerated deterioration in muscle strength has been observed (Phillips et al. 1993, Samson et al. 2000). These observations indicate that the female sex hormones might be involved in the early development of sarcopenia. However, using estrogen-containing hormone replacement therapy (HRT) does not totally prevent sarcopenia, since in a cross-sectional study on postmenopausal women using HRT 24% of the participants were reported to have low muscle mass (Kenny et al. 2003). The effects of postmenopausal HRT on skeletal muscle properties have been studied in randomized controlled trials (RCT). In some studies the use of HRT has been shown to increase muscle mass and to improve neuromuscular function (Skelton et al. 1999, Sipilä et al. 2001, Dobs et al. 2002, Taaffe et al. 2005), while other studies have concluded that HRT does not have beneficial effects on muscle mass or physical function (Ribom et al. 2002, Tanko et al. 2002b, Kenny et al. 2005). This discrepancy can partially be explained by differences in methodology and subjects' characteristics.

So far the best known prevention strategy against aging-related weakness and loss of muscle mass is physical exercise together with a healthy diet including adequate protein intake (Greenlund & Nair 2003, Taaffe 2006, Dillon et al. 2009, Visvanathan & Chapman 2010). Skeletal muscle maintains its ability to respond training, as demonstrated in studies in which sedentary nonagenarians have improved their muscle function following training interventions (Fiatarone et al. 1990, Fiatarone et al. 1994). Different training types have, however, different effects on muscle outcomes. For example, strength training increases muscle mass and maximal muscle strength (Frontera et al. 1988, Charette et al. 1991, Lexell et al. 1995, Sipilä et al. 1996), endurance training improves aerobic performance (Suominen et al. 1977, Coggan et al. 1992, Stiebellehner et al. 1998), and power training involving rapid force production improves muscle power production (Häkkinen et al. 1990, Paavolainen et al. 1999, Sipilä et al. 2001, Fielding et al. 2002, Kyröläinen et al. 2004). Nevertheless, even constantly physically active persons gradually lose their muscle mass and experience impaired performance, even if they may retain on levels which are comparable to those of sedentary, much younger persons (Klitgaard et al. 1990, Sipilä & Suominen 1991).

Despite the emerging amount of data on the physiological effects of aging and the effects of counteractive treatments on women's musculature, molecular level data remain scarce. In particular very little molecular level data has been published on the effects of postmenopausal HRT on gene expression or signal transduction in human skeletal muscle. Therefore, transcriptome-wide studies, such as those conducted for this thesis, which investigate the effects of HRT and power training on muscle gene expression in postmenopausal women, provide novel information about the factors involved in regulating women's muscle characteristics during aging. In addition, this study provides new knowledge on the molecular consequences of the withdrawal of ovarian steroid hormones on skeletal muscle properties among postmenopausal women.

2 REVIEW OF THE LITERATURE

2.1 Effects of aging, training and hormonal replacement on skeletal muscle

2.1.1 Overview of the structure and function of skeletal muscle

The human body contains three types of muscles, skeletal, cardiac and smooth, of which skeletal and cardiac muscles are striated, but only skeletal muscle is under voluntary, conscious control. Skeletal muscles are responsible for body movements, maintaining posture, stabilizing joints and generating heat. Importantly, skeletal muscle tissue is metabolically active, having major role in maintaining the energy homeostasis of the whole body and serving as a protein reservoir.

Skeletal muscle tissue is a mixture of different types of cells containing multinucleated, matured muscle cells termed muscle fibers, as well as quiescent satellite cells, fibroblasts embedded in the extracellular matrix, endothelial cells of vasculature and neuronal cells (Ross et al. 2003). The hierarchical anatomical structure of skeletal muscle comprises three connective tissue layers (Ross et al. 2003). The epimysium surrounds the entire muscle and is mainly composed of collagenous extracellular matrix in which collagen fibers become aligned towards the end of the muscle to form a tendon. Tendons connect muscles to the bones and transmit the force generated by a muscle to the bones or joints to generate movement. The epimysium is connected to the perimysial connective tissue layer, which surrounds muscle fiber bundles. Each muscle fiber bundle contains several multinucleated muscle cells, which are surrounded by a cell membrane, termed the sarcolemma and by two layers of collagenous membranes, the basal lamina and endomysium. Satellite cells, which are myogenic stem cells needed for the growth and repair of muscle fibers, are found between the sarcolemma and basal lamina (Mauro 1961, Zammit et al. 2006). Neurons, larger blood vessels and smaller capillaries also form a

continuous network sweeping between and around the muscle fiber bundles and fibers, providing, e.g., neuronal inputs, oxygen and energy supplies as well as endocrine signals to the muscle fibers. Muscle cells themselves contain hundreds of myofibrils, which are tightly packed in parallel to each other, almost entirely filling the cells. Each myofibril consists of a series of sarcomeres. The major contractile components of sarcomeres are actin and myosin proteins, forming thin and thick filaments, but several other proteins, e.g., troponin, tropomyosin, titin and nebulin, are also involved. The contraction of a muscle fiber occurs when a neuronal signal transmitted through the upper and lower motoneuron and finally across the neuromuscular junction, reaches the muscle fiber. This causes sarcolemmal depolarization, changes the cytoplasmic and sarcoplasmic reticulum calcium (Ca^{2+})-flux, induces cross-bridge cycling between actin molecules and the myosin head area, and finally activates actin and myosin filaments co-ordinately to slide between each other (Huxley & Niedergerke 1954, Huxley & Hanson 1954). This shortens sarcomeres and accordingly leads to contraction of the muscle fiber and finally of the entire muscle (Huxley & Hanson 1959, Huxley 1988).

Human skeletal muscle fibers can be classified into slow-twitch type I and fast-twitch type IIa and IIx fibers. The twitch properties are determined basically by the myosin heavy chain (MyHC) isoform which affects the speed by which adenosine triphosphate (ATP) is hydrolyzed enabling cross-bridge cycling to occur during the contractile process (Huxley 2000). The three main fiber types also differ regarding their metabolic profile (Brooke & Kaiser 1970): type I fibers use predominantly oxidative (aerobic) energy pathways for ATP production and are fatigue-resistant. Type IIa fibers are so-called intermediate fibers which utilize both oxidative and glycolytic (anaerobic) energy metabolism. Type IIx fibers have the highest capacity to use glycolytic, anaerobic energy production pathways. These fibers are the fastest twitching, but also fatigue quickly, and are recruited especially for the needs of short explosive muscle contractions. Normally, a single motor neuron innervates a bunch of muscle fibers with similar metabolic and contractile properties forming a motor unit which contracts co-ordinately (Huxley 1988). One motor unit may contain hundreds of muscle fibers, which are spread around the fiber bundle and form the well known mosaic structure of different fiber types seen in histological cross-sections of muscle samples (Cumming et al. 1994). In addition, hybrid fibers expressing more than one isoform of MyHC also exist (Andersen 2003).

2.1.2 Aging-related changes in skeletal muscle

Aging is often associated with reduced physical activity, reduced food intake, changes in hormonal milieu, and sometimes also illnesses, which all contribute to the changes occurring in skeletal muscle and eventually lead to impairments in functional capacity. According to a set of follow-up studies, after age of 50, the reduction rate of muscle mass and strength is ~1-2% per year (Winegard et

al. 1996, Rantanen et al. 1998, Hughes et al. 2001, Goodpaster et al. 2006) while according to cross-sectional studies, the loss of muscle power, i.e., ability to produce force quickly (force x velocity), may be even greater, that is, ~3-4% per year (Bassey & Short 1990, Skelton et al. 1994). There is also evidence indicating that muscle power is more important predictor of functional capacity than muscle strength (Foldvari et al. 2000, Suzuki et al. 2001, Bean et al. 2002, Cuoco et al. 2004, Sayers 2007, Sayers & Gibson 2010). During aging, fat infiltration between and within separate muscles, muscle fiber bundles and muscle fibers also increases concomitant with the increment in the amount of connective tissue leading to unfavorable changes in the composition of the skeletal muscles (Delmonico et al. 2009). This reduces the quality of muscle tissue and may contribute to the aging-related decrements in functional performance.

Two main mechanisms control the size of the whole muscle: an increase (hypertrophy) or decrease (atrophy) in muscle fiber size, as well as an increase or decrease in muscle fiber number. It is generally accepted that the number of fibers is principally determined during the perinatal period (Stickland 1981). Therefore the increase in muscle cross-sectional area (CSA) is primarily due to increase in muscle fiber diameter, which is a consequence of the accumulation of contractile proteins within the fiber (Nader 2005). In contrast, the loss of contractile proteins leads to reduced fiber CSA. Therefore the balance between protein synthesis and degradation is critical for determining the CSA of the whole muscle. However, the rate of muscle protein synthesis and breakdown may be reduced or impaired in old age (Balagopal et al. 1997, Combaret et al. 2009). Defects in protein degradation may lead to the accumulation of aberrant proteins and cell organelles, thereby affecting the metabolism of cells and leading to difficulties in responding to changing external demands such as training stimulus. Successful hypertrophy also requires an increase in the number of myonuclei in order to maintain the constant volume of cytoplasm supported by a single nucleus (Zammit et al. 2006). New myonuclei are provided by satellite cells, which can be activated in response to trauma or strenuous muscle load (Bischoff & Heintz 1994, Thornell 2011). After activation, satellite cells proliferate and fuse with existing muscle fibers. However, aging reduces the number and proliferating capacity of satellite cells, thereby limiting the rate of hypertrophy and muscle repair (Renault et al. 2002a, Renault et al. 2002b). Loss of muscle mass, strength and power are connected, but the association may not be straight forward. Instead it is likely to be rather complicated, and is not yet well understood. The whole process leading to a reduction in muscle strength or power is multifactorial. Neural factors are known to be significant contributors to the decline of both muscle strength and power (Lauretani et al. 2003, Kamen 2005, Christie & Kamen 2006). Also, mitochondrial dysfunction, accumulation of aberrant contractile and other proteins within muscle fibers as well as increased motor unit size, number of hybrid muscle fibers and loss of muscle fibers, especially fast-twitching type II fibers, are likely to contribute to this decline.

At the cellular level aging is accompanied by changes in muscle fiber size, number and organization. For example, the cross-section of muscle biopsy sample taken from a young person reveals a mosaic of different muscle fiber types while a similar cross-section from an older person shows grouping of fiber types and diminished CSA, especially of type II fibers (Larsson et al. 1978, Lexell & Downham 1991). In addition the number of hybrid fibers may increase during aging, probably due to the denervation-reinnervation process (Grounds 2002, Andersen 2003). This occurs when a motor neuron is lost. After losing of a neuron the neighboring motor neurons take the place of the lost one, also changing the fiber type to correspond to the type already belonging to that motor unit (Ellisman et al. 1978). This leads to the formation of very large motor units, which may lead to impaired muscle performance also at the whole organ level (Aagaard et al. 2010). Without reinnervation muscle fibers left without neuronal inputs degenerates and eventually die. Up to 30-40% of all muscle fibers may be lost by the age of 80 (Lexell 1995).

2.1.3 Effects of power training on skeletal muscle

Muscle tissue in older people responds well to mechanical stimulus in order to cope with external demands. This has been demonstrated in numerous studies investigating the physiological effects of different training modes on aging muscle. Older men in particular have been studied extensively, while older women have been a less popular subject of study. Different training modes improve overall fitness, but also have their own specific effects on muscle phenotype, possibly through distinct molecular signals. The most extensively studied training modes among middle-aged and older persons are strength and endurance training. Strength training is typically performed with the aid of resistance devices at near maximal load to increase muscle mass and strength, possibly by enhancing signaling through the IGF-1 pathway (Adamo & Farrar 2006). Endurance training such as walking, running and cycling, improves in particular aerobic capacity and fatigue resistance, possibly through activating AMPK- and calmodulin-related signaling pathways (Nader 2006) or by changing muscle gene expression related to metabolism and mitochondrial biogenesis (Wittwer et al. 2004, Mahoney et al. 2005, Timmons et al. 2005, Schmutz et al. 2006). While the benefits gained from strength and endurance training are important, there is little evidence of their ability to directly improve muscle power, which has been shown to be more critical than strength in many functional tasks relevant to daily life such as stair climbing, chair rising and correcting postural balance during slipping (Skelton et al. 1994, Foldvari et al. 2000, Suzuki et al. 2001, Bean et al. 2002, Bean et al. 2003).

Power training is a training mode that aims to improve the ability to produce force rapidly. Power has two components, velocity and force, of which velocity seems to decline faster during aging (Bosco & Komi 1980, De Vito et al. 1998). Therefore including high-velocity contractions in the training program might be more beneficial than traditional strength training in preventing or

reducing aging-related functional disabilities and improving performance in daily activities (Porter 2006, Hazell et al. 2007). In a systematic meta-analysis, traditional strength training was found to induce only a small improvement in functional tasks despite bringing considerable improvements in muscle strength (Latham et al. 2004). This result indicates that a critical variable in functional capacity may be the ability to produce force rapidly.

Power training can be performed in several ways. It may be done in a similar manner as resistance training but using lower weights and higher speed than employed in traditional strength training programs. Power training may also be performed by using one's own body as a resistance and producing rapid movements such as jumping activities. This type of power training is called plyometric power training and has extra benefit of loading bones in addition to muscles. Plyometric power training was also used in the Ex/HRT study from which a subsample was used in this thesis. In the year-long Ex/HRT study early postmenopausal women improved muscle power, assessed by vertical jumping height, by 6% compared to the decrease of 5% recorded in controls (Sipilä et al. 2001). Muscle quality assessed as attenuation coefficient was also shown to increase by 2% in the training group compared to a decrease of 1% in controls (Taaffe et al. 2005). The increased muscle attenuation value indicates a decrease in fat infiltration within muscle. In addition, improvement occurred in bone properties during intervention (Cheng et al. 2002). Plyometric power training may be a promising training mode, especially for postmenopausal women, who are at higher risk for osteoporosis (Walsh et al. 2006, Della Martina et al. 2008) in addition to increased risk for neuromuscular dysfunction (Phillips et al. 1993, Samson et al. 2000). The molecular mechanism underlying the effects of power training on skeletal muscle is currently unknown and no previous gene expression level studies using muscle samples from postmenopausal women have yet been published.

2.1.4 Effects of postmenopausal HRT on skeletal muscle

The discovery of estrogen receptors (ESR1 and ESR2) in human muscle tissue around the beginning of this century brought up the idea that skeletal muscle is a potentially estrogen responsive tissue (Lemoine et al. 2003, Wiik et al. 2003). Since that several studies have used postmenopausal women without endogenous estrogen production and with external estrogen replacement as a model to study the physiological effects of estrogen on aging muscle. The results obtained from RCTs have been controversial. In some studies the use of HRT has been shown to increase muscle mass and to improve muscle strength and power (Skelton et al. 1999, Sipilä et al. 2001, Dobs et al. 2002, Taaffe et al. 2005), while the other studies have concluded that HRT does not have beneficial effects on muscle mass or physical function (Ribom et al. 2002, Tanko et al. 2002b, Kenny et al. 2005). This discrepancy can partially be explained by differences in methodology and subjects' characteristics, such as the amount and content of the HRT preparation and treatment duration, as well as age, time

since menopause and physical characteristics of the study participants at the outset of the study.

In the year-long RCT used in this study, continuous, combined HRT or placebo was used by women during their first years of postmenopause (0.5 to 5 years since last menarche). HRT was found to increase the CSA of thigh muscle, knee extension strength, explosive muscle power, running speed and to increase the muscle attenuation coefficient, which indicates less fat infiltration within muscle (Sipilä et al. 2001, Taaffe et al. 2005). Furthermore, in a co-twin study performed in our laboratory, the identical twin sisters using HRT had better muscle composition, power and walking speed than their co-twin sister not on HRT (Ronkainen et al. 2009). Also a recent meta-analysis concluded that HRT improves muscle strength (Greising et al. 2009). In light of these studies HRT seems to have beneficial effects on muscle phenotype after menopause. The molecular level effects of postmenopausal HRT on muscle tissue are, however, not known, although some hints as to what the effects might be have been obtained from experimental animal studies.

2.2 Hormonal factors involved in the regulation of muscle properties during aging

Originally the word hormone was used to refer to a chemical compound made by a gland for export through the circulation to another part of the body where it had regulatory functions. Nowadays a hormone is more broadly understood to mean an organic or peptide compound that controls and regulates the activity of certain cells or organs, irrespective of whether it is produced and secreted by a special gland or not. The most dramatic change in women's hormonal status occurs during the transition from the premenopausal period to postmenopausal period. Menopause means cessation of menstruation and it is defined retrospectively, that is, at 12 months of following last menstrual flow, which occurs at a median age of 51 years (Grady 2006). During the menopausal transition, hormone levels may fluctuate, leading to irregular menstrual cycle. Finally estrogen levels decrease and production of the FSH increases. This leads to permanent cessation of ovulation, and women thereafter are defined as postmenopausal.

Hormones released into the circulation by specialized glands or certain tissues affect muscle properties by endocrine manner, that is, systemically. However, muscle properties may also be affected by local synthesis of hormones which directly activates signaling cascades within muscle tissue. Local functions of hormones are termed paracrine activities, if the hormones released from one cell influence the neighboring cells, and autocrine activities, if the released hormones influence the same cell which produced and released them in the first place, and intracrine activities, if the hormones function within

the cell which synthesized them without release into the pericellular compartment (Figure 1). Since in many instances it is difficult to detect which mode of action is ongoing, the terms paracrine or local activity will be used here to refer all three activity modes caused by local synthesis of hormones.

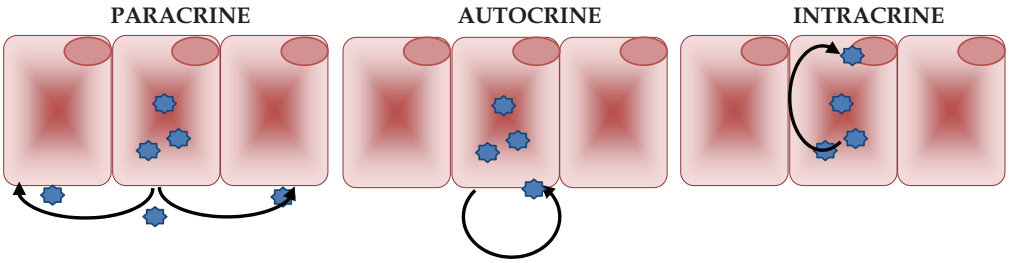

FIGURE 1 Modes of local hormonal actions.

Aging is accompanied by changes in both the systemic and paracrine factors which influence the properties of their target organs, including skeletal muscle. Examples of systemic and local factors which influence muscle properties and may be changed during aging include decrements in neural inputs (Aagaard et al. 2010), lowered concentration of peptide or steroid hormones (Veldhuis 2008), vitamin D deficiency (Ceglia 2009, Hamilton 2010), changes in IGF-1- and in myostatin signaling -related factors (Otto & Patel 2010), evolvement of so-called low grade inflammation (Degens 2010) and reduced response of exercise-induced myokines, (Brandt & Pedersen 2010) as well as microRNA-related regulation (Williams et al. 2009, Roth 2011).

This study focuses on understanding the regulatory processes occurring in the skeletal muscle of postmenopausal women in two treatment conditions, HRT and power training, and on the role of steroid hormones in the regulation of muscle properties in pre- and postmenopausal women. The literature concerning these regulatory processes, including systemic and paracrine actions of steroid and peptide hormones, are reviewed in the following sections.

2.2.1 Systemic steroid hormones

Steroid hormones are large class of organic, lipid-soluble compounds with four carbon atom rings fused together. The main source of sex steroid hormones into the circulation is the cortex of the adrenal glands and the gonads, i.e., testes in men and ovaries in women. The secretion of sex steroids is regulated by pituitary gland hormones such as FSH and LH.

Androgens include dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, testosterone (T) and dihydrotestosterone (DHT). The main estrogens are estriol, estrone (E_1) and 17β-estradiol (E_2). DHEAS, DHEA and androstenedione are precursor hormones with little biological activity of their own. They can, however, serve as precursors for the synthesis of more active T, DHT and E_2. The synthesis

route involves a cascade of steroidogenic enzymes and can be initiated with different precursor molecules (Figure 2). A simplified presentation of the whole cascade from DHEAS to the most potent androgens and estrogens is as follows (Labrie et al. 1998): Steroid sulfatase (STS) removes sulfate from DHEAS to form DHEA, which is then converted to androstenedione by 3β-hydroxysteroid dehydrogenases (HSD3Bs). Adrostenedione is either converted to T by 17β-hydroxysteroid dehydrogenases (HSD17Bs) or to E_1 by aromatase cytochrome P450 (aromatase). T can be converted to E_2 by aromatase or to DHT by 5α-reductases (SRD5As). E_2 can also be formed from E_1 by HSD17Bs.

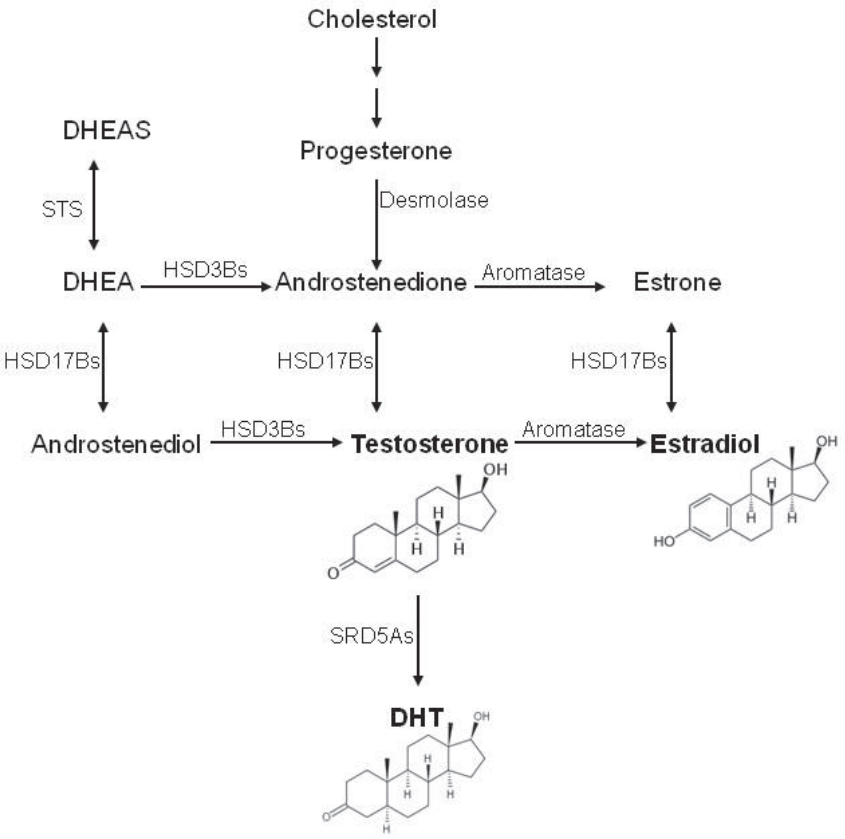

FIGURE 2 Simplified presentation of the steroidogenesis of androgens and estrogens. Figure is adapted and combined from the reviews by Labrie et al. (2001) and Du et al. (2006).

Although the precursor hormones DHEAS, DHEA and androstenedione are only weak androgens, they are much more abundant in the circulation than T and E_2 (Labrie 1991). Therefore, they may have a substantial role in the peripheral synthesis of active androgens and estrogens. The circulating levels of DHEAS, DHEA and androstenedione decline between ages of 20 and 80 years

in both men and women (Labrie et al. 1998). The decline in systemic levels of T and DHT in women is not as clearly defined as the decline in E_2 at menopause (Davison & Davis 2003). In one cross-sectional study it was indicated that the concentration of T decreases gradually in women after their thirties (Zumoff et al. 1995), while in a prospective longitudinal study no change in T levels across the menopausal transition was found (Burger et al. 2000).

The major reproductive function of T is to control the production and maturation of sperm and E_2 to promote the growth of the lining of the uterus in preparation for the implantation of a fertilized egg. However, these hormones also have a wide array of non-reproductive functions, including regulation of properties of peripheral tissues, and their receptors have been found in most of the human tissues, including skeletal muscle. T and DHT signaling is largely mediated by nuclear androgen receptor (AR), which acts as a transcription factor and is found in the skeletal muscle (Kadi et al. 2000, Sinha-Hikim et al. 2004). Due to the low expression levels of SRD5As, which convert T to DHT, T is considered to be the major androgen in skeletal muscle (Bhasin et al. 2003). T has been classified as an anabolic hormone, which increases muscle mass and strength due to changing the net balance of muscle protein synthesis and breakdown to favor synthesis (Ferrando et al. 1998). It may also function through activating satellite cells (Kadi et al. 1999, Sinha-Hikim et al. 2003).

The effects of E_2 on gene expression are delivered through nuclear receptors (ESR1 and ESR2), but the role of E_2-signaling in the regulation of muscle properties is not thoroughly understood. Current knowledge derives from the pioneering work of Jensen, Jacobson, Gorski and Toft, who initially discovered estrophillin, later named ESR1, to be a nuclear receptor, which upon binding to E_2 initiates transcription (Jensen & Jacobson 1962, Toft & Gorski 1966). The discovery of a second ESR, ESR2 (Kuiper et al. 1996) complicated the field, and an enormous amount of data has since been reported concerning both unique and redundant functions of ESRs in different tissues and cells. ESR2 may activate transcription of the same genes as ESR1, but it can also function as an inhibitor of ESR1 activity (Hall & McDonnell 1999). Upon activation by hormone binding, ESR1 and ESR2 form homo- or heterodimers and move to the nucleus to induce transcription of their target genes (Matthews & Gustafsson 2003). In addition, membrane-bound forms of ESRs, which induce rapid signals without direct transcriptional responses, may also be present (Razandi et al. 1999). Further diversification in the E_2 signaling pathway was found with identification of a new membrane receptor likely to deliver E_2 responses through G-protein coupled signaling (Carmeci et al. 1997, Filardo et al. 2002). Originally this new estrogen receptor was named GPR30, because it belongs to the family of G-protein coupled receptors, but is nowadays known as GPER; G-protein coupled estrogen receptor (Maggiolini & Picard 2010). However, the description of GPER as a functional estrogen receptor has also been criticized. It has been proposed that GPER only facilitates signal transductions from membrane ESR1 and does not itself function as an independent estrogen

receptor (Levin 2009). The possible subcellular locations of the estrogen receptors are presented in Figure 3. However, it is currently unknown if they all are also found in skeletal muscle.

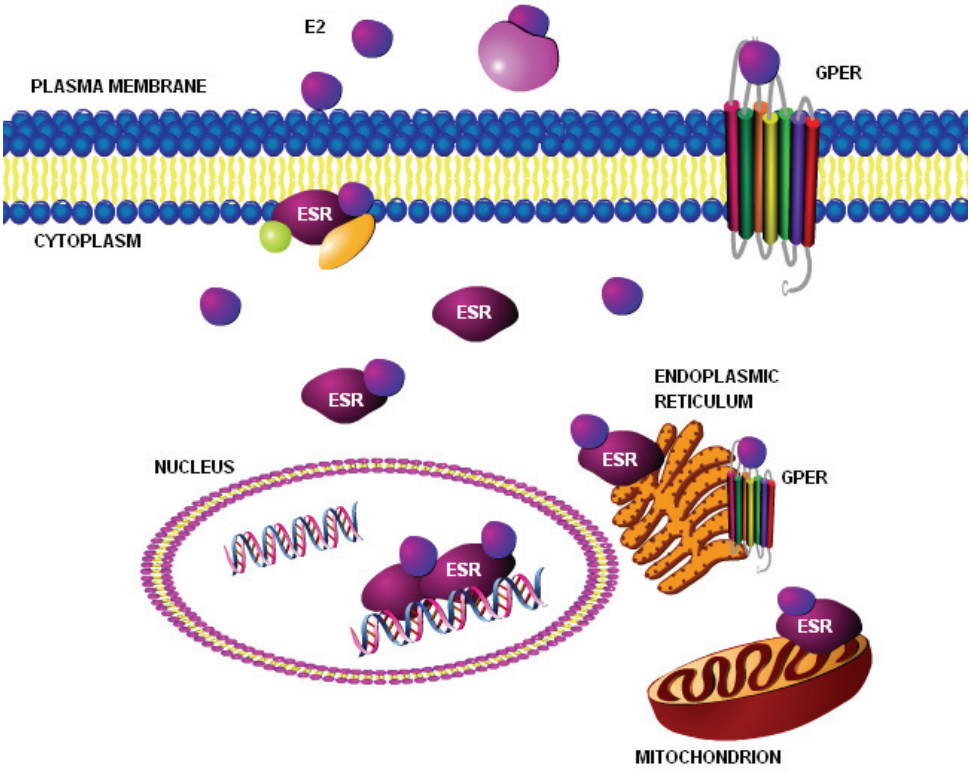

FIGURE 3 Possible subcellular locations of estrogen receptors. Modified according to Govind and Thampan (2003), Du et al. (2006), and Boland et al. (2008) and generated utilizing the Pathway Builder of ProteinLounge (www.proteinlounge.com).

Both ESR1 and ESR2 have been identified in human skeletal muscle (Lemoine et al. 2003, Wiik et al. 2003, Wiik et al. 2005, Wiik et al. 2009). Very recently, mRNA expression of GPER was also detected in murine skeletal muscle (Isensee et al. 2009, Baltgalvis et al. 2010), but information regarding human muscles continues to be lacking. In mouse, muscle ESR1, and to a lesser extent also ESR2 and GPER, show responsiveness to systemic E_2-levels (Baltgalvis et al. 2010). However as discussed above (see 2.1.4) the randomized trials on the effects of estrogen-containing HRT on muscle mass and strength have shown conflicting results and molecular level data on humans has not yet been obtained. On the other hand, the studies with rodent models are in line with human studies showing that specific force declines around the age at which ovaries fail in mice (Moran et al. 2006). Ovariectomized animals have been shown to be 10% to 20% weaker compared to estrogen-replaced and intact mice,

although not all studies have reported the same findings (Lowe et al. 2010). In light of the literature to date, it seems likely that menopause-related hormonal changes may be associated with worsening muscle properties. However, more research is needed to clarify the precise molecular mechanisms involved.

2.2.2 Estradiol as a paracrine factor

In addition to gonadal synthesis, steroids may be locally produced in numerous extragonadal sites, including the mesenchymal cells of adipose tissue, the osteoblasts and chondrocytes of bone, the vascular endothelium and aortic smooth muscle cells as well as numerous sites in the brain (Simpson 2003). Recently human and rodent skeletal muscle cells have been included in the growing list of steroidogenetic cells (Larionov et al. 2003, Aizawa et al. 2007). Particularly in women after menopause, the importance of paracrine actions and synthesis of steroid hormones, especially estrogens, is emphasized. The peripheral synthesis of estrogen is dependent on the external source of the precursor hormones, since extragonadal sites cannot initiate the synthesis route from cholesterol such as gonadal sites are able to do (Figure 2, Labrie et al. 1997, Labrie et al. 1998). The major prehormone for E_2 synthesis is circulating T, which can be converted to DHT or to E_2 in the target tissues (Figure 2). In postmenopausal women about 25% of the circulating T is secreted by the ovaries while the rest is formed in adrenal gland or in peripheral tissues to be locally used or to be released into the circulation (Simpson 2003). The main precursors for local T synthesis are DHEAS, DHEA and androstenedione. In postmenopausal women, all these precursors are of a higher order of magnitude than the circulating estrogens, although the levels are lower than in premenopausal women.

As stated previously, E_2 biosynthesis is catalyzed, in the last step, by the aromatase-enzyme encoded by *CYP19A1 cytochrome P450* gene (called aromatase throughout this thesis, Figure 2). The *aromatase* gene has several promoter regions enabling tissue-specific regulation. Thus its expression is regulated by FSH in the ovary, while glucocorticoids, some cytokines and TNFα control its expression in adipose tissue and bone (Simpson et al. 1997). As early as 1986, Matsumine and co-authors showed aromatase activity in skeletal muscle samples from men and postmenopausal women (Matsumine et al. 1986). Furthermore, Larionov et al. (2003) have shown that *aromatase*-gene is expressed in human skeletal muscle in similar manner as in adipose tissue, although its mRNA expression and enzymatic activity is relatively low. In addition, HSD3B, HSD17B and aromatase has been detected from murine skeletal muscle and shown to be activated upon acute exercise (Aizawa et al. 2007, Aizawa et al. 2008, Aizawa et al. 2010). Furthermore, resistance exercise has been shown not to induce differences in steroidogenesis between young men and women (Vingren et al. 2008). However, no previous studies have been reported in which the expression of steroidogenetic enzymes or the amount of steroid

hormones in muscle tissue has been studied in relation to the menopausal status of women.

2.2.3 Systemic peptide hormones

Peptide hormones are small proteins with regulatory functions. They include insulin, IGF-1, IGF-2 and GH. Insulin is pancreatic hormone, which regulates glucose, fat and protein homeostasis. In skeletal muscle it acts as an anabolic, energy-sensing hormone, which inhibits muscle protein breakdown and promotes synthesis, with some impairments occurring during aging (Boirie et al. 2001). Furthermore, insulin sensitivity seems to decrease after menopause and may be related to loss of muscle mass in women (Maltais et al. 2009). Insulin actions are linked to IGF-1, since it can bind to the IGF-1 receptor, which may explain some of the growth promoting effects of insulin (Kjeldsen et al. 1991). GH is secreted mainly by the pituitary gland and regulates several physiological processes ranging from somatic growth and development to carbohydrate, lipid and protein metabolism (Davidson 1987, Perrini et al. 2008a, Perrini et al. 2008b, Moller et al. 2009). It may act directly through specific GH receptors or indirectly by regulating the systemic or local production of IGF-1 (Hayes et al. 2001, Brill et al. 2002). GH levels decline progressively during aging (Hermann & Berger 2001) and postmenopausal decrease in circulating GH has been associated with changes in body composition (Leung et al. 2004). The anabolic effect of GH on skeletal muscle is modest and may come through activating IGF-1 (Sheffield-Moore & Urban 2004). IGF-2 is quite homologous to IGF-1 but its function in skeletal muscle is less well known. It regulates MYOD1-stimulated myocyte maturation, and therefore it is essential in muscle development and differentiation (Wilson et al. 2003, Wilson & Rotwein 2006). IGF-2 is also involved in the regulation of angiogenesis and its inappropriate expression has been associated with a growing number of diseases (Chao & D'Amore 2008).

The most potent anabolic peptide hormone IGF-1 is a single-chain peptide with three α-helices and three disulfide bonds. Despite the sequence similarity with IGF-2, both peptides have potentially divergent roles in human physiology (Rosenfeld & Hwa 2009). IGF-1 is major determinant of somatic growth. Mice null to IGF-1 expression have reduced birth weight, high mortality rate and less than one-third of the normal body size in adulthood (Baker et al. 1993, Powell-Braxton et al. 1993), while transgenic mice overexpressing IGF-1 in skeletal muscle have pronounced muscle hyperthrophy (Musaro et al. 2001). According to differentiated human muscle cell cultures, which contain myotubes and reserve cells, IGF-1 increases the size of the myotubes and recruitment of the reserve cells for fusion (Jacquemin et al. 2004, Jacquemin et al. 2007). Systemic IGF-1 is primarily synthesized in a GH-dependent manner in the liver (Gosteli-Peter et al. 1994), but IGF-1 can also be locally synthesized in muscle tissue as a response to GH (Hayes et al. 2001, Brill et al. 2002), androgens (Brill et al. 2002, Ferrando et al. 2002) and mechanical load (Yang et al. 1996, Bamman et al. 2001).

The amount of IGF-1 in circulation has been shown to decrease with menopause (Pfeilschifter et al. 1996). However, one-year of IGF-1 treatment did not improve body composition or increase lean body mass in postmenopausal women (Friedlander et al. 2001). Another RCT, on obese postmenopausal women, showed that combined GH and IGF-1 administration increased fat-free mass and reduced fat mass (Thompson et al. 1995). The effects of IGF-1 on skeletal muscle are mainly mediated by the IGF-1 receptor, which upon activation induces a phosphorylation cascade, changing the mode of action in muscle fibers and satellite cells (for more details see paragraph 2.3). IGF-1 can also bind to the insulin receptor, but the affinity is much lower than for the IGF-1 receptor (Pandini et al. 2002).

2.2.4 IGF-1 as a paracrine factor

In experimental animals, direct infusion of IGF-1 has been shown to increase the mass of the corresponding muscle as well as to block aging-related loss of muscle function (Adams & McCue 1998, Barton-Davis et al. 1998). Furthermore, transgenic mice with muscle-specific IGF-1 overexpression had significant myofiber hypertrophy without changes in circulating levels of IGF-1 (Coleman et al. 1995, Musaro et al. 2001). In contrast, transgenic mice ubiquitously overexpressing IGF-1 have increased serum concentration of IGF-1, but only a modest increment in muscle mass (Mathews et al. 1988). According to these results paracrine rather than systemic endocrine effects of IGF-1 are important for muscle hypertrophy.

The *IGF-1* gene has at least three splice variants with a common mature peptide fused to different C-terminal E-peptides (Figure 4): *IGF-1Ea*, which form its E-peptide from part of the fourth and sixth exon, is synthesized both by the liver and muscle and hence is known as systemic IGF-1. Apparently primate-specific *IGF-1Eb*, which has only the fifth exon in the C-terminus, has been shown to regulate cell growth of bronchial epithelial and neuroblastoma cells (Siegfried et al. 1992, Kuo & Chen 2002, Wallis 2009). *IGF-1Ec* has 49 bases from the fifth exon inserted between the fourth and sixth exon; this causes a reading frame shift, which in turn leads to a unique E-peptide. IGF-1Ec (named IGF-1Eb in rodents), has been called a mechano growth factor (MGF) or muscle IGF-1 due to its responsiveness to mechanical contraction (Yang et al. 1996). Based on the results obtained in an RCT studying the effects of GH, resistance training, or their combination, on the response time and rate of the mRNA expression of IGF-1 splice variants, the authors suggested that the IGF-1Ea may be required for the normal maintenance of muscle mass, but that after training-induced injury, the IGF-1Ec variant is required for satellite cell activation (Hameed et al. 2004). Previous data from the same authors also support this hypothesis and further suggests that mature peptides, which are common to all splice variants, may act through IGF-1 receptors to promote cell differentiation and growth, while splice variant-specific E-peptides may have their own biological functions (Yang & Goldspink 2002). It has also been suggested that

IGF-1Ec/MGF, may control muscle tissue repair, maintenance and remodeling through a distinct, possibly intracellular receptor, which has not yet been identified (Goldspink 1999), while IGF-1Ea is suggested to be more directly involved in regulation of muscle protein synthesis and degradation (Velloso 2008).

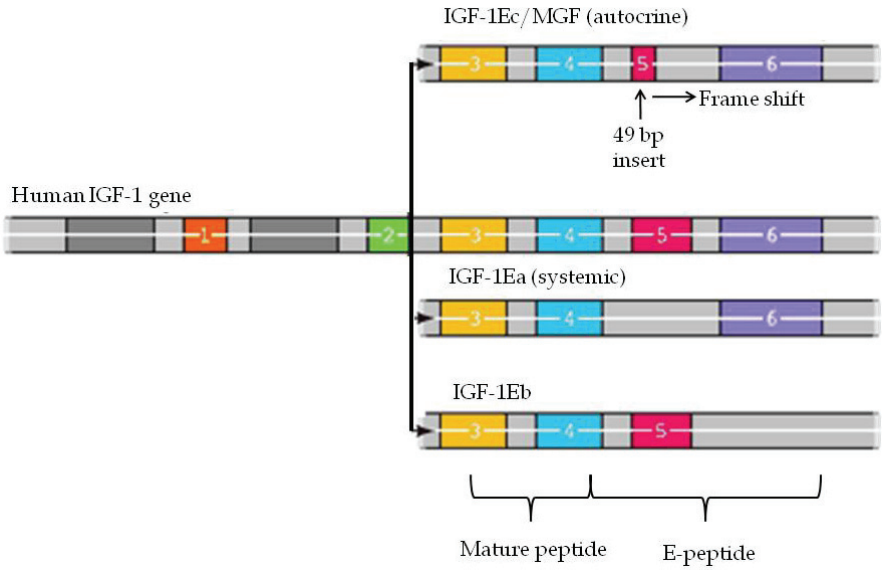

FIGURE 4 Splice variants of the IGF-1 gene. Colored areas represent the exon regions of the IGF-1 gene which remain in each splice variant after removal of the intron regions (grey). The figure was adapted and modified from the review by Prof. Geoffrey Goldspink (Goldspink 2005).

2.3 IGF-1- and estrogen-related signaling as the potential pathways for the maintenance of muscle mass during aging

2.3.1 Overview of the IGF-1/AKT signaling pathway

There are many overlapping and interacting signaling cascades involved in the regulation of muscle properties (Figure 5). One of the most prominent signaling cascades regulating muscle mass is the PI3K/AKT pathway because it activates protein synthesis and inhibits degradation thereby controlling the net balance of muscle protein turnover. The PI3K/AKT pathway can be activated by mechanical stimulus due to muscle contraction as well as by endo- or paracrine stimulus due to insulin or IGF-1 (Bodine et al. 2001, Rommel et al. 2001, Bolster et al. 2004).

A central factor on the pathway is serine-threonine kinase AKT (also known as PKB), which has three isoforms (AKT1, AKT2, AKT3) encoded by a different genes. AKT1 is important for growth regulation, whereas AKT2 is more important in glucose metabolism (Cho et al. 2001a, Cho et al. 2001b). The function of AKT3 is currently unknown. AKT1 and AKT2 are expressed in skeletal muscle, thymus, brain, heart and lung, while AKT3 is most abundant in brain and testes (Nader 2005). Another key factor on the PI3K/AKT pathway is the mTOR, which is known to be activated by AKT (Nave et al. 1999). mTOR can form protein complexes including the raptor (mTORC1) or rictor (mTORC2) proteins. mTORC1 is involved in regulating protein synthesis through translational regulators such as ribosomal protein kinase S6K1 and translation initiation factor binding protein 4E-BP1 (von Manteuffel et al. 1997, Saitoh et al. 2002). On the other hand, mTORC2 phosphorylates AKT and is involved in controlling the actin cytoskeleton (Jacinto et al. 2004, Sarbassov et al. 2004, Sarbassov et al. 2005). In addition to mTOR, AKT is known to phosphorylate and, in this case inactivate, the GSK3, a repressor of protein synthesis (Rommel et al. 2001).

AKT can also phosphorylate and thereby inactivate members of the FOXO transcription factors (Rena et al. 1999). FOXO1 and FOXO3 are regulators of protein degradation because they activate the gene expression of two ubiquitin protein ligases: atrogin-1 (also known as MAFbx and FBXO32) and MuRF-1 (Sandri et al. 2004, Stitt et al. 2004). Recently FOXO3 has also been shown to control autophagy, the degradation of intracellular proteins or organelles through the lysosomal machinery in order to maintain cellular homeostasis (Mammucari et al. 2007).

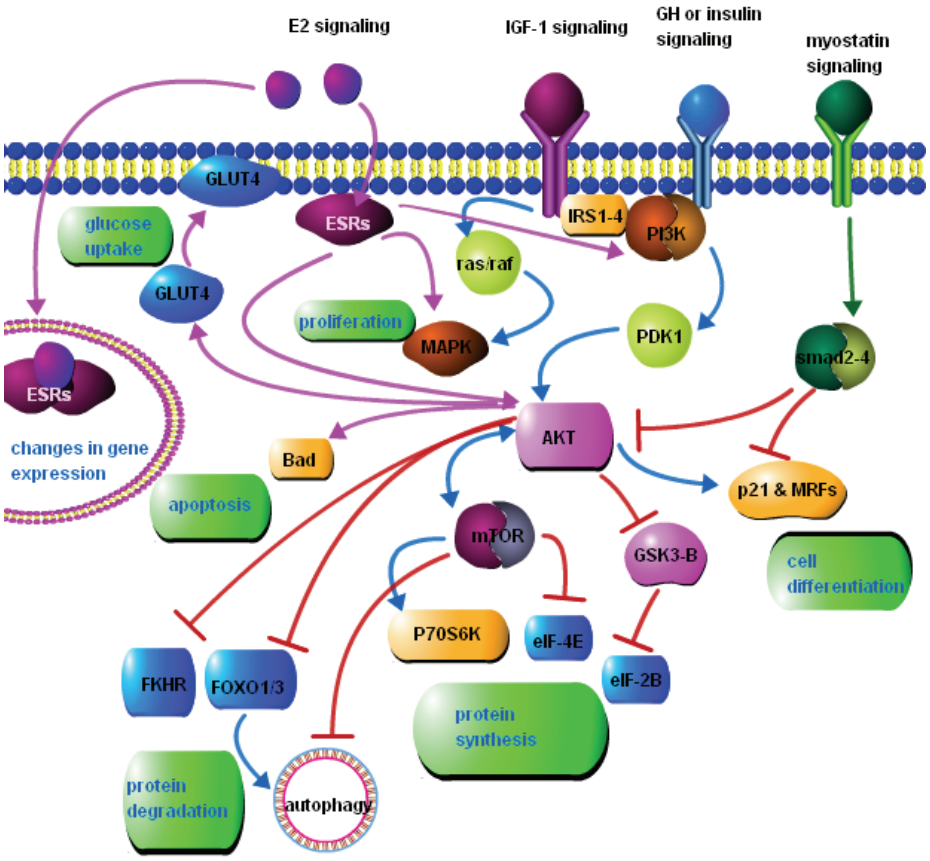

FIGURE 5 A simplified illustration of signaling pathways regulating muscle properties. Schematic figure is generated with the Pathway Builder of ProteinLounge (www.proteinlounge.com). This figure is a combined simplification of the signaling cascades presented in: Nader (2005), Jacquemin et al.(2007), Boland et al.(2008), Velloso (2008), Clemmons (2009), Ding et al. (2009), Galluzzo et al. (2009), Saini et al. (2009), Otto & Patel (2010), Perrini et al. (2010), Ronda et al. (2010), Schiaffino & Mammucari (2011).

2.3.2 Potential cross-talk between estrogen and IGF-1 signaling

Although the physiological effects of E_2, especially in the form of postmenopausal HRT, on aging human skeletal muscle have been studied in several RCTs (reviewed in chapter 2.1.4) only a limited amount of molecular level data are available on the effects of E_2 on human muscles. However, a relatively high amount of data has been gathered from other study settings, such as cell culture experiments, and in animal studies.

For example, in the kidney fibroblast cell line GPER has been shown to mediate rapid cell signaling events, including activation of the PI3K/AKT

cascade (Revankar et al. 2005). ESR1 and ESR2 may also have non-transcriptional effects involving PI3K/AKT signaling, as shown in endometrial cancer cells (Guo et al. 2006), and MAPK-related signaling, as shown in chondrocytes (McMillan et al. 2006). The MAPK pathways shown to be activated by E_2 in murine C2C12 myoblast cell culture are the ERK2 and p38 MAPK pathways (Ronda et al. 2010), and they may interact with the IGF-1 pathway through the Ras/Raf cascade. However, p38 was not activated by IGF-1 in human myotube culture (Jacquemin et al. 2007). Activation of the PI3K/AKT pathway may be ESR-dependent or –independent and was recently shown to occur at least in undifferentiated C2C12 cells in an ESR-dependent manner (Vasconsuelo et al. 2008). E_2-mediated signaling via the MAPK or PI3K/AKT axis has also been shown to increase eNOS activity in endothelial cells (Chen et al. 1999, Haynes et al. 2000, Simoncini et al. 2000). Furthermore, ESR1 was shown to directly interact with the PI3K-complex in endothelial cells (Simoncini et al. 2000). E_2- and IGF-1-signaling has also been shown to interact in breast cancer cells (Yee & Lee 2000, Oesterreich et al. 2001, Zhang et al. 2005) as well as in brain tissue (Mendez et al. 2003, Mendez et al. 2006). It remains to be seen whether E_2 and IGF-1 interactions also takes place in intact human skeletal muscle, as indicated by E_2-stimulated mRNA expression of *IGF-1* in bovine satellite cell cultures (Kamanga-Sollo et al. 2004, Kamanga-Sollo et al. 2008a). Furthermore, there is no previous knowledge of the possible effects of aging in E_2/IGF-1-related interaction in human muscle. In addition, there is strong line of evidence that E_2/ESR signaling also interacts with insulin signaling by involving partially the same components as are involved in the IGF-1 signaling pathway, further elucidating the complexity of the signaling events involved in the regulation of muscle properties (Foryst-Ludwig & Kintscher 2010).

Since no gene expression level data exist on the effects of E_2 or HRT on human skeletal muscle, the bulk of the current knowledge on the effects of E_2 on skeletal muscle has been gained through animal or cell culture studies. ESR1, ESR2 and GPER knockout mice have been quite uninformative regarding skeletal muscle structure or performance-related phenotypes. ESR1 and ESR2 knockouts survive to adulthood, exhibit defects in reproductive track and, especially in the case of ESR1 knockout mice, have increased body weight, possibly due to excess adipose tissue, and develop insulin resistance and impaired glucose tolerance (Couse & Korach 1999, Curtis Hewitt et al. 2000, Korach et al. 2003). The GPER knockout mice, on the other hand, show hardly any changes in phenotype (Levin 2009), indicating redundancy in the functions of GPER. Only one study has concentrated on the effects of ESR1, ESR2 or aromatase knockout on lower limb muscle mass and contractile function in female mice (Brown et al. 2009). The authors concluded that lack of E_2 or functional ESR1 has detrimental effects on the contractile quality of skeletal muscle, although the effect is not seen in all muscle groups. The statement is based on following observations: increased total body mass in ESR1 and

aromatase knockouts, increased *tibialis anterior* muscle mass in ESR1 knockouts and decreased peak tetanic tension per anatomical fiber CSA in *gastrocnemius* and *tibialis anterior* muscles of ESR1 and aromatase knockouts while no effects were observed in the *soleus* and *plantaris* muscles in the ESR1 and aromatase knockouts and none were observed in any of the studied muscle groups in the ESR2 knockouts. At the serum hormone level, ESR1 knockouts have increased serum E_2, T, LH and decreased serum IGF-1 levels, aromatase knockouts have elevated levels of T, LH, FSH and IGF-1 (in females) and no detectable E_2 while in the ESR2 knockout the serum levels of E_2, T, LH, FSH and IGF-1 are within the normal range (Vidal et al. 1999, Windahl et al. 2001, Öz et al. 2001, Korach et al. 2003), which may also affect the observed results.

Studies on ovariectomized rodents have proven to be useful in understanding the molecular effects of low circulating E_2 and its replacement on skeletal muscle. The animal studies conducted have shown that E_2 may reduce muscle inflammation and damage (Bär et al. 1988, Enns et al. 2008, Iqbal et al. 2008, Baltgalvis et al. 2010), modulate muscle fat and carbohydrate metabolism (Ahmed-Sorour & Bailey 1981, Puah & Bailey 1985, Kendrick et al. 1987, Campbell & Febbraio 2001, Beckett et al. 2002, Campbell et al. 2003, Kamei et al. 2005, Alonso et al. 2007), improve insulin sensitivity (Sugaya et al. 1999, Sugaya et al. 2000, Gonzalez et al. 2001, Campbell & Febbraio 2002, Gonzalez et al. 2002, Gonzalez et al. 2002, Park et al. 2004, Ordonez et al. 2007, Saengsirisuwan et al. 2009), change muscle mass or contractile properties (Fisher et al. 1998, McCormick et al. 2004, Schneider et al. 2004, Moran et al. 2006, Moran et al. 2007, Greising et al. 2009, Hou et al. 2010, Lowe et al. 2010, Greising et al. 2011), affect fiber CSA and fiber type distribution (Kobori & Yamamuro 1989, Kadi et al. 2002, Piccone et al. 2005, McClung et al. 2006, Liu et al. 2009, Velders et al. 2010) and increase myoblast proliferation (Thomas et al. 2010). Some of these effects may be delivered through IGF-1-, PI3K/AKT-, MAPK-, AMPK-mediated pathways (Ren et al. 2003, Sitnick et al. 2006, Tsai et al. 2007, Deng et al. 2008, Hatae et al. 2009, Wohlers et al. 2009, Moreno et al. 2010). However, it is unclear if the results obtained in animal studies are applicable in humans and, in particular in postmenopausal women.

2.4 DNA microarrays as a method for studying regulation of skeletal muscle properties during aging

High-density DNA microarrays have grown in popularity as they were long the only method providing a truly comprehensive view of genome-wide gene activities in a given sample. Nowadays commercial microarrays contain probes for tens of thousands of sequences, enabling characterization of most of the transcribed genes, i.e., transcriptome, present in a sample. Therefore microarrays are feasible vehicles for obtaining a snapshot of genome-wide

responses to, e.g., different physiological stimuli or to aging, and therefore interpreting the cellular events leading to physiological adaptation in studied tissue. This is based on the central dogma regarding gene expression: a gene encodes an mRNA, which in turn encodes a protein to produce the actions required in order to change the status of the cell. Of course, the whole picture is more complicated, involving a number of regulatory steps affecting the ultimate outcome. Therefore the results obtained from gene expression arrays do not always reflect the protein level events taking place in the cell. This problem may be more adequately addressed by using the recently developed next generation sequencing methods, which have an advantage over microarrays to enable simultaneous quantitative measurement of gene expression, discovery of novel transcribed regions and detection of alternative splice sites (Costa et al. 2010). It should be noted, however, that regardless of the method used to study the transcriptome, the tissue samples used to isolate RNA usually contain more than one cell type, which also affects the results. There also are several other issues which are needed to be considered before conducting a successful microarray study. These include proper study design, sampling, high quality RNA as well as adequate use of bioinformatic and statistic tools for the data preprosessing, normalization and mining of differentially expressed genes or gene groups (Bassett et al. 1999, Bowtell 1999, Brown & Botstein 1999, Quackenbush 2001, Holloway et al. 2002). Nevertheless, DNA microarrays have been proven to be powerful tools for gene expression profiling in wide array of tissues, including skeletal muscle (Timmons & Sundberg 2006).

2.4.1 Microarray studies related to aging

Several cross-sectional studies have utilized microarray technology to investigate the transcriptional changes associated with aging in human skeletal muscle (Welle et al. 2003, Welle et al. 2004, Giresi et al. 2005, Zahn et al. 2006). Welle et al. (2004) compared the muscle gene expression profiles of seven women aged 20-29 and eight aged 65-71 years and found 1178 probe sets to be differentially expressed with a false discovery rate (FDR) limited to 10%. These probes included several genes related to DNA damage, fiber regeneration and binding to pre-mRNA or mRNA. The older women had reduced muscle mass, strength and peak oxygen consumption compared to the younger women, but the authors did not provide direct evidence of how differences at the gene expression level would relate to the differences in phenotypes. Furthermore, the young women were in various stages of the menstrual cycle and four of them were taking oral contraceptives. Three of the older women were using HRT and one was taking raloxifene, which can act as an agonist or antagonist of ESRs. Therefore it is unclear whether the hormonal status of the participants affected the results. The same authors had previously conducted a similar experiment with eight men aged 21-27 and eight aged 67-75 years and found that the expression of several genes, involved in stress responses, hormone, cytokine or

growth factor signaling, control of the cell cycle and apoptosis as well as transcriptional regulation, to be affected by aging (Welle et al. 2003). As in the older women, the older men also had lower muscle mass and peak oxygen consumption, but not lower muscle strength, than the younger men. The differences obtained at the phenotype level may be due to differences at the gene expression level, although no direct evidence supporting this conclusion was presented. In both studies the observed age-related differences in the gene expression were quite modest, being less than 1.5-fold in magnitude for most of the genes, but were observed for quite a large number of genes. This might reflect the process of adaptation to slowly accumulating aging-related changes and increasingly sedentary lifestyle of older people.

Giresi et al. (2005) used muscle samples from ten men aged 19-25 and twelve aged 70-80 years to identify the aging signature, which for some reason was named as molecular signature of sarcopenia, despite the fact that sarcopenia was not assessed among the participants. At the phenotype level the older men had lower peak oxygen consumption, muscle strength and muscle power than the younger men. The identified molecular signature comprised 45 genes. This signature was able to classify 75% of the male samples used by Welle et al. (2003) as young or old solely based on the gene expression profiles.

Another type of approach to study gene expression associated with aging in human muscle was taken by Zahn et al. (2006). They analyzed the gene expression of 81 normal muscle samples obtained during surgery or other medical procedure independent of the condition of the muscle from which the sample was obtained. The patients participating in the study ranged in age from 16 to 89 years of age and included both men and women. Muscle samples were obtained from different locations including abdomen, arm, deltoid and thigh. Applying FDR of ≤13%, the authors found 250 genes to be age-associated. The possibility of the confounding effects of sex, anatomical origin of the sample or type of pathology associated with the patient or medication used was tested and found unlikely to have affected the results. The authors concluded that the overall difference which they found in the muscle gene expression between young and old persons was relatively small, indicating that age-related decline in cellular functions is caused by the accumulation of small changes in the regulation of gene expression. The aging signature obtained from muscle tissue was compared to previous results from the kidney and brain in order to obtain a common cellular signature for aging which would hold true in all three tissues. The common aging signature consisted of six pathways of which extracellular matrix, cell growth, complement activation and components of the cytosolic ribosome were upregulated, while genes involved in chloride transport and mitochondrial electron transport chain were downregulated with age. The authors also compared this common human aging profile to those of the mouse and fly and found that the electron transport chain pathway behaves similarly in all three organisms, suggesting that it may be a marker for aging across tissues and species.

2.4.2 Microarray studies related to physical training or exercise

The effects of plyometric power training or similar training type on the global gene expression of skeletal muscle had not been studied prior to the commencement of this thesis. However, several studies had investigated the role of single exercise bouts (Chen et al. 2003, Zambon et al. 2003), strength training (Roth et al. 2002) or endurance training (Wittwer et al. 2004, Mahoney et al. 2005, Teran-Garcia et al. 2005, Timmons et al. 2005) on the regulation of global gene expression in human skeletal muscle. Most of these studies had been conducted on fairly young men (Chen et al. 2003, Zambon et al. 2003, Wittwer et al. 2004, Mahoney et al. 2005, Timmons et al. 2005) or by pooling samples from men and women (Roth et al. 2002, Teran-Garcia et al. 2005). Furthermore, very different designs with different exercise modes had been used, including case-control studies comparing samples from the exercised and non-exercised legs (Chen et al. 2003, Zambon et al. 2003), untrained and professional athletes (Wittwer et al. 2004), before and after single exercise bouts (Mahoney et al. 2005) or high and low-responders to training (Teran-Garcia et al. 2005, Timmons et al. 2005). Therefore it is hard to draw general conclusions about the general effects of training on muscle gene expression, especially whether similar effects than found in other groups would also be found in muscles of older women. Nevertheless, differential expression of genes related to energy metabolism, mitochondrial functions, transcription and protein post-translational modification was reported in most studies, although the genes in question may differ from study to study.

One of the earliest studies to apply a microarray approach to study of the effects of training on human skeletal muscle was conducted by Roth et al. (2002), who used the earlier form of microarrays, i.e., filter microarrays containing probes for ~4000 genes, to study the influence of age, gender and strength training on muscle gene expression. They studied the influence of each variable on the transcriptome of skeletal muscle separately, finding over 200 differentially expressed genes between the male and female study participants. From these genes, 54 were identified as differentially expressed according to the age group of the participants and 69 according to the training status. These results demonstrate that gender, age and training status are all very important factors influencing the rate of gene expression in skeletal muscle, and therefore one should be very careful when generalizing across participants differing in these matters.

More microarray studies related to physical exercise or training have since been conducted in order to study how endurance training modulates the muscular transcriptome response to acute exercise (Schmutz et al. 2006), muscular transcriptome responses to mild eccentric ergometer exercise (Klossner et al. 2007) and muscular transcriptome response during recovery from eccentric exercise (Mahoney et al. 2008), and to analyze muscle gene expression in well-trained strength and endurance athletes (Stepto et al. 2009). Also these studies used samples obtained from fairly young males within the

age range from 22 to 38 years. The only new study, including females, examined whether resistance exercise could reverse the age-related differences in muscle gene expression by first determining the genes significantly differently expressed in samples from young and older participants and then analyzing whether resistance training performed by older participants had any effect on aging-related genes (Melov et al. 2007). The authors concluded from the results that healthy older adults show mitochondrial impairment and muscle weakness, which can be partially reversed by resistance training. Samples from men and women were studied together without dissecting possible gender-related differences.

2.4.3 Microarray studies related to functions of sex steroid hormones

Prior to the commencement of this thesis no microarray studies have yet been published on the effects of estrogens or androgens on human skeletal muscle. The only related study published outside of this thesis concerns the global gene expression profiles in skeletal muscle of postmenopausal female twins discordant for HRT, and was conducted in our laboratory (Ronkainen et al. 2010). According to the major findings, long-term use of HRT was associated with subtle, but significant differences in muscle transcript profiles, including regulation of cell structure, cell-matrix interactions, energy metabolism and utilization of nutrients. Not much more is known about the transcriptome-wide effects of T or DHT on skeletal muscle. So far the only human study has compared the global muscle gene expression of T- and placebo-treated men infected with immunodeficiency virus. The study found upregulation in the genes involved in myogenesis, muscle protein synthesis, immune regulation, metabolic pathways and chromatin remodeling (Montano et al. 2007). Yoshioka et al. performed two experiments utilizing the serial analysis of gene expression method to study the effects of castration and DHT in male mice and the effects of ovariectomy and DHT in female mice (Yoshioka et al. 2006, Yoshioka et al. 2007). The authors suggested that DHT promotes protein synthesis, cell signaling, cell proliferation, ATP production and muscle contraction and relaxation at the transcriptional level in male mice, while in female mice the transcripts of fast/oxidative fiber, oxidative phosphorylation and ATP production were repressed after DHT treatment, indicating gender differences in the effects of DHT on skeletal muscle.

The sexual dimorphism of skeletal muscle, which may be related to differences in steroid hormones, has been examined in only one study in which the skeletal muscle gene expression profiles of 15 men and 15 women (age range 20-75 yr) were compared (Welle et al. 2008). According to the findings, men have higher expression of genes encoding mitochondrial proteins, ribosomal proteins and some translation initiation factors while women have higher expression of two genes encoding important factors known to be involved in regulating muscle mass: *GRB10*, an inhibitor of IGF-1 signaling and *ACVR2B*, a myostatin receptor. These findings may be directly or indirectly

influenced by differences in sex steroid hormone-mediated regulation of skeletal muscle properties between men and women. However, it should be noted that other factors than gender-related differences in steroid hormones may also influence the results.

3 PURPOSE OF THE STUDY

The purpose of this study was to assess whether the use of estrogen-containing HRT or plyometric power training has an effect on the skeletal muscle properties of postmenopausal women by affecting transcriptome of the muscle. In addition, the endocrine and paracrine roles of steroid hormones in the regulation of muscle properties in pre- and postmenopausal women were investigated.

The specific aims of this thesis were:

1. To examine the transcriptome-wide and signaling cascade specific alterations in skeletal muscle gene expression during the early stage of postmenopause with and without HRT. (I, II)

2. To examine the specific and shared effects of physical training and HRT on the skeletal muscle transcriptome of early postmenopausal women. (III)

3. To determine whether sex steroid content and local steroidogenesis of skeletal muscle differs between pre- and postmenopausal women and to further examine the potential association of circulating and local steroid concentrations with muscle quality. (IV)

4 PARTICIPANTS, STUDY DESIGNS AND METHODS

4.1 Study designs and participants

The analyses included in this thesis were based on two separate study samples: an exercise and hormone replacement therapy intervention (Ex/HRT study) and a cross-sectional study on post- and premenopausal women (Post/Premenop. study). Detailed descriptions of the recruitment and design of both studies are presented in Figure 6 and in the following sections. The studies utilized in each original publication are summarized in Table 1.

THE EXERCISE AND HORMONE REPLACEMENT THERAPY STUDY (I, II, III). The Ex/HRT study (Sipilä et al. 2001) is a randomized, placebo-controlled 12-month trial (RCT) on bone and muscle structure and function in relation to exercise and hormone replacement therapy (Figure 6A, Current controlled trials registration number ISRCTN49902272). Population register of the city of Jyväskylä was used to take a random sample of 1333 women aged 50-57 years. Of the women contacted, 912 returned the questionnaire. At this point 794 were excluded due to refusing to participate or failing to meet the inclusion criteria (no serious medical conditions; no current or previous use of medication including estrogen, fluoride, calcitonin, biophosphonates or steroids; last menstruation at least six months but no more than 5 years ago; FSH level above 30 IU/l; and no contraindications for exercise and HRT). The eligible 118 women were invited to a clinical examination in where menopausal status was assured by serum steroid measurements (DELFIA, Wallac, Turku, Finland). Finally, 80 women fulfilling the inclusion criteria, i.e., women at the very early stage following menopause, were randomly assigned to one of four study groups: HRT (n=20), power training (PT, n=20), PT+HRT (n=20), and control (CO, n=20). Randomization was carried out manually by drawing lots.

The HRT intervention was carried out double blinded. All the study participants used either a continuous, combined HRT preparation containing estradiol (2 mg) and norethisterone acetate (1 mg) preparation (Kliogest, Novo Nordisk, Copenhagen, Denmark) or placebo (composed of lactose monohydrate, cornstarch, gelatin, talc, and magnesium stearate, which were auxiliary substances in the Kliogest tablet), one tablet every day. The HRT preparation used in the study does not induce menstrual flow and therefore it does not compromise the blinding of the participants. The PT participants underwent a progressive plyometric training program comprising two supervised sessions and four home-based unsupervised sessions per week. The training was performed in a circuit format and included bounding, drop jumping, hopping, and skipping performed at high velocity in order to improve muscle power production and to produce high-impact loading for bones. The training program progressed in the number of rotations performed, volume of work undertaken as well as height of obstacles for bounding and height for drop jumping. Each supervised session included three to four resistance training exercises for the upper body and commenced with a warm-up period and concluded with a cool-down period of stretching activities. The home exercise program was also performed in a circuit format including three rotations of skipping, hopping and drop jumping. In addition, exercises to strengthen the abdominal and lower back regions were included. HRT and CO subjects were advised to maintain their daily routines without altering their physical activity patterns.

In order to investigate the changes in muscle transcriptome in postmenopausal women using or not using HRT, the study population comprised the participants in the HRT and CO groups for whom both baseline and follow-up muscle samples were available (Table 1, Figures 6A and 7, I). To investigate the possible effects of HRT on the potential signaling cascade involved in the regulation of muscle mass, the same study participants with addition of four persons from the CO group who gave their consent later comprised the study population (Table 1, II). To further study the specific and shared effects of physical training and HRT on the muscle transcriptome, we also included the PT group (Table 1, Figure 8, III). Unfortunately, we had too few samples from the PT+HRT group to be able to include this group in the analyses.

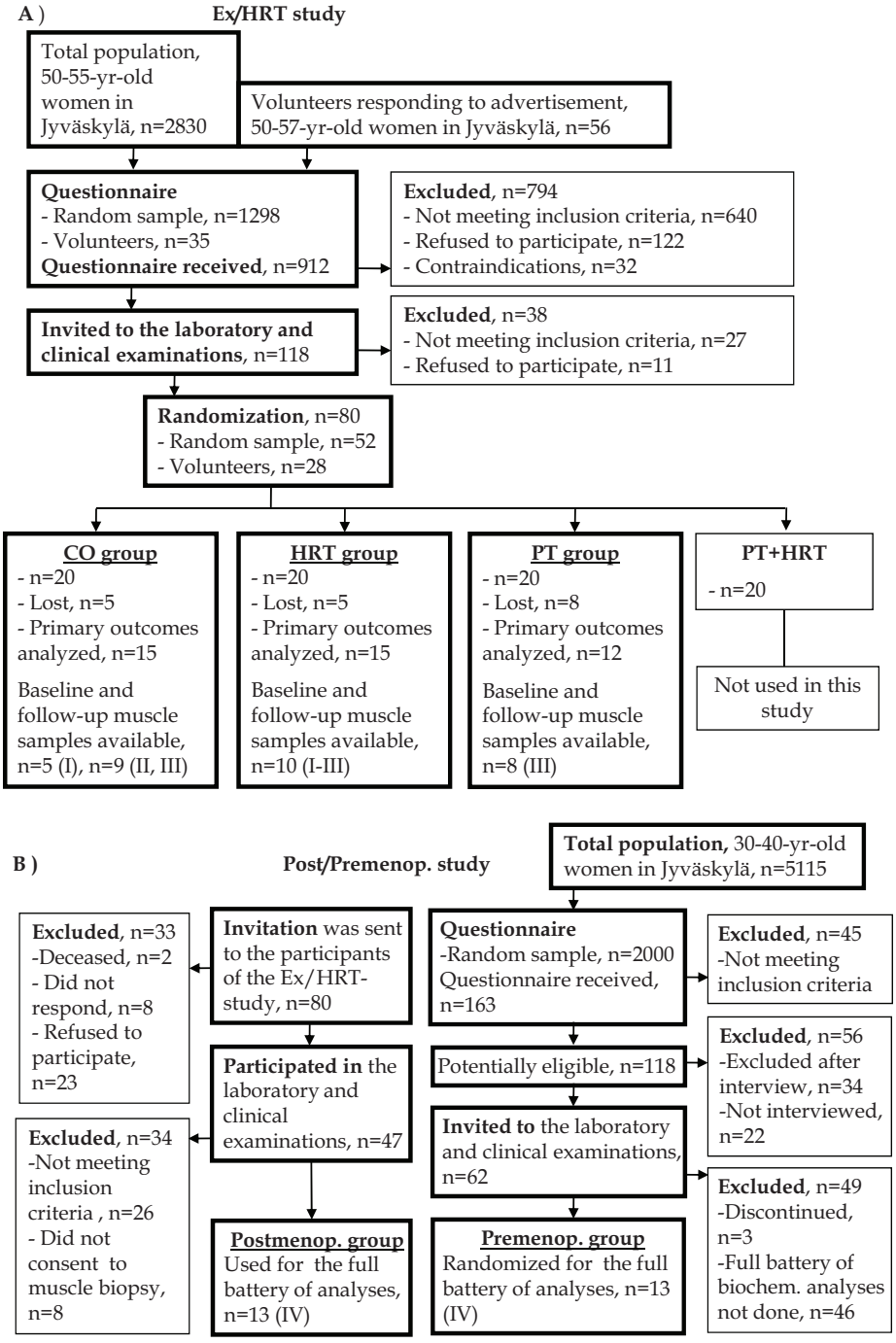

FIGURE 6 Recruitment protocol of the Ex/HRT study (A) and the Post/Premenop. study (B).

POST- AND PREMENOPAUSAL WOMEN'S STUDY (IV). In order to study possible differences in sex steroid content and the local steroidogenesis in the skeletal muscle of post- and premenopausal women we conducted a cross-sectional Post/Premenop. study (Figure 6B, IV).

All 80 women who participated in the baseline measurements of the original Ex/HRT trial were reinvited to the laboratory measurements and muscle tissue sampling 10 years later. Of these 80 women, two were deceased, eight did not respond and 23 refused or were unable to participate. Finally, 47 were invited to the laboratory. The past and current use of any estrogen-containing hormonal therapy as well as health was carefully evaluated by a physician. Of these 47 women, 13 reported having never or not within the past five years used HRT and consequently were invited to participate to the Post/Premenop. study as members of the postmenopausal group.

The premenopausal group comprised of 30-40 years old women not using any contraceptives and having a normal menstrual cycle. An invitation to the study was sent to two thousand women born in 1967-1977 (39.1% of the entire cohort) randomly selected from the entire 30-40 years old age cohort (born in 1967-1977) and living in the City of Jyväskylä. Screening of the study participants fulfilling the inclusion criteria was performed using a short questionnaire sent along with the invitation. The women were asked for their past and current history of being treated with hormonal contraceptives (contraceptive pills and plasters, hormonal intravaginal and hormonal intrauterine devices) or progesterone preparations. Altogether 163 questionnaires were received. Subsequently, 118 women fulfilling the inclusion criteria, i.e., not being treated with hormonal contraceptives or progesterone preparations within the past five years and willing to participate in the study, were contacted by telephone to further clarify their gynecological status, medication and potential contraindications for participation (chronic musculoskeletal diseases, type 1 or 2 diabetes, mental disorders, asthma with oral glucocorticosteroid treatment, cancer, drug or alcohol abuse, Crohn's disease). Irregular menstrual cycles, breast feeding and planned pregnancy were also used as exclusion criteria. Based on the interview, 62 women were eligible and willing to participate in the study. Of these, two discontinued and one was excluded by the physician due to a contraindication for muscle tissue sampling. For the full battery of biochemical analyses, a subgroup of 13 participants were randomly selected by using the random sampling feature of the PASW Statistics software (SPSS Inc., IBM, Chicago, IL, USA), for this study and used in the analyses reported in paper IV.

TABLE 1 Summary of data sets, study designs and outcomes used in original publications.

Original publication	Data set (design)	Physiological outcomes	Molecular biological outcomes
I	CO and HRT groups from the Ex/HRT– study (RCT)	• LBM (kg) • Fat mass (%) • $mCSA_{QF}$ (cm^2)	• Muscle transcriptome • Serum hormones
II	CO and HRT groups from the Ex/HRT study (RCT)	• BMI (kg/m^2) • LBM (kg) • Fat mass (%) • $mCSA_{QF}$ (cm^2)	• Gene expression of the components of the IGF-1 signaling pathway • Serum hormones
III	CO, HRT and PT groups from the Ex/HRT study (RCT)		• Muscle transcriptome
IV	Post/Premenop. study (cross-sectional)	• LBM (kg) • Fat mass (kg) • $tCSA_{QF}$ (cm^2) • $mCSA_{QF}$ (cm^2) • $fCSA_{QF}$ (cm^2) • muscle attenuation (HU) • KE strength (N) • Muscle force per $mCSA_{QF}$ (N/cm^2)	• Serum hormones • Muscle tissue hormones • mRNA expression of steroidogenesis-related genes

RCT, randomized controlled trial; LBM, lean body mass; $mCSA_{QF}$, lean cross-sectional area of the *quadriceps femoris* muscle; BMI, body mass index; $tCSA_{QF}$, total cross-sectional area of the *quadriceps femoris* muscle; $fCSA_{QF}$, cross-sectional area of fat tissue infiltrated within *quadriceps femoris* muscle.

4.2 Ethics

The studies included in this PhD study has been conducted in conformity with the guidelines laid down in the Helsinki declaration (World Medical Association, www.wma.net). The data collections were approved by the ethics committee of the Central Finland Health Care District. An informed consent explaining the possible risks and benefits associated with the examinations and permission to use the data for research purposes and in publications was signed by the study participants prior to performing the measurements.

4.3 Measurements

4.3.1 Physiological measurements

BODY AND MUSCLE COMPOSITION (I, II, IV). Body weight was measured with a beam scale and height with a stadiometer and the results used to calculate body mass index (BMI). Lean body mass (LBM) and body fat mass were assessed using bioelectrical impedance (Spectrum II, RJL Systems, Detroit, MI) in papers I and II, while in paper III a multifrequency bioelectrical impedance analyzer (InBody 720, Biospace, Seoul, Korea) was used. Computed tomography (CT) scans (Siemens Somatom Emotion Scanner, Siemens, Erlangen, Germany) were obtained from the mid-part of *m. vastus lateralis* and the scans were analyzed using BonAlyse 1.0 (I and II) or Geanie 2.1 software (IV). Both software were developed for cross-sectional CT image analysis and enables separation of fat and muscle tissue based on radiological density. Total CSA of the *m. quadriceps femoris* ($tCSA_{QF}$), muscle CSA of *quadriceps femoris* ($mCSA_{QF}$), and intramuscular fat area within *quadriceps femoris* ($fCSA_{QF}$) were measured. Skeletal muscle attenuation at $mCSA_{QF}$ was defined as the mean attenuation coefficient and expressed in Hounsfield units (HU). The interassay coefficient of variations (CV) between two consecutive measurements in our laboratory is 1-3% for $tCSA_{QF}$ and for $mCSA_{QF}$, 4-9% for $fCSA_{QF}$ and 1% for attenuation (Sipilä et al. 2001, Taaffe et al. 2005).

MUSCLE STRENGTH, POWER AND PHYSICAL ACTIVITY (IV). Maximal isometric knee extension strength (KE) was measured in a sitting position at a knee angle of 60° from full extension (Good Strength, Metitur, Palokka, Finland). After familiarization with the test, the participants were encouraged to produce maximal force. Three to six maximal efforts were conducted and the highest recording was used as the test result. Lower body muscle power, i.e. the ability of the neuromuscular system to produce the greatest possible force as fast as possible, was assessed as the height that a subject is able to elevate the body's centre of gravity during a vertical jump with counter-movement on a contact mat. Jumping height was calculated according to the equation by Bosco et al. (1983). The muscle force per $mCSA_{QF}$ was calculated by dividing KE by $mCSA_{QF}$. In our laboratory, the interassay CV between two consecutive measurements of KE has been 6% and of vertical jump 5% (Sipilä et al. 2001). Information concerning self-reported physical activity was collected using the six-point scale of Grimby (Grimby 1986) with slight modifications.

4.3.2 Collection of biological samples

MUSCLE BIOPSY SAMPLING (I, II, III, IV). Muscle biopsies were obtained from the mid-part of the *m. vastus lateralis* defined as the midpoint between the greater trochanter and the lateral joint line of the knee. To avoid variation due

to sampling, the biopsy protocol was standardized across the data sets used. All muscle biopsies were taken by the same experienced physician from the same site of the same thigh subjected earlier to CT scanning. Visible blood and fat were removed before muscle samples were snap frozen in liquid nitrogen and stored at -70°C pending analysis. The second part of the biopsy was mounted transversely on a cork with Tissue Tek Optimal Cutting Temperature compound (Sakura, Alphen aan den Rijn, Netherlands), and frozen rapidly in 2-methylbutane (Sigma-Aldrich Corporation, ST. Louis, MO, USA) pre-cooled to -160°C in liquid nitrogen and stored at -80°C. The histological evaluation of the samples did not reveal any signs of damage, such as to the central nuclei, in any of the samples.

SERUM SAMPLING (I, II, IV). In all studies blood samples were taken from the antecubital vein with the study participant in a supine position during the same morning as the muscle sampling was performed. From the premenopausal women, blood sample was collected also during the first follicular days (1-6 days) unless the muscle sample collection happened during this period. The aliquoted sera were stored in -70°C pending analysis.

4.3.3 Biochemical and microscopical analyses

SERUM HORMONE MEASUREMENTS (I, II, IV). In the Ex/HRT study, serum concentrations of SHBG, FSH, E_2 and T were measured by time-resolved fluoroimmunoassay method (DELFIA, Wallac) and used in papers I and II. The intra-assay CV for detection of E_2 and T was 3.8% and 8.2%, respectively.

In the Post/Premenop. study (IV) the serum concentrations of SHBG, FSH and LH were measured using solid-phase, chemiluminescent immunometric assays (Immulite 1000, Diagnostic Products, Los Angeles, CA, USA). Serum E_2 levels were determined in duplicates using an extraction radioimmunoassay as previously described (Ankarberg-Lindgren & Norjavaara 2008). E_1 was measured as a dansyl-derivative using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on API 4000 mass spectrometer as previously described (Nelson et al. 2004). Serum T (Turpeinen et al. 2008), DHT and androstenedione were measured using the LC-MS/MS method. Before the DHT and androstenedione analysis, 30 µl of 0.1 µM deuterated DHT or androstenedione in 50% (vol/vol) methanol (internal standards, IS) was added to 250 µl of serum before extraction with 5 ml of diethyl ether. After mixing for 3 min the upper layers were collected and evaporated to dryness under nitrogen. The residues were dissolved in 250 µl of 50% methanol. Calibrators containing 0.2-25 nmol/l of DHT or 0.5-50 nmol/l of androstenedione were prepared in 50% methanol. Forty µl (DHT) or 25 µl (androstenedione) of sample extracts and calibrators were analysed on an LC-MS/MS system equipped with an API 3000 triple quadrupole mass spectrometer (AB Sciex, Applied Biosystems, Foster City, CA, USA) with the electrospray ionisation probe and an Agilent series 1200 HPLC system with a binary pump. Separation was

performed on a SunFire C18 column (2.1 x 50 mm; Waters, Milford, MA, USA). The mobile phase was a linear gradient consisting of methanol and 100 mM ammonium acetate in water, at a flow rate of 250 µl/min. DHT and androstenedione were detected as protonated ions in the positive mode with the following transitions: m/z 287 to m/z 97 (A), m/z 294 to m/z 100 (IS) and m/z 291 to m/z 255 (DHT), m/z 295 to m/z 259 (IS), respectively. Data were acquired and processed with the Analyst Software (Ver 1.4; AB Sciex). E_2, E_1, T, DHT, androstenedione and SHBG concentration were used to calculate the corresponding free hormone levels (FE_2, FE_1, FT and FDHT) according to a recently presented spreadsheet method which takes into account the competitive binding of the different hormones present in sera (Mazer 2009). The CV was 19% for E_2 at 6 pmol/l, 7.8% for E_1 at 200 pmol/l, 5.2% for T at 4.7 nmol/l, 9.1% for DHT at 3.5 nmol/l, 5.5% for androstenedione at 3.2 nmol/l, 8.4% for SHBG at 32.4 nmol/l, 5.5% for FSH at 38.5 IU/l, 8.1% for LH at 30.0 IU/l and 4.7% for DHEAS at 5.2 µmol/l. In addition, the serum concentration of IGF-1 was measured using solid-phase, chemiluminescent immunometric assay (Immulite 1000) in all studies. The CV was 6.9% for IGF-1 at 25.5 nmol/l.

MUSCLE HORMONE MEASUREMENTS (IV). Muscle hormone measurements were done as previously described (Vingren et al. 2008). Briefly, muscle tissue samples were homogenized on ice in Tissue Extraction Reagent I-buffer (Invitrogen, Carlsbad, CA, USA; 15 µl buffer/mg muscle) containing 80 µl/ml PMSF, 40 µl/ml aprotinin, 40 µl/ml leupeptin and 1 µl/100µl Inhibitor Coctail I (all from Sigma-Aldrich Corporation, ST. Louis, MO, USA) using a plastic tissue grinder. The homogenate was gently mixed in rotation for 15 min at +4°C following centrifugation at 10,000 g for 15 min at +4°C. The supernatant (1:10-dilution) was used to measure the total protein concentration immediately following centrifugation using a Pierce BCA Protein Assay- kit (Thermo Scientific, Rockford, IL, USA). ELISA-tests from IBL-International (Hamburg, Germany) were used to determine E_2, T, DHT and DHEA concentrations in 1:10-diluted muscle homogenate supernatants in duplicate. The concentrations of all hormones were standardized using total protein concentration. The limits of quantification given by the manufacturer were 35.7 pmol/l for E_2, 287 pmol/l for T, 20.6 pmol/l for DHT and 374 pmol/l for DHEA. Interassay CVs for E_2, T and DHEA after correction for total protein concentrations as determined in our laboratory were 14%, 23% and 11%, respectively.

RNA PREPARATION FROM MUSCLE TISSUE SAMPLES (I, II, III, IV). Trizol-reagent (Invitrogen) was used to isolate total RNA from frozen muscle biopsy samples homogenized on FastPrep FP120 apparatus (MP Biomedicals, Illkrich, France). The RNA concentration and purity were measured spectrophotometrically using NanoDrop equipment (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Only pure, good-quality RNA was used in the following microarray and quantitative PCR (qPCR) analyses.

QUANTITATIVE PCR (I, II, IV). One to two micrograms of RNA from the muscle samples was reverse transcribed into cDNA for qPCR analysis by using TaqMan Reverse Transcription Reagents or High Capacity cDNA Reverse Transcription Kit (both from Applied Biosystems, Foster City, CA, USA). If the probes/primers did not cross the exon-intron boundary the RNA was subjected to DNase treatment (Turbo DNA-free kit, Applied Biosystems) before cDNA synthesis in order to avoid contamination from the genomic DNA. We used either commercial TaqMan gene expression assays (Applied Biosystems), custom-designed TaqMan assays (Applied Biosystems) or SYBR Green-based detection (iQ SYBR Green supermix-kit, Bio-Rad Laboratories, Hercules, CA, USA). The studied genes and qPCR-methods used are listed in Table 2. All the TaqMan assays were run with an Applied Biosystems' ABI 7300 unit using the standard PCR conditions recommended by the manufacturer: 1 cycle of 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. The CFX96 Real-Time PCR Detection Thermal cycler (Bio-Rad) was used for the SYBR Green-based assays for which the optimal annealing temperature was determined using temperature gradients before proceeding to qPCR. The qPCR was performed using the following program: 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 10 s, the predetermined optimum annealing temperature for each gene (STS: 61°C, HSD3B1: 62°C, HSD17B5: 61°C, aromatase: 62°C, SRD5A1: 62°C, SRD5A2: 62°C, GAPDH: 60°C, β-actin: 60°C) for 30 s, 72°C for 30 s following the determination of the dissociation curves: 95 for 10s, 65 to 95°C, with 0.5°C increment for 5 s. Each gene was run in a separate plate and with the same reference sample (a mixture of several muscle samples) to control run-to-run variation. Dilution series of the reference sample were used to determine the amplification efficiency for each gene. In all papers, the GAPDH (M value 0.6) was chosen to serve as reference gene to normalize variation due to differences in the initial cDNA amounts. The normalized, relative gene expression (RQ) was calculated using the sigmoidal curve fitting method (Liu & Saint 2002; I), the standard curve method (II) and the equation: $RQ=efficiency^{(Cq(reference\ sample) - Cq(gene\ of\ interest))}$ normalized with RQ of GAPDH where C_q stands for the quantification cycle determined in the PCR run (IV).

TABLE 2 The quantitative PCR methods used in this thesis.

Original paper	Gene name	Gene ID	Assay ID or forward primer (FP), reverse primer (RP), probe	Detection method
I and IV	*AR*	NM_000044	Hs00171172_m1	TaqMan
I	*DDX52*	NM_152300	Hs00294711_m1	TaqMan
I and IV	*ESR1*	NM_00125	Hs01046812_m1	TaqMan
I	*ESR2*	NM_001040275	Hs01100358_m1	TaqMan
I	*FBXO11*	NM_001190274	Hs00251516_m1	TaqMan
I	*FBXO32*	NM_058229	Hs00369714_m1	TaqMan
I, II and IV	*GAPDH*	NM_002046	Hs99999905_m1	TaqMan
I	*MGEA5*	NM_001142434	Hs00201970_m1	TaqMan
I	*USP1*	NM_001017415	Hs00163427_m1	TaqMan
I	*USP2*	NM_004205	Hs00899199_g1	TaqMan
I	*USP15*	NM_006313	Hs00378613_m1	TaqMan
I	*USP50*	NM_203494	Hs01596824_m1	TaqMan
I	*OGT*	NM_181672	Hs00269228_m1	TaqMan
I and II	*18S*	NR_003286	Hs99999901_s1	TaqMan
II	*IGF-1Ea*	NM_000618	FP: AGCGCCACACCGACATG RP:TCCCTCTACTTGCGTTCTTCAAA probe: CAAGACCCAGAAGGAAGTA	TaqMan
II	*IGF-1Eb*	NM_001111285	FP: GAGGAGCAGACAGCAAGAATGA RP: CCAGCAGGCCTACTTTTCTTCA probe: AAGCAGAAAATACAATAGAGG	TaqMan
II	*IGF-1Ec*	NM_001111283	FP: CACGAAGTCTCAGAGAAGGAAAGG RP: CTTGTTTCCTGCACTCCCTCTAC probe: AAGTACATTTGAAGAACGCA	TaqMan
IV	*aromatase*	NM_000103	FP: GTGGACGTGTTGACCCTTCT RP: GCCATGCATCAAAATAACCTTGGA	SYBR Green
IV	*β-actin*	NM_001101	FP: GACAGGATGCAGAAGGAGATCACT RP: TGATCCACATCTG CTGGAAGGT	SYBR Green
IV	*GAPDH*	NM_002046	FP: CCACCCATGGCAAATTCC, RP: TGGGATTTCCATTGATGACAA	SYBR Green
IV	*HSD3B1*	NM_000862	FP: TTGTCAAATAGCGTATTCACC TTC RP: AGCTTGTGCCCTTGTCACTTT	SYBR Green
IV	*HSD3B2*	NM_000198	FP: TACTTTG GATTGGCCACGAT RP: CATCAATGATACAGGCGGTG	SYBR Green
IV	*HSD17B1*	NM_000413	FP: AGCTGGACGTAAGGGACTCA RP: GTGGGCGAGGTATTGGTAGA	SYBR Green
IV	*HSD17B3*	NM_000197	FP: TGCGTGAGATTCTCCAGATG RP: AATGGCTTGGGAGA AGGTTT	SYBR Green
IV	*HSD17B5*	NM_003739	FP: CCAGTTGACTGCAGAGGACA RP: TCGCTAAA CAGGACGGATTT	SYBR Green
IV	*SRD5A1*	NM_001047	FP: ATGTTCCTCGTCCACT ACGG RP: GCCTCCCCTTGGTATTTTGT	SYBR Green
IV	*SRD5A2*	NM_000348	FP: CTCAGGAAGCCTGGAGAAAT RP: AAATGCAAATGCAAGTGCTG	SYBR Green
IV	*STS*	NM_000351	FP: GGAAGGCCTTTTTCTTCACC RP: AGGGTCTGGGTGTGTCTGTC	SYBR Green
IV	*GPER*	NM_001039966	Hs01922715_s1	TaqMan
IV	*megalin*	NM_004525	Hs00189742_m1	TaqMan

IMMUNOFLUORESCENCE ANALYSIS (IV). Frozen muscle tissue samples were cut by cryostat into 8 µm sections, fixed with acetone (10 min at -20°C), and air-dried. To prevent non-specific staining, the sections were blocked with 10% donkey normal serum for 30 min at room temperature and incubated with primary antibodies against STS, aromatase and SRD5A1 (sc-33499, sc-14245 and sc-20396, respectively, all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Sections were washed with phosphate buffer solution and exposed to the fluorescence secondary antibody AlexaFluor donkey anti-goat 568 (Invitrogen). Sections were again washed with phosphate buffer and stained with DAPI to visualize nuclei. The specificity of the staining was controlled by omitting primary antibodies from the staining protocol.

4.3.4 Microarray experiments and data mining

HYBRIDIZATION TO THE BEADCHIPS (I, II, III). Biotin-labeled cRNA from 500 ng of total RNA was produced (Illumina RNA amplification kit, Ambion, Austin, TX, USA) and quality controlled (Experion, Bio-Rad) before hybridizations. Before the first microarray study (I), the quality of the samples was further tested with the Illumina protocol to obtain biotin-labeled cRNA, and the test hybridizations were performed using Sentrix-16 BeadChips (BD-15-101, Illumina, San Diego, CA, USA) with Human Sampler probe set for 517 genes. TestChip data have been deposited in the public NCBI' Gene Expression Omnibus (GEO) database (www.ncbi.nlm. nih.gov/geo/; (Edgar et al. 2002)) under series code GSE6378. HumanRef-8 v1.0 BeadChips were used in paper I and both HumanRef-8 v1.0 and HumanWG-6 v1.0 BeadChips (All chips from Illumina Inc.) were used in papers II and III. Hybridization to the BeadChips as well as washing and scanning was performed according to the Illumina BeadStation 500x manual (revision C). Both samples (baseline and follow-up) from each study participant were always hybridized onto the same chip. The chips were scanned by confocal laser scanning system (Illumina BeadReader Rev. C, Illumina Inc.). The data were acquired by the BeadStudio Direct Hybridization V.1.5.0.34. The Turku Centre for Biotechnology (Turku, Finland) carried out the cRNA generation, array hybridizations, and quality control of the raw data. The data discussed in this thesis have been deposited in GEO database and are accessible through GEO Series accession numbers GSE6348 (I) and GSE16907 (II and III). The MIAME guidelines were followed during array data generation, preprocessing, and analysis.

PREPROCESSING AND DATA MINING (I, II, III). The intensity of gene expression in the BeadChips was determined by calculating the average signal intensity, excluding beads that fall outside three normal standard deviations of median intensity. After this hybridization quality control, the high quality signals were quantile normalized in order to perform cross-chip analysis. Median normalization was also inspected and found to give results similar to those of quantile normalization.

In the study on transcriptome-wide alterations in skeletal muscle gene expression during the early stage of postmenopause with and without HRT (I) only the HumanRef-8 v1.0 BeadChips containing probes for 24,000 NCBI RefSeq-genes (on average, 30 beads to each probe) were used and hybridized with samples from 10 HRT users and 5 CO women. All data were filtered with a background value such that upregulated genes had to be above the background value in the follow-up samples and downregulated genes above the background value in the baseline samples. Each BeadChip contains 778 negative control probes, with no match to the human genome. The individual background value for each BeadChip was determined by averaging the signal intensities of these negative controls. To be recognized as up- or downregulated, among the HRT users or COs not using HRT, the gene had to meet the following criteria: the change between the follow-up and baseline condition had to be >0 (upregulated genes) or <0 (downregulated genes); the change had to be in the same direction in all but one subject; and the change had to be significantly different between the HRT and CO groups. The difference between study groups was statistically identified using t-test for independent samples with a threshold p-value of ≤0.05. The t-test was adjusted for multiple comparisons using the Benjamini-Hochberg FDR-correction (Benjamini & Hochberg 1995). Genes with FDR-corrected p-values of ≤0.05 were considered differentially expressed. The approach maximized stringency in avoiding false positive error in the data analysis, but allowed minor variability between study participants. In practice, this means that 90% of the subjects in the HRT group and 80% of the subjects in the CO group needed to have the same directionality in the expression of the gene before the gene was taken into account in the data analysis. Identical analyses were done separately for the HRT and CO groups by using R/Bioconductor (www.r-project.org, www.bioconductor.org) and Inforsense KDE 3.1 software (Guildford, United Kingdom).

For the studies on the effects of HRT on the gene expression of the components of the IGF-1 signaling pathway (II) and on the specific and shared effects of PT and HRT on skeletal muscle transcriptome (III), the raw gene expression data from eight PT (used only in III) and four CO participants were obtained by using Human WG-6 BeadChips, while HumanRef-8 BeadChips were utilized for ten HRT and five CO participants. The raw data from both BeadChips were combined, and only the probes, which were identical in both platforms, were included in the further analysis. Consequently, over 24,000 probes for approximately 21,000 NCBI RefSeq-transcripts were included. Quality control and visualization of the combined raw data were performed with aid of box plots, hierarchical clustering, correlation matrix, and principal component analysis. Microarray data from the study groups were normalized separately using the quantile normalization method implemented in the Affy-package (Gautier et al. 2004) of the R/Bioconductor analysis software. Samples (n=6) from three CO participants were hybridized onto both Illumina platforms in order to compare the performance of the array types with the Pearson

correlation coefficient test. Although the correlation of the data produced by the same samples on different Illumina platforms was high (r=0.88–0.94), a clear batch effect caused by two different platforms was visible. This was corrected in the statistical analyses by using chip type as a covariate. After preprocessing of the microarray data, the Limma-package (Smyth 2004) of R was used to identify differentially expressed genes within each condition. The fold changes (FC) and p values calculated by Limma methods were used as filtering criteria in order to detect genes up- or downregulated within each study group. The repeated measurements design and the batch effect caused by the usage of two different chip types were taken into account in the statistical testing. At this point, we chose to use relatively low stringency thresholds (p value <0.05 and |FC| >1.2) in order to list a reasonable number of the genes most likely to be differentially expressed. Decreasing or increasing the thresholds resulted in poorer cluster structures in the clustering of the filtered genes.

ENRICHMENT ANALYSIS OF FUNCTIONAL CATEGORIES AMONG DIFFERENTIALLY EXPRESSED GENES (I, III). In paper I, the two lists of genes significantly up- or downregulated in the HRT and CO groups (FDR-corrected p of <0.05) were searched for possible enrichment in certain biological processes defined by the Gene Ontology (GO) annotation association (Ashburner et al. 2000) by using Web-based ONTO-Express software (http://vortex.cs.wayne.edu/projects.htm; Khatri et al. 2002, Draghici et al. 2003). The number of genes corresponding to each GO category among the differentially expressed genes in the HRT and CO groups was compared with the number of genes expected for each GO category on the basis of their representation on the HumanRef-8 BeadChip. Significant differences from the expected numbers were calculated using a two-sided binomial distribution with FDR-correction. Only GO categories with FDR-corrected p values of <0.05 were reported.

In paper III, the genes determined to be differentially expressed within at least one of the three study groups (CO, HRT or PT) were used in the further analyses, as with this approach it is possible to identifying co-regulated gene clusters, i.e., genes, which show consistent behavior being either up- or downregulated within each study group. First, the hierarchical clustering with Pearson's metrics was applied to form clusters of the most similarly behaving genes. The Pearson's correlation thresholds were set to 0.95. Additionally, extensive analysis of functional categories, i.e., GO terms (Ashburner et al. 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa et al. 2006), was carried out to clarify whether certain gene clusters are enriched with particular functional categories. Hypergeometric test and FDR calculation were applied to identify the most significant categories. GenoSyst Ltd (Turku, Finland) carried out the hierarchical clustering with enrichment analysis. The results obtained were further analyzed and processed in our laboratory. PatternViewer 2.39 (GenoSyst Ltd, Turku, Finland) was used to visualize the results.

4.4 Statistics

All the descriptive statistical results are presented using mean ± standard deviation (SD) and the level of significance is set at $p \leq 0.05$. Normal distribution of the data was checked using the Shapiro-Wilk test. Differences in the physiological outcomes within the Ex/HRT intervention (I, II and III) were tested by analysis of co-variance (ANCOVA) with the baseline measurement as covariate or with one-way analysis of variance (ANOVA) when the baseline measurements of the study groups were compared. When appropriate, the Bonferroni *post hoc* procedure was employed to locate the source of significant differences in means. In a cross-sectional Post/Premenop. study the parametric independent samples *t*-test or the non-parametric Mann–Whitney *U* test was used to test the significance of the differences between study groups. Pearson's correlation coefficient was used to assess the associations between the muscle quality and hormone concentrations. Data analyses were carried out using PASW Statistics software (SPSS Inc.).

5 RESULTS

5.1 Muscle phenotype and hormonal status of the study participants

5.1.1 General characteristics and muscle phenotypes

Two data sets, the Ex/HRT study (I, II, III) and the Post/Premenop. study (IV), were used in this thesis. The general and muscle phenotype characteristics that were analyzed in the original publications are presented in Table 3 for the participants of the Ex/HRT study (I and II) and in Table 4 for the participants of the Post/Premenop. study (IV).

The analysis presented in Table 3 includes all three study groups with nine CO, ten HRT and eight PT participants. The mean age of the Ex/HRT women was 53 years. No significant difference was observed between the study groups in level of self-reported physical activity at the baseline. The activity score 2 refers to walking activities 1-2 times per week, score 3 refers to walking activities several times per week and score 4 refers to sweating-inducing activities 1-2 times per week. The participants in this study with a score around 3 can thus be considered moderately active. Weight, BMI and body fat mass remained similar during the one year study period and did not differ between groups. The change in LBM did not quite reach statistical significance ($p=0.057$, the p-values for the pair-wise comparisons were as follows HRT vs. CO $p=0.091$ and PT vs. CO $p=0.145$). The use of HRT significantly increased $mCSA_{QF}$ (8.6% HRT vs. 1.7% CO $p=0.017$, 1.9% PT vs. 1.7% CO $p=1.0$). The muscle attenuation and power (measured as vertical jumping height) were found to be affected in the original Ex/HRT study (Sipilä et al. 2001, Taaffe et al. 2005), but were not statistically significant in a smaller study sample used in the gene expression studies. However, the changes in the subgroup analysis were parallel to the results obtained from the larger Ex/HRT study. In the subgroup analysis the change in $fCSA_{QF}$ was 5% in the HRT, 5% in the PT and 25% in the CO group,

the change in muscle attenuation was 3% in the HRT, 2% in the PT and -1% in the CO group, the change in KE was 3% in the HRT, 8% in the PT and -3% in the CO -3% group and change in jumping height was 7% in the HRT, 1% in the PT and -4% in the CO group.

TABLE 3 Phenotype characteristics of the Ex/HRT study participants (I-III).

Ex/HRT study	**Age** (years)	**Physical activity**	**Height** (cm)	**Weight** (kg)
CO (n=9)				
baseline	53.2±2.1	3.6±1.2	161.9±4.9	68.0±7.1
follow-up				68.4±7.6
HRT (n=10)				
baseline	53.5±1.6	3.1±1.5	161.1±6.9	73.2±11.7
follow-up				72.4±10.3
PT (n=8)				
baseline	52.8±0.9	2.8±0.9	164.8±4.4	68.9±12.3
follow-up				70.6±13.0
p-value	*0.650*§	*0.434*§	*0.380*§	*0.348*
Ex/HRT study	**BMI** (kg/m^2)	**LBM** (kg)	**Body fat** (%)	**mCSA$_{QF}$** (cm^2)
CO (n=9)				
baseline	25.9±2.1	47.0±4.6	30.6±3.6	46.3±6.8
follow-up	26.1±2.3	46.7±3.4	31.3±4.8	47.1±6.6
HRT (n=10)				
baseline	28.1±2.9	47.5±4.0	34.3±7.8	47.5±6.1
follow-up	28.0±2.7	48.5±4.0	32.5±6.4	51.6±7.7#
PT (n=8)				
baseline	25.3±4.0	46.3±4.9	32.3±7.3	48.1±7.7
follow-up	26.0±4.3	47.4±4.6	31.8±7.4	49.0±7.1
p-value	*0.623*	*0.057*	*0.430*	*0.013*

Values are mean ± standard deviation; p-value is obtained by ANCOVA or by §one-way ANOVA; #significant pair-wise comparison in mCSA$_{QF}$: HRT vs. CO p=0.017; BMI, body mass index; LBM, lean body mass; mCSA$_{QF}$, muscle cross-sectional area of QF; QF, *quadriceps femoris*.

The Post/Premenop. study consisted of two study groups, one with a mean age of 64 years (postmenopausal group) and the other with a mean age of 33 years (premenopausal group). No differences between the groups were found in body composition or in self-reported physical activity level (Table 4). The premenopausal women had 14% larger mCSA$_{QF}$ (p=0.052), 13% higher muscle attenuation value (p<0.001), 33% greater KE (p=0.002), 84% greater jumping height (p<0.001) and 19% better muscle force per mCSA$_{QF}$ (p=0.011) than the postmenopausal women.

TABLE 4 Phenotype characteristics of the Post/Premenop. study participants (IV).

	Postmenopausal women (n=13)	**Premenopausal women** (n=13)	*p-value*
Age (years)	63.6±2.1	32.8±2.5	*<0.001*
Height (cm)	163.8±4.5	163.7±3.7	*0.944*
Weight (kg)	68.0±13.6	66.9±15.4	*0.857*
BMI (kg/m^2)	25.3±4.5	25.0±5.6	*0.890*
LBM (kg)	46.1±4.8	46.6±3.6	*0.754*
Body fat (%)	30.6±7.8	28.0±10.1	*0.463*
Physical activity	3.6±0.8	3.8±1.1	*0.681*
$mCSA_{QF}$ (cm^2)	46.8±7.2	53.1±8.3	*0.052*
$fCSA_{QF}$ (cm^2)	3.1±1.2	2.5±1.4	*0.304*
Muscle attenuation (HU)	54.5±4.0	61.3±3.9	*<0.001*
Muscle force per $mCSA_{QF}$ (N/cm^2)	8.3±0.9	9.9±1.6	*0.0116§*
KE (N)	386.4±57.7	513.5±115.2	*0.002*

Values are mean ± standard deviation; p-value obtained by independent samples t-test; §p-value obtained by Mann–Whitney *U* test; BMI, body mass index; LBM, lean body mass; $mCSA_{QF}$, muscle cross-sectional area of QF; QF, *quadriceps femoris*, $fCSA_{QF}$, fat cross-sectional area within QF.

5.1.2 Participants' hormonal status

The results obtained from the measurements of circulating hormones are presented in Table 5. As described in the methods, the methodology used to study serum hormone concentrations differed between the studies. Therefore the values presented in Table 5 are not directly comparable across the different data sets.

As expected, among the participants of the Ex/HRT study the use of HRT clearly increased the serum concentration of E_2 (change HRT: 201%, PT: -35%, CO: -78%, ANCOVA p-value <0.001; significant pairwise comparisons: HRT vs. CO and HRT vs. PT p<0.001 for both comparisons) and decreased the concentration of FSH (change HRT: -65%, PT: -5%, CO: 9%, ANCOVA p-value <0.001; significant pairwise comparisons: HRT vs. CO p<0.001, HRT vs. PT p<0.001). The serum concentrations of T, SHBG or IGF-1 were not affected.

The extensive analyses of the serum and muscle concentrations of steroid and other hormones were conducted in order to compare differences in

steroidogenesis between the post- and premenopausal women (IV). According to these analyses, the serum concentrations of E_2, E_1, androstenedione, DHEAS and IGF-1 were significantly lower in the post- than premenopausal women (for all comparisons $p<0.001$) while the concentration of FSH was significantly higher ($p<0.001$, Table 5). The muscle concentration of E_2 was 15% ($p=0.030$) and T 23% ($p=0.010$) higher in the post- than premenopausal women (Table 6).

TABLE 5 Hormone concentrations in serum samples.

	Estrogens		**Androgens**	
Data set	E_2 (pmol/l)	E_1 (pmol/l)	T (pmol/l)	DHT (pmol/l)
Ex/HRT study				
CO (n=9)				
baseline	33.3±84.4		663.4±521.8	
follow-up	6.9±14.7		735.1±549.5	
HRT (n=10)				
baseline	73.8±125.5		1068.4±817.5	
follow-up	222.5±50.8*		1018.4±917.2	
PT (n=8)				
baseline	3.1±8.8		894.6±501.1	
follow-up	2.0±5.7		897.3±545.7	
p-value#	*<0.001*		*0.601*	
Post/Premenop. study				
Postmenopausal women (n=13)	22.9±7.6	95.8±27.0	920.8±312.2	347.5±279.5
Premenopausal women (n=13)	400.3±313.1	371.8±215.3	1093.8±430.1	431.5±257.8
p-value§	*<0.001*	*<0.001*	*0.293*	*0.165*

	Prehormones		**Other hormones**		
Data set	A (nmol/l)	DHEAS (µmol/l)	SHBG (nmol/l)	FSH (IU/l)	IGF-1 (nmol/l)
Ex/HRT study					
CO (n=9)					
baseline			50.7±12.7	65.0±19.2	15.0±4.5
follow-up			55.8±14.1	70.9±15.1	16.5±7.2
HRT (n=10)					
baseline			53.3±16.3	62.5±26.6	15.0±3.7
follow-up			48.7±23.5	22.4±11.0*	16.7±4.4
PT (n=8)					
baseline			65.6±31.4	80.4±19.8	14.8±1.5
follow-up			63.3±30.7	75.9±24.7	13.7±3.1
p-value#			*0.056*	*<0.001*	*0.368*
Post/Premenop. study					
Postmenopausal women (n=13)	2.2±0.9	2.2±1.0	69.1±17.6	78.4±22.6	13.2±2.9
Premenopausal women (n=13)	4.8±1.7	4.8±1.3	54.7±30.0	5.8±2.2	23.7±5.9
p-value§	*<0.001*	*<0.001*	*0.149*	*<0.001*	*<0.001*

Values are mean ± standard deviation; #p-value is obtained by analysis of co-variance using baseline measurement as co-variate; *significant pairwise comparisons in E_2 and FSH : HRT vs. CO and HRT vs. PT $p<0.001$ for all comparisons; §p-value obtained by Mann–Whitney *U* test; E_2, 17β-estradiol; E_1, estrone; T, testosterone; DHT, dihydrotestosterone; A, androstenedione; DHEAS, dehydroepiandrosterone sulfate; SHBG, sex hormone binding globulin; FSH, follicle stimulating hormone; IGF-1, insulin-like growth hormone 1.

TABLE 6 Hormone concentrations in muscle tissue of post- and premenopausal women.

Post/Premenop. study	**E_2** (nmol/µg prot)	**T** (nmol/µg prot)	**DHT** (nmol/µg prot)	**DHEA** (nmol/µg prot)
Postmenopausal women (n=13)	1.5±0.2	13.2±1.8	0.6±0.1	70.2±7.7
Premenopausal women (n=13)	1.3±0.2	10.7±2.6	0.5±0.1	66.4±12.4
p-value	*0.030*	*0.010*	*0.140*	*0.356*

Values are mean ± standard deviation; #p-value is obtained by independent samples t-test; E_2, 17β-estradiol; T, testosterone; DHT, dihydrotestosterone; DHEA, dehydroepiandrosterone

5.2 Muscle gene expression in postmenopausal state with or without HRT and PT

Two microarray studies (I, III) investigating the global changes in the gene expression of skeletal muscle and one IGF-1 signaling cascade-specific study (II) were conducted. In the first microarray study, the effects of HRT were studied by analyzing the transcriptome-wide changes within CO (n=5) and HRT (n=10) participants (Figure 7, chapter 5.2.1, I). Since the increase in $mCSA_{QF}$ was found to be due to HRT, the gene expression related to a potent anabolic signaling cascade, the IGF-1 signaling, was also studied within the CO (n=9) and HRT (n=10) women (chapter 5.2.2, II). In the second microarray study, hierarchical clustering analysis with enrichment assay was performed in order to determine unique and shared effects of HRT and PT on the global gene expression by using muscle samples from the CO (n=9), HRT (n=10) and PT (n=8) groups (Figure 8, III). By setting the statistical threshold $p<0.05$ and $|FC|>1.2$ in at least one of the three study groups, a non-redundant list of 665 differentially expressed genes was determined. These genes were organized into 8 hierarchical clusters, which represented clusters of genes specific to the effects of HRT (chapter 5.2.1) or PT (chapter 5.2.3), clusters with effects parallel in both HRT and PT (chapter 5.2.4) and clusters with similar effects in the HRT, PT and CO groups (chapter 5.2.5).

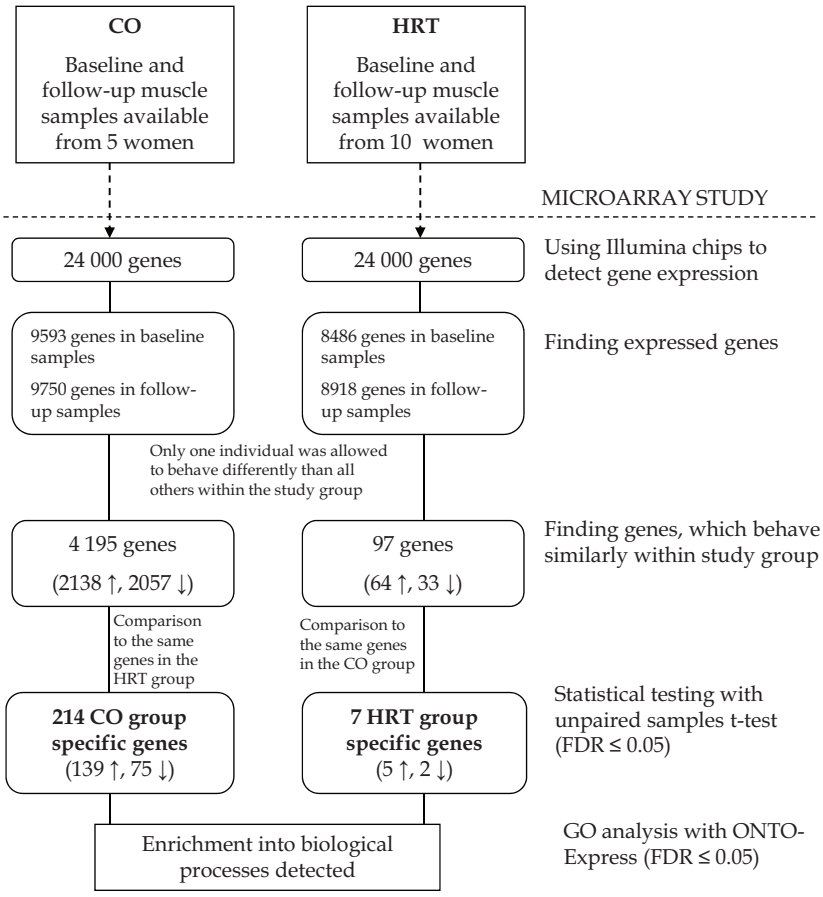

FIGURE 7 Flow chart presenting the experimental design and data mining of the microarray study on the effect of HRT on global muscle gene expression (I).

5.2.1 Effects of postmenopausal HRT on global muscle gene expression (I, III)

Ninety-seven genes were found to have a similarly up- or downregulated expression profile in the samples from the HRT group (Figure 7, I). Of these, 64 were upregulated and 33 downregulated. Only seven of these genes were significantly differently expressed when compared to their expression profile in the CO group indicating HRT-specific alteration. These genes were *OGT* (change: HRT 6%, CO 30%, $p<0.001$), *AR* (change: HRT 13%, CO -6%, $p=0.01$), *RNASEH2C* (change: HRT 13%, CO -18%, $p<0.001$), *atrogin-1* (change: HRT 24% CO -3%, $p<0.001$), *SULT1A1* (change: HRT 10%, CO -4%, $p<0.001$), *IL4I1* (change: HRT -6%, CO 9%, $p<0.001$) and *RAB31* (change: HRT -11%, CO 7%, $p<0.001$). Specific enrichment was observed in the following GO terms: protein

amino acid glycosylation, proteolysis and peptidolysis, catecholamine metabolism and cell proliferation.

Changed gene expression was found for 4195 genes in the CO group (Figure 7, I). Of these, 214 were significantly differently expressed when compared to the expression of the same genes in the HRT group, indicating that HRT was able to alter the expression of these genes to resemble the baseline situation. Of these 214 genes, 139 genes were upregulated while 75 were downregulated during the intervention. The list of affected genes is too long to be presented here, but according to the enrichment analysis it contained, e.g., genes related to RNA metabolism (9 genes), protein post-translational modifications (8 genes), cellular catabolic processes (18 genes) and other biological processes (11 genes). The remaining genes (168) did not show significant enrichment into particular functional categories.

The microarray analysis in which all three study groups were included found differential expression for 54 genes within the HRT group (Figure 8, III). Of these, 34 genes were upregulated and 20 downregulated. The clustering analysis, which included all 665 genes initially found to be differentially expressed in at least one of the study groups (CO, HRT or PT), placed 76 genes into the HRT-specific gene clusters. The number of genes was higher than originally found in the HRT group, since some genes up- or downregulated in both CO and PT groups were also clustered together with HRT genes showing potential co-regulation. Eleven genes in the HRT-specific clusters did not have GO or KEGG annotation. Specific enrichment among the upregulated genes was observed for GO term "mitochondrion", including four genes encoding mitochondrial ribosomal proteins (*MRPS12, MRPS36, MRPL27*, and *MRPL33*), one encoding mitochondrial chaperon (*HSPE1*), four genes involved in energy metabolism through oxidative phosphorylation (*ATP5H* and *COX7A2*), citric acid cycle (*IDH3A*) or fatty acid oxidation (*PECI*), and three genes involved in other mitochondrial functions (*MTFP1, PTPMT1*, and *TST*). Enrichment in the GO term "regulation of cell growth" was observed among the downregulated genes (*ING2, CHAD*, and *UBE2E3*). No HRT-specific enrichment of KEGG-annotated pathways was observed.

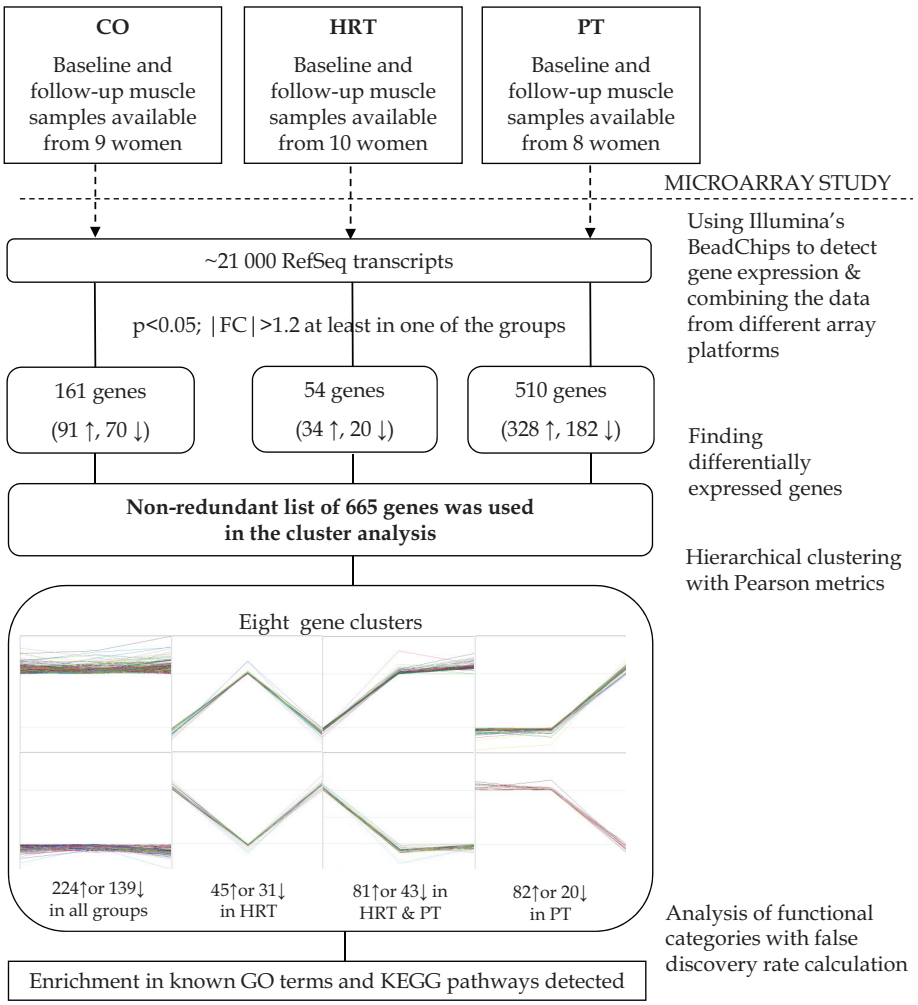

FIGURE 8 Flow chart presenting the experimental design and data mining of the microarray study on unique and combined effects of HRT and PT on global muscle gene expression (III).

5.2.2 Effects of postmenopausal HRT on IGF-1 -related gene expression (II)

According to the microarray data, the intramuscular expression of *IGF-1* gene was upregulated among the HRT users when compared to the CO (change: HRT 13%, CO -16%, p=0.014, II). All three splice variants of the *IGF-1*, the expression of which was determined with qPCR, were upregulated among the HRT users, but the change between the study groups was significant only for *IGF-1Ec* (change: HRT 58%, CO -31%, p=0.003). The change in the gene expression for *IGF-1Ea* was 62% in the HRT and -30% in the CO group (p=0.10) and for *IGF-1Eb* it was 10% in the HRT and -61% in the CO group (p=0.31).

Other genes along the IGF-1 signaling pathway were also analyzed from the microarray data. Of these, significant changes in gene expression were observed for *AKT1* (change: HRT -8%, CO 1%, p=0.036), *mTOR* (change: HRT 2%, CO -13%, p=0.043), *FOXO3* (change: HRT 2%, CO -9%, p=0.021) and *AR* (change: HRT 12%, CO -8%, p=0.001).

5.2.3 Effects of plyometric PT on global muscle gene expression (III)

In the initial analysis of global gene expression, 510 genes changed expression in the PT group (Figure 8, III). Of these genes, 328 were upregulated and 182 downregulated. The clustering analysis reduced the list to 82 specifically upregulated and 20 specifically downregulated genes in the muscles of the PT participants. Of the upregulated genes, 13 had no GO or KEGG annotation. According to the enrichment analysis, the largest GO term found to be enriched was cytoplasm, constituting 42% of the whole cluster. The other significantly enriched functional categories were actin binding, insulin signaling pathway and adipocytokine signaling pathway. No significant categories were found among the downregulated genes. This probably reflects the fact that the cluster was small and 40% of the genes did not have a GO or KEGG annotation, meaning that the functions of these gene products are unknown.

The significant PT-specific genes included *STARS* (change: PT 50%, HRT -16%, CO -46%), *MKNK2* (change: PT 24%, HRT -7%, CO -11%), *GLUT4* (change: PT 22%, HRT -2%, CO -8%), *FASN* (change: PT 22%, HRT -7%, CO -3%), *AKT2* (change: PT 22%, HRT -2%, CO -6%), *PRKAR2A* (change: PT 27%, HRT -4%, CO -7%) and *STK11* (change: PT 29%, HRT -8%, CO -19%) among others. *STARS* is involved in linking the contraction-induced changes in sarcomere structure with regulation of muscle gene expression (Kuwahara et al. 2005) while *MKNK2, GLUT4, FASN, AKT2, PRKAR2A* and *STK11* are involved in insulin or adipocytokine signaling.

5.2.4 Parallel effects of HRT and PT on global muscle gene expression (III)

Parallel upregulation in the HRT and PT groups was observed for 81 genes and parallel downregulation for 43 genes (Figure 8, III). According to the enrichment analysis, nine KEGG pathways were significantly upregulated. These pathways included genes related to carbohydrate metabolism and calcium signaling. Only one pathway, axon guidance, was found to be significantly enriched among the downregulated genes. Of the genes in this cluster, 56% did not have a known annotation.

One of the most dramatically affected genes was *ALDOA* (change: PT 70%, HRT 20%, CO -10%), which encodes an important enzyme needed for energy production during glycolysis. The other upregulated genes involved in glycolysis or insulin signaling were *PKM2* (change: PT 27%, HRT 16%, CO -5%), *TPI1* (change: PT 27%, HRT 12%, CO -7%), *PHKG1* (change: PT 30%, HRT 15%, CO -13%), *FOXO1* (change: PT 21%, HRT 1%, CO -9%), *CALM1* (change: PT

28%, HRT 7%, CO -3%) and *CALM3* (change: PT 25%, HRT 1%, CO -5%). Of these, *CALM1, CALM2* and *PHKG1* are also involved in the Ca^{2+}-signaling pathway together with *DCTN1* (change: PT 24%, HRT 3%, CO -14%), *CaMK2A* (change: PT 23%, HRT 12%, CO -1%), *CaMK2B* (change: PT 24%, HRT 1%, CO -4%), *FZD7* (change: PT 30%, HRT 2%, CO -2%) and *DVL1* (change: PT 32%, HRT 8%, CO -1%).

5.2.5 Global muscle gene expression in postmenopausal women with no effect of HRT and PT (III)

Uniform upregulation within all study groups was observed for 224 genes (Figure 8, III). Of these, significant enrichment was observed in the GO terms "muscle development", "muscle contraction" and "energy metabolism", and in the KEGG pathways related to energy metabolism and VEGF and epithelial cell signaling. There were 23 genes without a GO or KEGG classification. Uniform downregulation within all study groups was observed for 139 genes. Of these genes, 20 were unclassified according to the GO and KEGG databases. Of the classified, downregulated genes, enrichment was observed for common terms related to mitochondrial energy metabolism, oxidation-reduction reactions, extracellular matrix as well as regulation of muscle contraction.

5.3 Differences in steroidogenesis in post- and premenopausal women

5.3.1 The expression of steroidogenesis-related genes and proteins in skeletal muscle of post- and premenopausal women (IV)

The gene expression of steroidogenesis-related genes was studied utilizing the muscle samples of post- and premenopausal women obtained in the Post/Premenop. study. The studied genes included *STS, HSD3B1, HSD17B5, aromatase, SRD5A1, SRD5A2*, which are needed for the synthesis of active steroid hormones from pre-cursor molecules. Also the measurement of the gene expression of steroid hormone receptors, *ESR1, AR* and *GPER*, as well as the endocytic receptor called *megalin* (official name: *LRP2*) was included in the analysis. The difference in gene expression between the study groups was statistically significant for *HSD3B1* (mean FC=20, p=0.007) and *aromatase* (mean FC=1.8, p=0.018), and in the border line of significance for *ESR1* (mean FC=1.9, p=0.057).

The immunofluorescence staining of the key enzymes in steroidogenesis revealed rather uniform staining over the cross-sectioned muscle samples (Figure 9). STS antibody showed staining at or close to the sarcolemma with some sarcoplasmic staining between myofibrils. Both aromatase and SRD5A1 antibodies stained either the sarcolemma or subsarcolemmal zone. None of the antibodies showed muscle cell type-specific staining patterns.

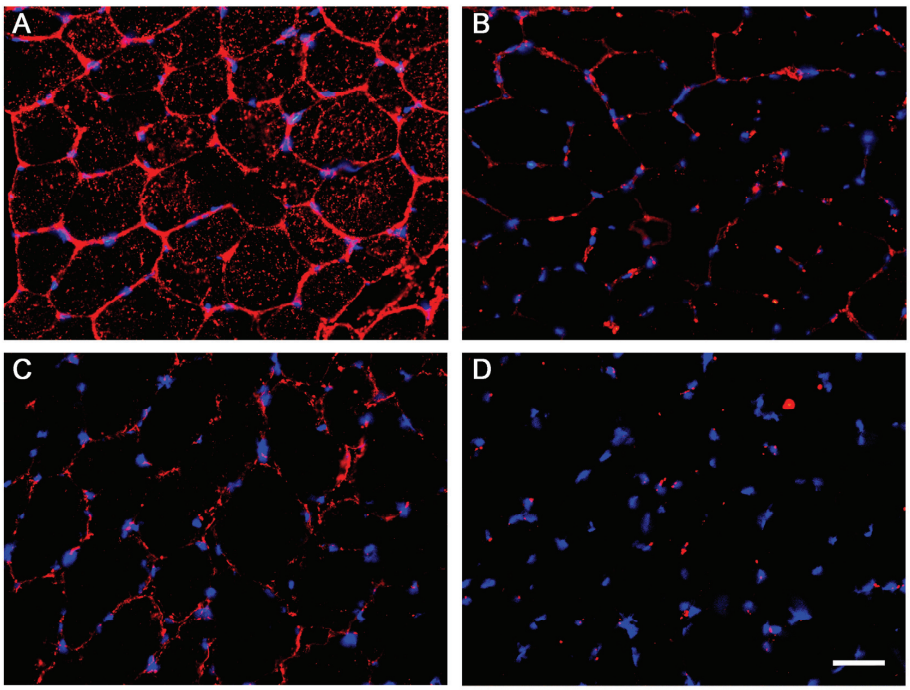

FIGURE 9 Immunofluorescence detection of STS, aromatase and SRD5A1 protein in the skeletal muscle. A) Representative image of immunofluorescence for STS (red) shows intensive staining at or close to sarcolemma of muscle cells as well as some sarcoplasmic staining. B) Representative image of immunofluorescence for aromatase (red) shows some staining at thin subsarcolemmal zone. C) Representative image of immunofluorescence for SRD5A1 (red) shows staining at subsarcolemmal zone at close proximity to sarcolemma. D) Omission of primary antibody showed no immunoreactivity in the skeletal muscle. Nuclei are stained blue in all figures. Bar = 50 µm.

5.3.2 The association of systemic and local hormone levels with muscle quality (IV)

Pearson's correlation was used to evaluate the association between systemic and local hormone concentrations with muscle quality variables: muscle force per $mCSA_{QF}$ and muscle attenuation (Table 7). A positive correlation with serum concentrations of E_2, E_1, DHEAS and IGF-1 was observed with muscle force per $mCSA_{QF}$ and with muscle attenuation. In addition, androstenedione was positively and SHBG negatively associated with muscle attenuation. Serum androgens T and DHT were not significantly associated with muscle quality. Of the measured muscle hormones, the E_2 and T concentrations were negatively associated with muscle attenuation.

TABLE 7 Association of systemic and local hormone levels with indicators of muscle quality.

Serum hormones	**Muscle force per $mCSA_{QF}$ (n=25)**	**Muscle attenuation (n=25)**
E_2[#]	r=0.411 p=0.046	r=0.674 p<0.001
E_1	r=0.596 p=0.002	r=0.535 p=0.006
T	r=0.143 p=0.496	r=0.062 p=0.769
DHT	r=0.327 p=0.119	r=-0.214 p=0.316
A	r=0.360 p=0.077	r=0.503 p=0.010
DHEAS	r=0.508 p=0.009	r=0.545 p=0.005
SHBG	r=-0.037 p=0.859	r=-0.417 p=0.038
IGF-1	r=0.451 p=0.024	r=0.437 p=0.029
Muscle hormones	**Muscle force per $mCSA_{QF}$ (n=25)**	**Muscle attenuation (n=25)**
E_2	r=-0.142 p=0.499	r=-0.469 p=0.018
T	r=-0.050 p=0.811	r=-0.573 p=0.003
DHT	r=-0.130 p=0.537	r=-0.337 p=0.099
DHEA	r=0.228 p=0.273	r=-0.381 p=0.060

[#]For the premenopausal women the normal basal levels of E_2 were used in the analysis. The basal levels, i.e., the lowest levels during the menstrual cycle were determined from the serum samples obtained during the early follicular phase of the menstrual cycle. QF, *quadriceps femoris*; $mCSA_{QF}$, muscle cross-sectional area of QF; E_2, 17β-estradiol; E_1, estrone; T, testosterone; DHT, dihydrotestosterone; A, androstenedione; DHEAS, dehydroepiandrosterone sulfate; SHBG, sex hormone binding globulin; IGF-1, insulin-like growth hormone 1; DHEA, dehydroepiandrosterone.

5.4 Summary of the results

This thesis is based on two data sets comprising the year-long Ex/HRT intervention with three study groups and the cross-sectional Post/Premenop. study. The Ex/HRT women were on average 53 years old at baseline while the average ages for the two groups forming Post/Premenop. study were 64 and 33 years. The women in both studies had very similar body composition and physical activity levels. When the postmenopausal group was compared to the premenopausal group, it was obvious that the postmenopausal women had smaller thigh muscles, higher fat infiltration within muscle tissue and lower muscle strength and power than the premenopausal women. Furthermore, within the Ex/HRT intervention, the use of HRT increased $mCSA_{QF}$.

Two separate microarray studies, in which the transcriptome-wide changes in skeletal muscle gene expression were investigated using the muscle samples obtained from the CO and HRT groups or from the CO, HRT and PT groups of the Ex/HRT study, were performed. According to the microarray studies, a rather wide range of changes occurs in the muscle transcriptome during the first postmenopausal years. These gene expression level changes occur in particular GO- and KEGG-annotated functional categories, including RNA metabolism, cellular catabolic processes, energy metabolism, and muscle development and contractions. However, both HRT and PT seemed to balance or slow down some of these changes and can therefore be considered as counteractive treatments. Furthermore, PT induced transcriptional changes in the genes involved in the GO/KEGG terms cytoplasm, actin binding, insulin signaling and adipocytokine signaling while HRT affected in particular the GO/KEGG terms protein amino acid glycosylation, proteolysis and peptidolysis, catecholamine metabolism, cell proliferation and mitochondrial functions. Also, combined effects of PT and HRT were observed. These included changes in the gene expression related to carbohydrate metabolism and calcium signaling. In addition, year-long HRT was found to affect the gene expression of several genes related to the IGF-1 signaling pathway.

The analysis of steroidogenesis in post- and premenopausal women revealed that despite the extensive loss of steroid hormones in circulation, the skeletal muscle tissue of postmenopausal women has even higher concentrations of steroids than that of the premenopausal women. E_2 and T concentrations in muscle tissue were associated with muscle attenuation, indicating that the amount of infiltrated fat has an influence on local steroidogenesis. As an indicator that local steroidogenesis truly takes place in muscle tissue, the steroidogenesis-related enzymes were observed in skeletal muscle at both mRNA and protein level. However, the postmenopausal women showed poorer muscle characteristics than the premenopausal women. Furthermore, the higher circulating concentrations of steroid hormones were associated with better muscle quality, while muscle hormone concentrations were associated with higher fat infiltration in the muscle.

6 DISCUSSION

This thesis examined the effects of hormone replacement therapy and power training on skeletal muscle gene expression among postmenopausal women. In addition, the endocrine and paracrine roles of steroid hormones and their association with muscle quality in post- and premenopausal women were investigated.

According to the results, the early stage of postmenopause, defined as 0.5-5 years since last menarche, is characterized by changes in the muscle transcriptome, which in turn can be affected by HRT and PT. These treatments can be considered to function as counteractive treatments, as they have the effect of stabilizing or slowing down some of the gene expression level changes naturally occurring in skeletal muscle after menopause. In particular, the transcriptome-wide studies conducted in this thesis showed that gene expression related to many functional categories important for the plasticity of skeletal muscle was affected, including proteolytic processes, mitochondrial functions, energy metabolism, Ca^{2+}-signaling and remodeling-related genes. Furthermore, HRT affected, at least at gene expression level, the IGF-1 signaling pathway, which is a potential signaling cascade regulating muscle mass.

This thesis also examined the possible differences between post- and premenopausal women in circulating and muscular sex steroid hormones in relation to muscle quality. The results suggested that despite the extensive systemic deficit of estrogens, as well as prehormones, in the postmenopausal compared to the premenopausal women, the levels of steroid hormones in skeletal muscle do not follow the same trend. In contrast to the systemic levels, the skeletal muscle of the postmenopausal women had higher concentrations of E_2 and T than the skeletal muscle of the premenopausal women. Furthermore, the circulating hormone concentrations were positively associated with muscle quality, while the hormone concentration of the muscle reflected the amount of infiltrated fat in the muscle tissue.

6.1 Association between muscle phenotype and hormone or training status

In the original Ex/HRT study, which concentrated on the effects of HRT and PT on muscle phenotype characteristics, both hormone and training status were found to have beneficial effects on muscle phenotype (Sipilä et al. 2001, Taaffe et al. 2005). A significant 7% increase in muscle power in the HRT and 6% in the PT group, compared with a 5% reduction in the CO participants without any treatments was observed during the intervention. Running speed increased by 4% in both HRT and PT, and decreased by 2% in the COs. CSA of the knee extensor muscle increased significantly by 6% in HRT compared to 2% in PT and 0.4% in CO. Moreover, the knee extensor muscle attenuation coefficient increased significantly following HRT and PT, reflecting beneficial changes in muscle quality. Due to lack of muscle samples, smaller sample sizes were used in the gene expression studies in this thesis. Therefore, despite the parallel results obtained in the original sample and in the subsample, the statistical testing performed here lacked the power to locate all the previously observed significant results on muscle phenotypes, especially in the performance-related variables. However, no significant differences were found between the subsample used here and the entire Ex/HRT study in any of the muscle phenotype variables or in changes in the variables during the intervention. Therefore it is justified to conclude that the results obtained here at the gene expression level represent well the situation observed at the phenotype level in the original Ex/HRT study.

As expected, in the Post/Premenop. study, the postmenopausal women had lower muscle strength (KE) and power (jumping height) as well as smaller thigh muscle CSA than the premenopausal women. The CSA of intramuscular fat in QF did not differ between the study groups, but muscle quality, which was measured both as muscle attenuation and as muscle force per $mCSA_{QF}$, was lower in postmenopausal than premenopausal women. Both measures are widely used indicators of muscle quality, since attenuation determined by CT provides an estimate of the lipid content of muscle, and muscle force per $mCSA_{QF}$ provides an estimate of the force per unit cross-sectional area (Lynch et al. 1999, Metter et al. 1999, Goodpaster et al. 2000, Schroeder et al. 2003, Delmonico et al. 2009, Ali et al. 2011). The concentration of estrogens (E_1 and E_2), DHEAS, androstenedione and IGF-1 in circulation was found to be associated with higher muscle force per $mCSA_{QF}$ and less fat in QF (higher attenuation values), but not directly with $mCSA_{QF}$ or $fCSA_{QF}$. Therefore a high amount of circulating hormones seems to be associated with better muscle quality, as has also been concluded in other studies (Ronkainen et al. 2009, Lowe et al. 2010). On the other hand, the E_2 and T concentrations in muscle tissue were negatively associated with muscle attenuation, pointing towards a connection between fat infiltration and local steroidogenesis. The result accords well with the previous

understanding that visceral and subcutaneous adipose tissue is able to take up and convert steroid hormones for intracrine as well as endocrine purposes. (Belanger et al. 2002). However, it seems that the higher E_2 and T concentrations in the muscle tissue of the postmenopausal women compared to the premenopausal women were not able to counteract the aging-related decrements in muscle phenotype. Therefore it is not wholly clear what the functional consequences of local steroidogenesis are on skeletal muscle.

6.2 Possible molecular mechanisms behind the changes in postmenopausal muscle phenotype

As explained above, the postmenopausal women not using HRT or taking part in PT had a poorer muscle phenotype compared to that of the postmenopausal women on HRT or PT. Moreover older, postmenopausal women had a compromised muscle phenotype compared to the younger, premenopausal women. A plausible explanation may be unearthed from the results obtained in this thesis, including the changes in global gene expression (I, III), defects in the IGF-1 related signaling (II) or inefficiency in sex steroid signaling (IV).

The plasticity of skeletal muscle during aging involves a complex network of signaling pathways forming a regulatory scheme which we are only beginning to understand. Therefore it is essential to gain further information on the cellular events associated with the effects of different treatments and conditions shown to improve muscle function in order to draw a complete picture of all interactions possibly leading to aging-related defects in skeletal muscle. The following sections attempt to do so by interpreting the meaning of the transcriptional and other results obtained in this thesis in relation to other studies in the field and their association with muscle phenotype.

6.2.1 Protein degradation and synthesis

Muscle size is mainly regulated by a continuous fluctuation between muscle protein synthesis and degradation which defines the state of muscle fiber. If degradation exceeds synthesis, muscle size decreases and vice versa. On the other hand, if the disposal of aberrant proteins or cell organelles is decelerated, the functionality of the cell suffers. Therefore an adequate balance between catabolic and anabolic processes is needed to maintain or increase muscle size without impairing its functionality. There are at least four proteolytic pathways, namely autophagy-lysosomal, Ca^{2+}-dependent, caspase-dependent and ubiquitin-proteosome-dependent, which may be altered during aging (Combaret et al. 2009). Lysosomal pathway may directly break down proteins, but it also eliminates whole cell organelles by generating vesicles called autophagosomes, that engulf a portion of cytoplasm containing impaired organelles or protein aggregates. Autophagosomes are then fused with

lysosomes for degradation of their content. The most potent inhibitor of autophagy in skeletal muscle is protein kinase AKT, while FOXO3 functions as an activator (Mammucari et al. 2007, Zhao et al. 2007). On the other hand, IGF-1/AKT-mediated signaling also controls protein synthesis through mTOR-mediated signaling and ubiquitin-proteosome-dependent protein degradation by inactivating FOXO1 and FOXO3, which in turn control the transcription of ubiquitin ligases *atrogin-1* and *MuRF1* (Nader 2005). Proteosome-dependent protein degradation proceeds, first, by polyubiquitination of the target proteins, which needs the sequential functions of ubiquitin-activating, ubiquitin-conjugating and ubiquitin-ligating enzymes and, secondly, by recognition and degradation by the 26S proteosome (Attaix et al. 2005).

In this study, the gene expression of 18 genes directly related to cellular catabolic processes was found to change in the CO group from the Ex/HRT study. These included, e.g., four ubiquitin proteases (*USP1*, *USP2*, *USP15*, *USP50*), one ubiquitin-conjugating enzyme (*UBE2G2*), one ubiquitin protein ligase (*FBXO11*), and *CASP9*. In addition, use of HRT significantly upregulated the gene expression of another ubiquitin ligase, *FBXO32,* which is better known as *Atrogin-1*. It has also been suggested that the multifunctional OGT, whose gene expression was found to increase by 22-30% among the COs and by only 6% in the muscles of the HRT users, inhibits the activity of the ubiquitin-proteosome system (Zhang et al. 2003), thereby increasing the list of affected protein degradation-related genes.

Use of HRT also affected gene expression along the IGF-1-signaling cascade which may potentially have influenced the observed increment of muscle mass. According to the results, the gene expression of *IGF-1*, *mTOR*, *FOXO3* and *AR* were significantly upregulated in the HRT users' muscle compared to the COs while *AKT* was downregulated. In other words, the gene expression of three anabolic (*IGF-1*, *mTOR* and *AR*) and one catabolic (*FOXO3*) factor increased, while the gene expression of one anabolic factor (*AKT*) decreased. Somewhat contrary results were observed in a cross-sectional case-control study comparing over 55-year-old postmenopausal obese current HRT users to non-users. In that study the expression of *FOXO3*, *Atrogin-1* and *MuRF-1* was lower in the muscles of current HRT users than non-users (Dieli-Conwright et al. 2009). It is, however, possible that the difference between our results and those obtained by Dieli-Conwright and colleagues may be explained by differences in subjects' characteristics (e.g., age and body composition), the HRT specimens used (both estrogen only and combined preparations were included) and the time of HRT use. Furthermore, there was an intrinsic difference in body composition: the HRT users had 42% greater body mass than the non-using controls participating in the study by Dieli-Conwright et al. (2009). To complicate the issue further, in orchiectomized male mice, 5 weeks of E_2 or DHT treatment was shown to increase the expression of *atrogin-1*, while the expression of *IGF-1* decreased (Svensson et al. 2010).

In the present study possible changes in the gene expression of specific splice variants of the *IGF-1* gene were also studied. We found that the expression of *IGF-1Ec* was upregulated in the HRT group compared to the CO group. Furthermore the change in *IGF-1Ec* expression was positively associated with the size of the thigh muscle measured at post-intervention. *IGF-1Ec*, which was first identified as a factor responding to muscle contraction, is nowadays regarded as a major activator of muscle satellite cells as well as a direct growth factor (Goldspink & Harridge 2004). Also, two animal and three cell culture experiments investigating the influence of HRT or estrogen on exercise-induced myogenesis or on satellite cell proliferation concluded that the preservative function of HRT on muscle mass may emerge from improved muscle regeneration due to activation of satellite or other adult stem cells involving IGF-1-related signaling (Kamanga-Sollo et al. 2004, Enns & Tiidus 2008, Enns et al. 2008, Kamanga-Sollo et al. 2008a, Kamanga-Sollo et al. 2008b). Although the synthetic E-peptide identical to the C-terminus of IGF-1Ec has been shown to promote cellular proliferation and to delay the onset of senescence of human myoblasts (Kandalla et al. 2011), the actual role or even existence of the endogenous Ec-peptide has recently come under criticism (Matheny et al. 2010). However, there is no doubt that *IGF-1Ec* mRNA exists and coincides with improvements in muscle mass, and that it may induce growth-promoting signaling althought whether this is due to the Ec-peptide or the whole pro-IGF-1Ec remains to be resolved.

Taken together, gene expression level changes in the proteolytic machinery as well as in the IGF-1-related signaling pathway seem to occur in postmenopausal women's muscle, and this may partially explain the muscle phenotype differences observed between HRT users and non-users. However, to be able to conclude whether the net sum of all the observed gene expression level changes generate increased or decreased proteolysis, increased or decreased protein synthesis or weather the efficiency of protein turnover is enhanced or reduced in the muscles of postmenopausal women, further studies are needed.

6.2.2 Mitochondrial functions

Mitochondria are essential for the metabolism and function of skeletal muscle since they produce the majority of ATP needed, e.g., for cellular signaling events, protein synthesis and degradation or myosin-actin-interactions leading to muscle contraction (Romanello & Sandri 2010). In addition to ATP, muscle contractility also needs proper control of Ca^{2+}-delivery and removal, which in turn is linked to the ATP production, as Ca^{2+} uptake into the mitochondria stimulates oxidative phosphorylation and mitochondrial ATP production (Dirksen 2009). Importantly, mitochondria are also involved in the regulation of cell death through apoptosis (McBride et al. 2006). During aging, impairments in mitochondrial integrity and function have been shown to accumulate,

contributing to the loss of muscle mass and function (Short et al. 2005, Chabi et al. 2008).

The enhanced negative transcriptional regulation of oxidative phosphorylation and other mitochondrial functions with advancing age in skeletal muscle has been discovered in previous microarray studies (Welle et al. 2003, Welle et al. 2004, Zahn et al. 2006, Melov et al. 2007, Welle et al. 2008). A considerable number of gene expression level changes related to mitochondrial functions were also observed in this study: 29 genes with GO annotation "mitochondrion" or related to "oxidoreductase activity" were downregulated in the muscle samples of the postmenopausal women in the HRT, PT and CO groups of the Ex/HRT study. This finding indicates that neither PT nor HRT were able to counteract the changes occurring postmenopause. However, the downregulation of mitochondrion-related genes was only minimal among the HRT women: 0–13% for most of the genes and 16–23% for nine genes related to oxidation events. Much more profound downregulation was observed in the PT (5–55%) and CO (3–46%) groups. Moreover, there was specific upregulation of another set of 12 "mitochondrion"-genes after HRT treatment. Although the upregulation of these genes was modest (0–13%) among the HRT women, the same genes were substantially downregulated among the PT (12–53%) and CO (2–33%) women. Therefore it seems that mitochondria start to suffer accumulating negative transcriptional regulation already in the early postmenopausal years and that HRT may aid in maintaining rather than increasing the proper functionality of mitochondria.

Further evidence on the importance of mitochondrion-related gene regulation in postmenopausal women has come from another study conducted at our laboratory in which the association between global muscle gene expression and muscle properties was investigated in twin-pairs discordant for long-term use of HRT. It was found that "cofactor catabolic processes", including several mitochondrion-related genes, explained 26% of the variance observed in the relative proportion of thigh muscle and 20% of the variance in jumping height (Ronkainen et al. 2010). Furthermore, contrary to resistance (Melov et al. 2007) and endurance training (Mahoney et al. 2005), the plyometric PT used in the present study was not effective in resisting the negative transcriptional regulation of mitochondria. Therefore it seems that HRT aids in maintaining muscle mitochondrial function via transcriptional regulation while PT was not particularly effective.

6.2.3 Energy metabolism

Skeletal muscle is a highly glucose-sensitive tissue and therefore the maintenance of glucose homeostasis is one of the major determinants of muscle performance. Estrogen signaling has been shown to affect glucose homeostasis and insulin sensitivity in humans and rodents (Colacurci et al. 1998, Karjalainen et al. 2001, Alonso et al. 2006, Ropero et al. 2008), although the exact mechanisms are not understood. The uptake of glucose into muscle cells is mainly mediated by

GLUT4, which can be activated by insulin- and/or exercise-induced signal transduction (Ryder et al. 2001). In both animal and human studies, estrogen has been shown to modulate insulin action by affecting insulin secretion (Godsland 2005) and to participate in the regulation of *GLUT4* expression in the skeletal muscle of mice (Barros et al. 2006).

Interestingly, the overexpression of *OGT*, which we found to be highly increased at the mRNA level upon menopause, and which was most prominent within the CO women (22%), and less obvious among the PT (12%) and HRT (6%) women, has also been shown to induce insulin resistance in mice (McClain et al. 2002, Yang et al. 2008). OGT catalyzes the attachment of O-linked N-acetylglucoseamine (GlcNAc) to proteins, providing post-translational modification comparable to, and often competitive to phosphorylation. The rate of O-GlcNAcetylation is highly dependent on the availability of intracellular glucose, of which 2–5% is converted into UDP-GlcNAc, the donor substrate for GlcNAcetylation (Marshall et al. 1991). Therefore, OGT functions as an energy sensor, capable of regulating signal transduction, transcription as well as proteosomal degradation (Wells et al. 2003a, Wells et al. 2003b, Copeland et al. 2008) and its functionality is directly dependent on the availability of glucose.

Some other mediators of the insulin signaling or glycolysis were also found to be upregulated at gene expression level by PT (five genes) or by both PT and HRT (6 genes), suggesting overlapping effects of these two interventions. PT specifically increased the gene expression of *AKT2*, several other kinases, and the expression of *GLUT4*. Of the three isoforms of AKT kinase, AKT2 is the most important in modulating glucose homeostasis by regulating GLUT4 translocation into the plasma membrane in skeletal muscle (Gonzalez & McGraw 2009). These results may indicate that the import of glucose into muscle cells increased in the PT women, assuming that enhanced mRNA expression does in fact lead to a larger amount of GLUT4 proteins. Fourteen additional genes related to insulin signaling or glycolysis were upregulated among the PT, HRT and CO women. The expressions of all major enzymes (*PGM1*, *PFKM*, *FBP2*, *ALDOA*, and *TPI1*) needed for conversions of glucose to C3-carbohydrates were upregulated at the mRNA level. Also the gene expression of *PKM2*, which is a glycolytic enzyme that catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate to adenosine diphosphate, thus generating ATP (Ikeda & Noguchi 1998), was upregulated in the PT and HRT groups. Therefore, it seems that the tendency for increased transcription of genes related to carbohydrate metabolism after menopause is further enhanced by PT and HRT, and may finally be manifested as better muscle performance. It is also possible that after menopause, excess sugar metabolites start to accumulate, which produces a need to enhance both insulin signaling and glycolysis. Further studies are needed to verify if this strain observed here at the gene expression level is due to an increment in energy need per se, due to truncation in energy production or due to inefficiency in energy utilization after menopause, and whether the increments at mRNA level will lead to actual increments of corresponding proteins/enzymes.

6.2.4 Calcium-signaling

Myoplasmic Ca^{2+} is a well-known secondary messenger in skeletal muscle. It is involved in muscle contraction (Melzer et al. 1995) and in an array of cellular functions including carbohydrate metabolism, gene transcription, protein synthesis, and mitochondrial biogenesis, further increasing the diversity of the pathway (Hook & Means 2001, Wu et al. 2002, Wright 2007, Rose & Richter 2008, Illario et al. 2009, Rose et al. 2009).

In this study, five members of the calcium signaling pathway, namely two calmodulins: *CALM1* and *CALM3*, and three calmodulin and/or Ca^{2+}-activated kinases: *PHKG1*, *CaMK2A*, and *CaMK2B*, showed consistent transcriptional activation within the PT and HRT groups. PT increased the expression of these genes by 20–30%, and the use of HRT by 0–15%, while the same genes were slightly downregulated in the CO group. It is well established that contraction-induced elevation in Ca^{2+}-concentration leads to the rapid activation of CALMs, which in turn activate multifunctional CaMK kinases, among other downstream targets (Walsh 1983). These results suggest that the transcription of CaMK2, the most abundant CaMK in skeletal muscle, is regulated by physical exercise and that year-long PT leads to elevated basal mRNA levels. This is in line with protein level studies in which the activity of CaMK2 has been shown to be rapidly induced by exercise in an intensity-dependent manner (Rose et al. 2005). It is also widely acknowledged that physical exercise increases the mRNA and protein expression of GLUT4. The mechanism for an exercise-induced increment is not entirely clear, but it has been suggested that it may go through an increase in intracellular Ca^{2+} content, which activates CaMK and calcineurin, in turn activating specific transcription factors including MEF2 (Holmes & Dohm 2004). These results support the hypothesis and suggest that PT also directly affects the transcription of *CaMK*, but not that of *calcineurin*, the transcription of which was not induced in this study. The mechanism by which HRT is able to resist decrements in *CALM1*, *CALM3*, *PHKG1*, *CaMK2A*, and *CaMK2B* transcription, which was seen among the CO women, is not known.

STARS is a muscle specific actin-binding protein responding to muscle contraction-induced changes in Ca^{2+}-signaling (Kuwahara et al. 2005). The gene expression of *STARS* was 50% upregulated among the PT women compared to the downregulation observed among the HRT (-16%) and CO (-46%) participants. Significant upregulation of *STARS* has been recently shown also upon resistance training (Lamon et al. 2009) and upon a single bout of eccentric exercise (MacNeil et al. 2010). These findings indicate that *STARS* expression is induced by contraction per se and that the signaling through STARS can be directed to different pathways either to directly promote hypertrophy as suggested by Lamon and co-authors (2009), or to improve muscle composition, power and performance, as observed among the participants of this study (Taaffe et al. 2005).

6.2.5 Remodeling-related gene expression

The parallelly affected functional categories among all the study participants included eight "muscle development"-related genes and 16 "muscle contraction"-related genes. For example, from the four myogenic regulatory factors (MYOD1, myogenin, MYF5, and MYF6), both *MYOD1* and *MYF6* were found to be upregulated within the PT, HRT and CO participants. In another study, comparing muscle gene expression between current HRT users and non-users, the expression of all four myogenic factors were found to be higher among the HRT users than non-users (Dieli-Conwright et al. 2009). These transcription factors have both specific and redundant functions during muscle development, and their activation is required for satellite cell differentiation during myogenesis and muscle regeneration (Yablonka-Reuveni & Rivera 1994, Yablonka-Reuveni et al. 1999). Also, the MEF2 transcription factor family includes four genes which have sequential expression patterns during muscle development. The *MEF2*-gene, which we found to be activated among all study participants, was *MEF2D*, whose binding to the promoter of *MYOD1* is required for the increase and maintenance of *MYOD1* expression during differentiation into myotubes (L'honore et al. 2007). Taken together, it seems that transcriptional regulation of myogenesis and muscle regeneration is activated in early postmenopausal women.

Furthermore, the functional categories "regulation of contraction," "muscle contraction," and "extracellular matrix" were enriched among all the study groups (including 11 upregulated and 14 downregulated genes). In addition, PT specifically affected another group of genes that are known to respond to contraction (nine upregulated genes). In the studies by Welle and co-workers (Welle et al. 2003, Welle et al. 2004), *MYH8*, encoding the perinatal MyHC, was found to be upregulated with aging. Similarly, we found that *MYH8* was upregulated in all three study groups, although only barely in the HRT group (2%) and highly pronounced in the PT (84%) and CO (79%) groups. Expression of *MYH8* dominates the early development of skeletal muscle, and it is re-expressed in regenerating muscles (d'Albis et al. 1988, Weiss et al. 1999). Regeneration requires the activation of quiescent satellite cells to proliferate and differentiate into myoblasts, which eventually fuse with existing muscle fibers to repair damage or to help fibers to cope with physiological demands (Cooper et al. 2006). Despite the expression level of perinatal *MYH8* was low compared to the adult *MYH* genes encoding MyHC I, MyHC IIa, and MyHC IIx, the observed substantial increment in expression in the PT and CO groups may indicate increased demand for myofiber regeneration, although possibly for different reasons. In the PT group, the exercise itself may have led to increased regeneration while in the CO group it may be due to aging-related changes in muscle tissue. Furthermore, the demand of enhanced regeneration appeared to be minimal among the women using HRT, possibly because they were the only group able to maintain and even increase their muscle mass.

6.2.6 Steroidogenesis

There are four main pathways through which steroid hormones may interact with muscle fibers: circulating hormones may diffuse freely through the sarcolemma, may be actively internalized through endocytosis or may interact with membrane receptors without necessarily entering cell interior, in addition to the possibility that hormones may be produced within muscle fibers through local steroidogenesis. According to the free hormone hypothesis, only the unbound fractions of hormones in circulation are readily accessible to peripheral tissues, while hormones bound to SHBG are considered inactive and need to be dissociated from their carriers before they can enter target cells (Mendel 1989). The precursor hormones DHEAS, DHEA and androstenedione, which are only weakly or not at all bound to the SHBG, are considered to be freely accessible to the target cells. The alternative pathway for the uptake of steroid hormones is active endocytosis, as reported to occur through the endocytic megalin receptor (also known as LRP2 or GP330) (Hammes et al. 2005). However, the role of megalin in the delivery of sex steroids to target tissues has met with both criticism (Rosner 2006, Rosner et al. 2010) and support (König et al. 2008). Yet another tempting mode of action to consider, which does not necessarily involve the internalization of sex steroid hormones at all, has been proposed and reviewed by Rosner et al. (2010). Accordingly, first, SHBG itself binds to a membrane receptor, after which the receptor is activated through interaction with steroid hormones, leading to G protein-coupled signal transduction inside the target cell (Nakhla et al. 1999). Nevertheless, it remains unclear whether megalin or an equivalent membrane receptor for SHBG exists in skeletal muscle. In this study qPCR was used to assess the mRNA expression of *megalin* in the muscle samples of pre- and postmenopausal women, without, however, finding a detectable signal.

If skeletal muscle relies totally on free diffusion of the hormones, the muscle concentrations should closely follow the free serum concentrations, which in the Post/Premenop. study were 1.7-3.5% of the total concentrations for estrogens and 0.5-1.4% for androgens. Therefore, the muscle concentration of E_2, E_1 and T should be substantially lower and the concentration of DHT similar in postmenopausal compared to premenopausal women. This was the case only for DHT. Although the expression of *megalin* was not detected in this study, this does not exclude the possibility that active endocytosis of SHBG-bound sex steroids occurs in muscle tissue instead of free diffusion. However, in that case, a positive association between the hormone levels in serum and in muscle tissue should also be evident, unless the endocytosis is strongly enhanced in postmenopausal compared to premenopausal women. An explanation for the higher concentrations of E_2 and T in the muscle of the post- compared to premenopausal women, despite the contrary situation in the circulating levels, would require active and upregulated endocytosis of E_2 and T and/or upregulated steroid synthesis in muscle tissue.

The synthesis route from DHEAS to T/DHT and E_2 requires several steroidogenesis-related enzymes. Here, the gene and protein expression of these enzymes were studied by qPCR and immunofluorescence microscopy. According to the results at the mRNA level, most of the enzymes were more abundant in the muscle tissue of the post- than premenopausal women. However, as also stated by other investigators (Bhasin et al. 2003), they were expressed at quite a low level in the muscle tissue, rendering accurate qPCR determination difficult for some of the enzymes. Nevertheless, statistically significant differences were found in the mRNA expression of *HSD3B1* and *aromatase*. The immunofluorescence staining of skeletal muscle samples with antibodies specific to aromatase, SRD5A1 and STS revealed staining at or close to the sarcolemma. Staining was evenly distributed across the sections without muscle fiber-type specificity or obvious differences between age groups. The presence of steroidogenetic enzymes at the mRNA and protein level indicates that steroidogenesis occurs in muscle tissue, which could also explain the higher concentrations of T and E_2 observed in the muscle of the postmenopausal women. However, based on the current experiments, it is not possible to exclude a confounding effect via actively regulated endocytosis of steroid hormones from the circulation or from the surrounding tissues.

As already discussed, no associations between the measured muscular hormone concentrations and specific muscle force were observed. Instead the higher amount of infiltrated fat in muscle tissue (lower attenuation values) was associated with the higher E_2 and T concentrations in muscle tissue. In contrast, the serum hormone concentrations correlated positively with muscle quality, i.e., higher attenuation values and higher force per $mCSA_{QF}$. Differences in the associations indicate that systemic and local steroids may have their own distinct modes of action in skeletal muscle. At the molecular level systemic and local steroids may involve activation of distinct signaling routes in the target tissue. Systemic signaling may favor activation of membrane-bound receptors, as proposed elsewhere (Rosner et al. 1999), while the intracrine effects of steroid hormones may involve other types of receptors or signaling. One possible route for circulating hormones to affect muscle cells is SHBG binding to an as yet undetermined membrane receptor, following activation by interaction with circulating androgens or estrogens. Such interaction was shown to occur in the cell culture experiment with COS-1 cells, but the possible receptor has remained unknown (Nakhla et al. 1999). Similarly the interaction of E_2 with GPR30 has been shown to induce cAMP and mediate intracellular signaling, changing the metabolic fate of the cell (Maggiolini & Picard 2010). GPR30 belongs to the family of G-protein coupled membrane-receptors and has been recently recognized as a membrane-bound ESR and designated with new official name GPER. In addition to nuclear estrogen and androgen receptors, skeletal muscle expresses also *GPER*, as shown in mouse (Baltgalvis et al. 2010) and here in human muscles. In the present study the gene expression of *GPER* tended to be lower in the muscle of the postmenopausal than premenopausal women, which

could indicate impairments in the GPER-coupled signaling. However, the difference was not statistically significant. The opposite trend was observed for the muscle expression of *ESR1*, which tended to be higher in the postmenopausal than premenopausal women. Other studies have also shown that lower systemic levels of estrogens tend to increase the expression of *ESR1* in muscle (Baltgalvis et al. 2010) and in adipose tissue (Lundholm et al. 2008), which may indicate menopause-related withdrawal of negative regulation on the expression of *ESR1*. Nevertheless, changes in systemic and paracrine steroid hormones are clearly not the only factors involved in determining muscle function, mass and quality. Instead the whole concept is defined by complex interactions between several factors and tissue types involving functions of the central and peripheral nervous system as well as cardiovascular and musculoskeletal systems, which makes it difficult to dissect the precise molecular mechanisms behind the observed aging-related changes in skeletal muscle.

6.3 Limitations and perspectives

The present study is the first attempt to use explorative microarray analysis to identify the changes that take place in the skeletal muscle of postmenopausal women in relation to the hormonal and training status. Microarrays have been used in some previous studies to analyze the effects of training status on muscle transcriptome, but not with a type of training (plyometric PT) or age group comparable to those investigated in this thesis. The present study also provides the first transcriptome-wide study on the effects of HRT on skeletal muscle. Furthermore, despite the recognized association between menopause-related reductions in systemic hormone concentrations and decrements in skeletal muscle function, the actual hormonal status of skeletal muscle tissue has not been previously investigated in post- vs. premenopausal women. Therefore, this thesis both broadens the concept of the regulation of skeletal muscle gene expression through external stimuli and also provides novel information about muscle steroidogenesis and its possible effects on muscle properties in postmenopausal women.

One of the limitations of the gene expression studies included in this thesis is the relatively small sample size (n=8-10 per group in I-III) used. This is, however, quite typical of the microarray studies, partially owing to experimental costs and the invasive nature of muscle sampling. Similar sample sizes have successfully been used in other microarray studies (Chen et al. 2003, Rome et al. 2003, Welle et al. 2003, Zambon et al. 2003, Welle et al. 2004, Wittwer et al. 2004, Giresi et al. 2005, Mahoney et al. 2005, Teran-Garcia et al. 2005, Timmons et al. 2005, Timmons & Sundberg 2006, Mahoney et al. 2008). On the other hand, the RCT design with repeated-measures analysis and using a single HRT preparation in double-blinded fashion, strengthens this study by

eliminating physiological and genetic differences in responses to training or HRT. Therefore this study enables the detection of true intra-individual responses. The specimen of HRT used contained both estradiol (2 mg) and norethisterone acetate (1 mg). Therefore it was not possible to dissect possible differences in the effects of the two components. On the other hand, the combination of estrogenic and progestrogenic compounds studied here is commonly used as postmenopausal HRT and may therefore deliver results with practical relevance.

The year-long duration of the Ex/HRT intervention enabled us to examine true adaptation to treatments both at the phenotype and gene expression level. Therefore, it was not surprising that the observed changes in the gene expression were rather low compared to the acute responses reported in other studies. The modest responses found here may also be due to the true nature of aging-related change in global gene expression, which is characterized by the accumulation of numerous small transcriptional changes, as noted by two cross-sectional microarray studies investigating the influence of aging on the muscle transcriptome (Welle et al. 2004, Zahn et al. 2006). On the other hand, samples were taken at only two time points, at baseline and after one year, which prevents monitoring of the adaptation process and assessment of the possible fluctuations in gene expression during the study period.

To date, most of the expression array studies investigating the association of aging with muscle properties (Welle et al. 2003, Welle et al. 2004, Giresi et al. 2005) are cross-sectional and compare old (65-80 years) to younger (19-30 years) participants, neglecting the middle-aged population. Furthermore, thus far only a few studies using whole genome microarrays to study aging-related issues have specifically studied women (Welle et al. 2004, Ronkainen et al. 2010) or included samples from men and women (Zahn et al. 2006, Welle et al. 2008). Therefore, this study with on early postmenopausal 50- to 57-year-old women extends the work undertaken in previous microarray studies. The only other microarray study investigating the association of HRT with muscle transcriptome was conducted in our laboratory with samples from female twins discordant for HRT (Ronkainen et al. 2010). In that study the focus was on the effects of long-term use of HRT (2-16 years) on the muscle transcriptome of 55- to 62-year-old postmenopausal women. In the present study the gene expression level changes in muscle tissue were studied using the precise age group which, according to earlier studies (Kallman et al. 1990, Phillips et al. 1993, Samson et al. 2000), is just beginning to accumulate deterioration in their muscle function. Therefore, this age range is optimal for detecting the first alterations in the transcriptome before they are fully translated to the phenotype.

The nature of microarray studies is to be explorative and to be able to generate hypotheses rather than prove them. The generated hypotheses should then be further tested in different setups preferentially by moving from RNA level to protein level interactions. Unfortunately, we were unable to do this for

other than the MyHC proteins due to lack of muscle samples. Another approach is to concentrate on certain candidate genes or a signaling cascade likely to affect the phenomenon under investigation. That is what we did in the study on the effects of HRT on IGF-1-related signaling in which we combined the gene expression level results from human data with cell culture experiment (II).

In the study focusing on possible differences in the endocrine and paracrine effects of steroid hormones in post- and premenopausal women rather small sample sizes were also used, possibly reducing the statistical power of the study (IV). Both study groups had 13 participants. In addition the low mRNA levels of steroidogenesis-related enzymes in the skeletal muscle technically limited the amount of successful measurements, which may have affected the power and significance of the statistical testing. Yet another limitation was that it was not possible to define the origin of the sex steroid hormones measured in skeletal muscle tissue as these could reflect systemic endocrine or local paracrine processes possibly originating from surrounding or infiltrated adipose tissue instead of direct production in muscle cells. The overall body composition did not differ between the study groups and all the fat visible to naked eye was removed from the muscle samples before the hormone measurements. However, as shown by muscle attenuation, the postmenopausal women had more fat infiltration between and within the muscles. Such fat can also be a source for steroids, and therefore it is impossible to distinguish the role of muscle cells from that of infiltrated adipose cells in the local production of steroid hormones. However, according to the immunofluorescence assays, the steroidogenetic enzymes STS, aromatase and SRD5A1 are expressed in muscle cells evenly staining the cross-sections of muscle samples. With respect to the ELISA analyses, some inborn cross-reactivity may be present in the tests used, although according to the manufacturer the amount is not substantial. If occurring, cross-reactivity may falsely elevate the muscle concentration of the measured hormone. However, if so, the error will be similar in both study groups. Therefore, it is unlikely that such inborn methodological limitations have seriously affected the tissue level observations or the interpretation of the results.

6.4 Future directions

This thesis provides information about gene expression level changes taking place in postmenopausal women's skeletal muscle with an emphasis on the effects of HRT and PT. In addition, the differences between post- and premenopausal women in systemic and local steroidogenesis in relation to muscle quality have been revealed. The results clearly demonstrate the complex nature of the regulatory networks influencing the properties of muscle fibers and eventually the properties of whole muscle. Since this thesis is mostly

concerned with RNA level information, the next logical step is to move to protein level experiments. Many possibilities exist for such approach, among which the most interesting would be proteome-wide methods, as these maximize the information that can be gathered from valuable and often limited muscle samples from human participants. Another possibility is to concentrate only on some of the genes found here to be affected. Several affected genes were found for which only limited or zero amount of information is known concerning their functions in general, not to mention specifically in postmenopausal women's muscle. Such functional assays could in the first instance be performed with aid of cell culture and/or animal experiments before moving back to human studies to verify their applicability in humans.

The role of local steroidogenesis in the regulation of skeletal muscle properties should also be fully studied. At the moment it is not known what happens to the steroidogenesis in skeletal muscle if the reduction of systemic sex steroid hormones is compensated for with HRT. Also unknown is how the duration of time since menopause affects the local steroidogenesis in skeletal muscle. It is possible that systemic steroid hormones affect skeletal muscle by inducing changes in cell signaling while intracrine hormones may directly regulate gene expression by activating nuclear hormone receptors. This would explain the differential associations of systemic and local hormones with muscle properties, as observed in this thesis. However, at the moment this remains only a hypothetical explanation and should be tested in a series of controlled experiments.

7 MAIN FINDINGS AND CONCLUSIONS

The main conclusions of this thesis are:

1. Steroid hormones participate in the regulation of skeletal muscle properties by affecting gene expression. Especially, during the early postmenopausal stage which is characterized by low systemic estrogen levels, muscle transcriptome changes concomitant with decrements in muscle properties. In particular gene expression related to cellular catabolic processes, energy metabolism and muscle remodeling was affected. The use of estrogen-containing hormone replacement balances or slows down some of the transcriptome level changes and may also specifically enhance IGF-1-related signaling along with improvements in muscle properties.
2. Plyometric power training affects global gene expression in the skeletal muscle of postmenopausal women by changing the expression of energy metabolism and muscle contraction-related genes. PT also had effects parallel to those of HRT, including on genes related to energy metabolism and Ca^{2+}-signaling.
3. Despite the extensive systemic deficit of sex steroids in postmenopausal women, the amount of estradiol and testosterone in skeletal muscle is higher in post- than premenopausal women, which may be explained by the enhanced paracrine synthesis of these hormones in the skeletal muscle of postmenopausal women. However, local steroidogenesis was not sufficient to compensate for other possible aging-related changes so as to maintain muscle properties at the same level as those of premenopausal women.

Based on the present findings and recent literature, both hormone replacement therapy and power training can be considered to improve muscle properties by affecting the gene expression of skeletal muscle in postmenopausal women.

YHTEENVETO (FINNISH SUMMARY)

Normaaliin ikääntymiseen liittyy asteittainen lihasmassan pienentyminen, lihasvoiman väheneminen ja lihaskudoksen laadun heikentyminen. Ikääntymiseen liittyvät luurankolihaksen ominaisuuksien muutokset ovat monimutkaisia eikä kaikkia niiden taustalla olevia syitä vielä tunneta. Naisilla nämä muutokset näyttäisivät olevan yhteydessä mm. vaihdevuosiin liittyviin muutoksiin steroidihormonien synteesissä. Vaihdevuosia lähestyttäessä munasarjojen toiminta heikkenee ja lopulta lakkaa kokonaan, jolloin verenkierron hormonipitoisuudet laskevat voimakkaasti. Samaan aikaan muutokset kehon koostumuksessa ja lihaskudoksen ominaisuuksissa nopeutuvat verrattuna samanikäisiin miehiin tai hormonikorvaushoitoa (hormone replacement therapy, HRT) käyttäviin naisiin. Toisaalta fyysinen aktiivisuus vaikuttaa suotuisasti lihasten ominaisuuksiin liikkujan iästä riippumatta. Joissakin tapauksissa myös HRT:n on todettu parantavan lihasten ominaisuuksia. Ikääntymiseen liittyviä muutosten taustalla olevia molekyylibiologisia mekanismeja tai HRT:n ja fyysisen aktiivisuuden vaikutuksia näihin mekanismeihin ei kuitenkaan vielä tunneta kovin tarkasti.

Tämän tutkimuksen tarkoituksena oli selvittää vaikuttavatko estrogeeniä sisältävä HRT tai hyppelyharjoitteita sisältävä tehoharjoittelu (plyometric power training, PT) vaihdevuosi-iässä olevien naisten luurankolihasten ominaisuuksiin muuttamalla geenien luentaa ko. kudoksessa joko koko genomin laajuisesti tai erityisesti liittyen IGF-1 signalointireittiin. Tämän lisäksi tutkittiin systeemisten ja paikallisesti tuotettujen steroidihormonien merkitystä lihaksen ominaisuuksien säätelyssä. Tutkimuksissa käytettiin kahta erilaista tutkimusaineistoa: Ex/HRT-tutkimus oli vuoden mittainen satunnaistettu, lumekontrolloitu koe, johon osallistuneet 50–57-vuotiaat naiset (vaihdevuosista kulunut 0,5-5 vuotta) satunnaistettiin kontrolli-, HRT- ja PT-ryhmiin. Post/Premenop.-tutkimus oli poikkileikkausasetelmalla toteutettu tapausverrokkitutkimus, johon osallistui 26 ulkoisia hormoneja käyttämätöntä naista, joista puolet oli 61–67-vuotiaita vaihdevuosi-iän ohittaneita naisia ja puolet 29–38-vuotiaita naisia, jotka eivät vielä olleet saavuttaneet vaihdevuosi-ikää. Tämä väitöskirjatutkimus pohjautuu tutkittavilta otettuihin lihaskudosnäytteisiin, joista määritettiin geenien luennan eroja ja jälkimmäisessä tutkimusaineistossa myös hormonien määrää lihaksissa. Lisäksi mitattiin tutkittavien kehon koostumus, reisilihasten koko, koostumus, voima ja voimantuottoteho.

Ex/HRT-tutkimuksessa havaittujen tulosten mukaan tämän ikäryhmän naisilla, joiden vaihdevuosista oli siis kulunut 0,5-5 vuotta, tapahtuu melko paljon muutoksia lihaskudoksessa ilmentyvien geenien luennassa. Erityisesti muutoksen kohteena näytti olevan solun katabolisiin prosesseihin, energiametaboliaan ja lihaskudoksen uudelleen järjestäytymiseen liittyviä geenejä. Koska HRT ja PT näyttivät tasapainottavan tai hidastavan havaittuja muutoksia, niitä voidaan pitää lihasten kannalta myönteisinä hoitoina. Lisäksi PT aiheutti geenien luennan muutoksia solulimaan, aktiinin sitoutumiseen ja insuliinisignalointiin liittyvissä geeneissä. Sen sijaan HRT vaikutti proteiinien translaation jälkeiseen muokkaukseen, proteiinien hajotukseen, solun kasvuun ja mitokondrioiden

toimintaan liittyvien geenien luentaan. PT:n ja HRT:n vaikutukset hiilihydraattien metaboliaan ja kalsiumsignalointiin liittyvien geenien luentaan olivat samansuuntaisia. Steroidihormonien synteesin tutkiminen Post/Premenop.-tutkimuksen aineistolla osoitti, että paikallinen hormonien synteesi on voimakkaampaa vaihdevuosi-iän ohittaneiden kuin sitä nuorempien naisten lihaksissa. Vanhempien naisten lihasten estrogeeni- ja testosteronipitoisuus oli suurempi kuin nuoremmilla naisilla, vaikka steroidihormonien määrä verenkierrossa oli päinvastainen. On huomattava, että osa lihasnäytteistä mitatuista hormoneista voi olla peräisin kudosta ympäröivästä tai lihassolujen väliin sijoittuvasta rasvakudoksesta. Osoituksena siitä, että paikallinen steroidihormonien synteesi ei riitä kompensoimaan kaikkia ikääntymiseen liittyviä muutoksia, vanhempien naisten lihasten ominaisuudet olivat heikommat kuin nuoremmilla naisilla. Toisaalta ainakin vaihdevuosia seuraavien ensimmäisten viiden vuoden aikana käytetty HRT tai toteutettu PT aiheuttivat hyödyllisiä muutoksia lihasten geenien luentaan, mikä näkyi myös fenotyyppitasolla paremmin säilyneinä tai jopa parantuneina lihaksen ominaisuuksina verrattuna kontrolliryhmään.

Tämän väitöskirjatutkimuksen tulosten perusteella voidaan todeta, että vaihdevuosiin liittyvät lihasten ominaisuuksiin vaikuttavat geenien luennan muutokset tasoittuvat HRT:n tai PT:n vaikutuksesta. Lihaskudos voi myös tuottaa steroidihormoneja itse tai hyödyntää ympäröivän rasvakudoksen tuottamia hormoneja ylläpitääkseen omaa toimintaansa. Tämä ei kuitenkaan riitä säilyttämään lihasten ominaisuuksia nuorempia naisia vastaavalla tasolla. Lisää tutkimuksia tarvittaisiinkin, jotta täsmälliset molekyylibiologiset toimintamekanismit ja mahdollisesti HRT:n tai fyysisen harjoittelun vaikutukset näihin mekanismeihin selviäisivät.

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