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Towards Powerful Old Age

Association between Hormone Replacement Therapy and Skeletal Muscle

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ABSTRACT

The purpose of this study was to investigate the association between postmenopausal hormone replacement therapy (HRT) and the condition of skeletal muscle, and especially the mechanisms underlying the plausible link. To assess the role of HRT in preserving adequate quality and function of muscle tissue, identical 54-62-year-old female twin pairs (n=15 pairs) discordant for the long-term use of postmenopausal HRT were invited to the laboratory. Muscle biopsies were drawn to carry out microarray analysis (n=11 pairs) and to build up gene expression profiles reflecting the use of HRT. A special focus was laid on investigating the contribution of IGF-1 signaling pathway on improved muscle mass. This was achieved by examining the effects of year-long HRT use (n=10) compared to placebo (n=9) on the gene expression along the IGF-1 signaling cascade, and by studying the influence of the effective agents in the respective HRT preparation on gene expression and protein phosphorylation along the respective route in C2C12 myotubes. Also the association of two estrogen-related single nucleotide polymorphisms (SNPs), namely variation within catechol-*O*-methyltransferase (COMT) and estrogen receptor α, with muscle properties directly or in combination with physical activity were investigated in women aged 63 to 76 years (n=434). HRT was associated with better mobility and muscle power as well as favorable body and muscle composition among postmenopausal women. Gene expression analysis suggested that these links were at least partly modulated by enriched expression of biological processes concerning regulatory actions on cytoskeleton, intramuscular extracellular matrix and energy metabolism. Also IGF-1-related activation of PI3K/Akt pathway is suggested to represent one player behind the connection between HRT and muscle mass. When it comes to genetic variation a functional SNP within the *COMT* gene, affecting the activity of the enzyme, was associated with muscle mass. Furthermore, sedentary individuals with potential high enzyme activity were the most prone to muscle weakness, but also appeared to benefit the most from physically active lifestyle underscoring the notion that genetic predisposition into unfavorable muscle properties may be compensated for by lifestyle. Altogether, the results imply that HRT is positively associated with the condition of skeletal muscle and may represent one factor underlying the deterioration of muscle properties in postmenopausal women. The results suggest that molecular mechanisms related to regulation of cellular integrity, intramuscular extracellular matrix, energy metabolism, and perhaps to IGF-1 signaling at least partly modulate the observed association between the HRT use and muscle properties.

Keywords: skeletal muscle, hormone replacement therapy, estrogen, aging, muscle weakness, gene expression, twin study, candidate gene

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Jyväskylä, November 2010

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals I-IV.

I   Ronkainen, P.H.A, Kovanen, V., Alén, M., Pöllänen, E., Palonen, E-M., Ankarberg-Lindgren, C., Hämäläinen, E., Turpeinen, U., Kujala, U.M., Puolakka, J., Kaprio, J., Sipilä, S. 2009. Postmenopausal hormone replacement therapy modifies skeletal muscle composition and function: a study with monozygotic twin pairs. Journal of Applied Physiology 107, 25-33.

II   Ronkainen, P.H.A., Pöllänen, E., Alén, M., Pitkänen, R., Puolakka, J., Kujala, U.M., Kaprio, J., Sipilä, S., Kovanen, V. 2010. Skeletal Muscle Transcriptome and Postmenopause: A Study with Identical Female Twin Pairs. Aging Cell 9, 1098-1110.

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ABBREVIATIONS

Akt	v-Akt Murine Thymoma Viral Oncogene also called protein kinase B (PKB)
APP	amyloid protein beta precursor protein
AR	androgen receptor
BAD	Bcl-2 associated death agonist
BIA	bioelectrical impedance
BMI	body mass index
CO	control
COMT	catechol-O-methyl transferase
cDNA	complementary DNA
cRNA	complementary RNA
CSA	cross-sectional area
CytB	cytochrome B
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified eagle medium
DZ	dizygotic
E_1	estrone
E_2	17β-estradiol
ERα/ESR1	estrogen receptor α/1
ERβ/ESR2	estrogen receptor β/2
FDR	false discovery rate
FOXO	forkhead box O
FSH	follicle-stimulating hormone
GEE	generalized estimating equations
GLUT4	glucose transporter 4
GPR30/GPER	G-protein coupled ER1
HGS	hand grip strength
HRT	hormone replacement therapy
HWS	habitual walking speed
IGF-1	insulin-like growth factor 1
IPD	intrapair difference
KES	knee extension strength
LBM	lean body mass
LEP	leg extension power
MGF	mechano growth factor
MWS	maximal walking speed
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
MZ	monozygotic
NETA	norethisterone acetate
PCR	polymerase chain reaction
PDC	pyruvate dehydrogenase complex

PI3K	phosphatidylinositol 3-kinase
pQCT	peripheral quantitative computed tomography
QCT	quantitative computed tomography
qPCR	quantitative PCR
RFLP	restriction fragment length polymorphism
RCT	randomized controlled trial
SDH	succinate dehydrogenase
SHBG	sex hormone-binding globulin
SNP	single nucleotide polymorphism
T	testosterone
VEGF	vascular endothelial growth factor

CONTENTS

1 INTRODUCTION

Aging is inevitably orchestrated by muscle wasting and weakness, processes referred to as sarcopenia. Hormonal, neuronal, immunological, nutritional and physical activity-related mechanisms precede the development of a sarcopenic phenotype bearing consequences such as overall frailty and fragility, loss of independence, hospitalization and even mortality. Aside from its sequela in deteriorating individuals' quality of life, sarcopenia is also a tremendous societal burden estimated to be associated with 1.5 % of total health care costs in the United States. Thereby, the poor condition of skeletal muscle can readily be regarded as a severe geriatric syndrome.

According to current predictions by year 2030 approximately one out of eight persons will exceed the age of 65 years. Rather than concentrating solely on the investigation aiming at extending longevity, recent research involves a vast amount of attempts to extend health span and thus promote the concept of healthy aging among older citizens. The importance of the absolute mass of the musculature and the agents governing it is justified, since muscle serves as a major reservoir for proteins needed to withstand resistance to disease conditions, is an essential player in respiration and a site for various metabolic activities and also aids the body in recovering from critical illnesses.

At this point understanding the mechanisms underlying the improvement or preservation of muscle function comes into play. During the last decades, the usability of a portfolio of interventions with variable treatments to combat sarcopenia and mitigate the problem with physical frailty has been carefully studied. Studying the mechanisms underlying the deterioration or preservation of muscle properties is, however, not that simple. The mammalian cells are under constant bombardment of variable signals from the surroundings and should have the capacity to respond properly. Several growth factors, for instance, predispose the target cell to a situation, in which the decisions related to growth, differentiation or proliferation, or alternatively to atrophy or apoptosis have to be made. Thus far, the most promising results come from interventions concerning with physical activity and especially resistance

training. To decisively improve public health strategies in this area also other interventions have earned the focus of the scientific community worldwide.

Albeit the age-related decline of multiple physiological systems including the musculature is universal in the human population, women are more prone to loosing functional independence, since they typically have longer lifespan and at any given age exhibit lower muscle mass and strength than men. The menopausal transition, characterized by a decline in the production of estrogen from ovaries, and the following postmenopausal era entail rapid loss of muscle properties. The knowledge on how estrogen actually affects the phenotype of one of its non-classical target tissues, the skeletal muscle, is so far somewhat inconsistent not to mention the related molecular mechanisms, which are merely poorly identified. Data from human studies show that estrogen receptors (ERs) are found in skeletal muscle tissue in both the muscle fibers and in the capillaries. Studies utilizing animal models or cell cultures have suggested that estrogen is an important factor in the maintenance of glucose homeostasis, functions at least partly through mitochondria, may aid the muscle in recovering from damage, and may have some anti-apoptotic influence in the milieu of skeletal muscle. The data on the biological processes affected by estrogen in human skeletal muscle are, however, scanty.

Hormone replacement therapy (HRT) is a worthwhile option for women to relieve menopausal symptoms. Estrogen-based therapies were first introduced more than 60 years ago and have since been widely utilized by women in their postmenopausal era. Although highly beneficial in relieving typical postmenopausal symptoms, the use of HRT decreased even 20-60% in various countries after the publication of the disputed WHI trial. A 26% decline was observed in Finland being the most dramatic in 50-59-year-old women. The findings easily prompted women undergoing the expected midlife event, i.e. the menopause, to believe that HRT may indeed be detrimental to health. The post-mortem of the publication has, however, included discussion, whether the results in WHI were somewhat too drastically presented. Later reports concerning the WHI trial showing for instance that the participant starting the use of HRT between the ages of 50 and 59 years had 30% lower mortality in comparison with the women with placebo have to some extent escaped the notice of media. Aside from studying health risks or predisposition to diseases, postmenopausal women using HRT and their counterparts with no treatment represents a strong study model to be reckoned with if the link between the absence or the replacement of estrogen and a given phenotype is investigated.

Towards assessing the potential of postmenopausal HRT as an element of powerful aging, the focus of the present study was to elucidate the association between HRT and the condition of the musculature and especially to identify the biological processes possibly creating the respective link. A through understanding concerning the biomolecular mechanisms triggering the age-related impairments of the musculature has to be achieved in order to reach the goal of maintaining adequate functional capacity and related independence until the individual limit of chronological age.

2 REVIEW OF THE LITERATURE

2.1 Sarcopenia

The original anecdotal recognition concerning the loss of skeletal muscle mass with age emerges from Shakespeare's monologue "The Seven Ages of Man" from the 17th century. This process was further brought to the forefront of science in 1989 by Dr. Irwin Rosenberg, who pointed out that the decline in lean body mass is the most dramatic and potentially the most functionally significant decline of the systems in the human body upon aging and suggested the process be called sarcopenia, a term from the Greek referring to "poverty of flesh" (Rosenberg 1989, Rosenberg 1997).

To date, instead of covering only the loss of muscle mass, sarcopenia is used rather loosely as a catch-all term to encompass the age-related decline of both muscle mass and strength. Aside from sarcopenia, a novel term dynapedia, referring to "poverty of strength" has been introduced (Clark & Manini 2008), but it is yet to be seen, whether the scientific community will approve this term to be utilized in practice. Only recently, the European Working Group on Sarcopenia in Older People (EWGSOP) gathered experts from its four participant organizations with the aim of developing a practical, thus far lacking, clinical definition and diagnostic criteria for sarcopenia. The authoritative panel decided to recommend the term sarcopenia to describe both low muscle mass and low muscle function, more specifically strength or power (Cruz-Jentoft et al. 2010). Although the exact terminology in this arena is only beginning to become exact, sarcopenia may be regarded to represent an important phenomenon related to aging of the neuromuscular system and to include the decline of both muscle mass and strength.

2.1.1 Overview of the structure and function of skeletal muscle

In order to understand the characteristics of sarcopenia and the underlying processes the basic structure and function of skeletal muscle, a highly specialized organ, should be clarified. Muscles are organized into fascicles

containing several muscle fibers, i.e. muscle cells (Figure 1). Muscle fibers lie parallel to each other and extend along the entire length of the muscle or are arranged in a feather-like manner. Individual fibers contain well-defined contraction units – the myofibrils – in their cytoplasm, while mitochondria and smooth endoplasmic reticulum among others occupy the intermediate space between the myofibrils (Cross & Mercer 1993, Guyton & Hall 2006). Longitudinal examination of a myofibril reveals a precise striated organization of actin and myosin, two important proteins in muscle contraction. During muscle contraction the thick filaments, composed of myosin molecules, and the thin filaments, consisting of actin molecules, slide past each other. According to this accepted sliding filament theory the so-called myosin heads attach to actin and propel them in a new location using the energy derived from the hydrolysis of adenosine triphosphate (ATP) (Huxley & Hanson 1954, Huxley & Niedergerke 1954, Huxley 1958). The traditional classification of muscle fibers includes three categories, which hydrolyze ATP in different speeds: the slow type I, the fast type IIa and the fastest type IIx fibers (Brooke & Kaiser 1970, McComas 1996, Harridge 2007)**.** Each muscle fiber, whatever the MHC type, is surrounded by sarcolemma and basal lamina. Small neuronal branches and capillaries are embedded in endomysium, a fine layer of connective tissue just above basal lamina. Perimysium, another layer of connective tissue, holds together a bundle of muscle fibers forming a fascicle and contains the arterioles, venules and nerves. A sheath of connective tissue, which envelopes all the fascicles, is called the epimysium (Ross et al. 2003).

Skeletal muscle has the primary function of producing force and power for locomotion. The musculature is able to execute a wide array of movement patterns in everyday life reflecting the elegant design and plasticity of skeletal muscle. These stem from the intricate control that the nervous system has over the musculature, the diversity of muscle fiber types, the carefully designed architectural arrangements, interplay with elastic elements, and ability to utilize an array of fuels as energy (Saltin & Gollnick 1983, Harridge 2007). During voluntary contraction, an action potential activating a group of muscle fibers is originally generated at the motor cortex and reaches the neuromuscular junction through upper motor neuron following the activation of an efferent α-motor neuron, which propagates the signal quickly to the target cells. Finally, the signal evokes calcium release from the sarcoplasmic reticulum in these target cells and initiates a synchronous contraction of the muscle fibers constituting the motor unit via interaction of actin and myosin on the edge of the sarcomere (Huxley 1988, Fluck & Hoppeler 2003).

Muscle increases its size via mechanisms mediated by satellite cells, a pool of myogenic precursor cells lying underneath the basal lamina, just above sarcolemma and responding to wear and tear of exercise. A group of mechanical, hormonal and nutritional signals result in increased protein synthesis and employment of new nuclei into the growing fibers. This process aims at maintaining the proper ratio between the cytoplasm and the myonuclei

i.e. the myonuclear domain and thus at responding to the increased demand of transcriptional activity (Allen et al. 1999, Kadi et al. 1999, Harridge 2007).
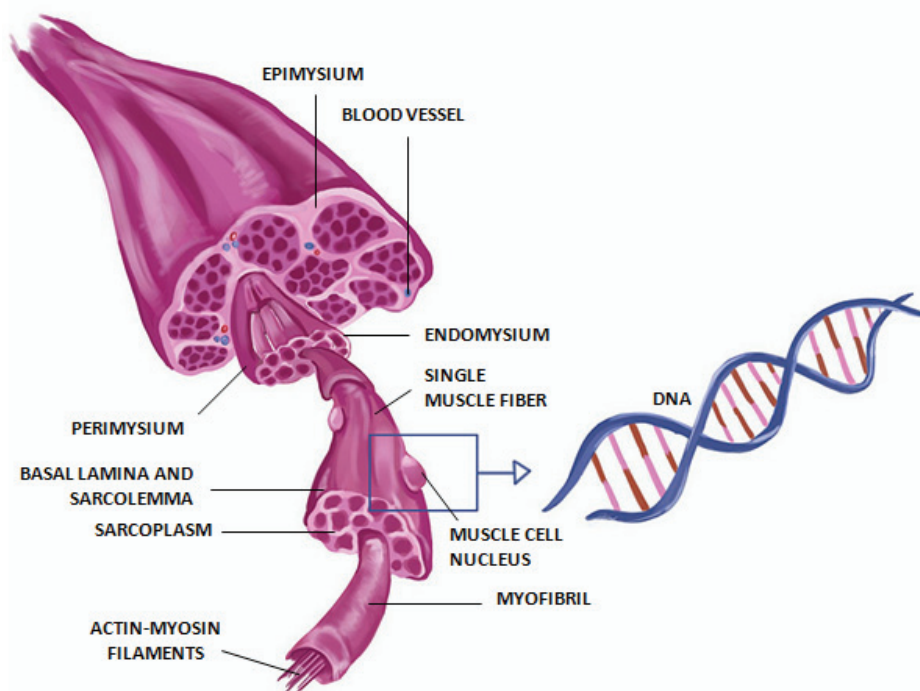
FIGURE 1   Schematic figure of the structure of skeletal muscle with multinucleated muscle fibers. Artistic view was created by Julia Vornanen.

2.1.2 Loss of muscle mass with age

The best possible preservation of muscle mass throughout the human life span is of immense importance, since this tissue, constituting about 40% of the total mass of the body, is a highly specialized contractile organ moving the entire human body. According to a classical, cross-sectional study of high repute authored by Lexell and colleagues, the decline in muscle cross-sectional area (CSA) was estimated to average 40% over the era of sixty years beginning from the age 20 years (Lexell et al. 1988). Elsewhere, the decline in muscle mass has been estimated to average 0.4 to 0.8 kilograms per decade, starting at 20 years of age (Gallagher et al. 1997). The prevalence of sarcopenia, when defined as the loss of muscle mass greater or equal than two standard deviations (SDs) below the young reference mean, is reported to range between six and twenty-seven percent depending on the age group and the study (Baumgartner et al. 1998, Melton et al. 2000, Janssen et al. 2002, Castillo et al. 2003). Some of the earlier studies suggest that the decrease in lean body mass (LBM) is higher in the lower limbs (~15%) compared to the upper limbs (~10%) (Janssen et al. 2000), while women have considerable lower muscle mass than men at any given time. A

study by Baumgartner and colleagues, for instance, showed a sarcopenia prevalence of 24% in postmenopausal women (<70 years old), while the corresponding number was 15% in men (<70 years old) (Baumgartner et al. 1998). Whatever the exact speed of the respective process, it most certainly is of high importance and is characterized by deterioration of the quality of the tissue as well. Studies utilizing various imaging techniques have clearly pointed out that the loss of muscle tissue itself is observed along with infiltration of fat and connective tissue (Sipilä & Suominen 1994, Sipilä & Suominen 1995, Jubrias et al. 1997, Harridge et al. 1999, Kent-Braun et al. 2000).

Loss of muscle mass involves the reduction in both muscle fiber size and number. Plausible documentation shows that type II fibers are more vulnerable to atrophy in comparison with type I fibers (Larsson et al. 1978, Lexell et al. 1983, Klitgaard et al. 1990b). In addition to this process, fibers expressing both type I and type II myosin heavy chains are more pronouncedly observed in aged compared to young muscle (Klitgaard et al. 1990a, D'Antona et al. 2003). Andersen has even reported that muscle fibers of very old persons in comparison with young muscle switch fiber type along the length of the fiber or exhibit areas or myonuclear domains with MHC composition different from the rest of the fiber (Andersen 2003). On average 30-40% of all muscle fibers are lost by the age of 80 years, partially due to apoptosis (Dirks & Leeuwenburgh 2002, Dirks & Leeuwenburgh 2005). According to the current general opinion, the loss of fiber number is phased in that up to late 70s primarily type II fibers and after 80s also type I fibers are lost and finally a new "balance" in fiber type composition is reached (Andersen 2003). Besides the changes in the size and the total number of the muscle fibers, also the entire architecture of the muscle tissue is modified upon aging. More precisely, not only is the length of the fiber fascicles, predicting the loss of muscle shortening velocity, diminished, but also their angle of insertion into the tendon aponeurosis, predicting deterioration in muscle force generating potential, decreased with age (Narici et al. 2003, Thom et al. 2007). Due to the fact that muscle power is the product of force and velocity, the above-mentioned changes in muscle architecture probably play a noteworthy role during the aging process and the development of disability (Thom et al. 2007). The contribution of muscle mass to loss of muscle strength is discussed in the following chapter.

2.1.3 Loss of muscle strength and power with age

There are numerous factors beyond the loss of muscle mass that ultimately are associated with muscle weakness among older population (Clark & Manini 2008). Studies dissecting the changes in strength owing to increased or decreased physical activity level underline the disassociation between mass and strength. Firstly, the early phases of resistance training program are characterized by improved muscle strength, while morphological changes in the muscle tissue required for increased force generating capacity are not yet elicited (McDonagh et al. 1983, Young et al. 1985). Secondly, leg unloading with

the duration of four weeks has been shown to result in 15% loss of muscle strength and 9% loss of mass (Clark et al. 2006). In the same study "muscular factors", primarily the function of sarcolemma, were reported to explain ~40% of the loss of strength, whereas "neurologic factors", mainly the deficit in central activation, accounted for 50% of the respective process.

In healthy adults, the first signs of naturally occurring muscle-related deterioration is manifested as mild impairment in contractile function becoming apparent in the fourth decade of life (Doran et al. 2009). Cross-sectional data suggest that the annual decline of isometric muscle strength is on average 0.5-1.0% starting from about forty years of age (Hortobagyi et al. 1995, Lindle et al. 1997, Samson et al. 2000, Akima et al. 2001, Lauretani et al. 2003). A set of follow-up studies have indicated an even greater annual decline of strength ranging from 1.5% to 4.1% after 60 years of age (Winegard et al. 1996, Rantanen et al. 1998, Hughes et al. 2001, Goodpaster et al. 2006). The decline in muscle power, on the other hand, starts earlier and progresses faster compared to the loss of strength (Bassey & Short 1990, Bassey et al. 1992, Metter et al. 1997, Bean et al. 2002). Cross-sectional analyses concerning the loss of muscle power with age have resulted in an estimation of 3.5% decline annually (Bassey & Short 1990, Skelton et al. 1994).

The etiology of or the interrelationship between the loss of muscle mass and strength are still somewhat enigmatic and have actually proven to be quite intricate processes. The loss of muscle mass in particular can be considered to reflect a progressive withdrawal of anabolism and increased catabolism accompanied by reduction in muscle regeneration capacity. Aside from being a burden due to added inert mass, this loss of muscle quality due to infiltrated fat is also suggested to maintain sarcopenia via the release of pro-inflammatory cytokines and adipokines by infiltrated macrophages (Neels & Olefsky 2006). Reviews on the issue have summarized that a concomitant 2-4-fold increase in serum levels of inflammatory markers among the elderly individuals creates a catabolism-favoring environment suggested to be associated with reduced muscle mass (Ferrucci et al. 2002, Roubenoff 2003, Krabbe et al. 2004). The process referred to as sarcopenic obesity is closely connected to accelerated decline in functional capacity and increased risk of diseases and mortality. Ultimately, a vicious circle characterized by further loss of muscle mass and strength, progressive insulin resistance and a severe risk for developing metabolic syndrome has begun (Roubenoff 2004).

Neural factors are quite certainly known to significantly affect the decline of both muscle strength and power (Häkkinen et al. 1998, Lauretani et al. 2003, Kamen 2005, Christie & Kamen 2006). In addition to inflammation and defects in the nervous system, a menu of other possible mechanisms underlying the chain of events resulting in, or accelerating the progression of sarcopenia has been described, but the precise contribution of each is largely tentative. The list of events involves for example individual genetic background, early life developmental influences, sedentary lifestyle, poor nutritional intake, boosted oxidative stress, mitochondrial dysfunction, chronic diseases, certain drug

treatments and last, but not the least, hormonal changes (Baumgartner et al. 1999, Roubenoff & Hughes 2000, Doherty 2003, Dirks et al. 2006, Lee et al. 2007, Paddon-Jones et al. 2008, Sayer et al. 2008, Buford et al. 2010). At the level of individuals, several mechanisms underlying the sarcopenic phenotype may exist concomitantly, while the relative contributions of each may change over time. Moreover, people age at different rates in that some remain fit and strong longer, whereas others become frail and weak at relatively young age.

The decline in muscle strength and power is especially noteworthy as it may eventually predispose older people to mobility limitation and consequent falls and fractures having enormous impact on overall disability (Roubenoff & Castaneda 2001, Doherty 2003, Greenlund & Nair 2003, Lauretani et al. 2003, Rolland et al. 2008). Muscle weakness is clearly associated with impaired ability to perform mobility tasks such as rise from a chair (Alexander et al. 1997), walk fast (Bassey et al. 1992), and climb stairs (Jette & Jette 1997), thereby contributing to decreased independence and quality of life. Muscle weakness has even been related to increased mortality (Guralnik et al. 2000), again reinforcing the significance of the poor condition of skeletal muscle as a severe geriatric syndrome.

The special focus of this thesis is to assess the association between HRT and skeletal muscle – and especially the underlying mechanisms – in this highly complex area and thus the following chapters describe the general features of estrogen signaling followed by description of the current knowledge concerning estrogen, partly in the form of HRT in the milieu of skeletal muscle.

2.2 Estrogen signaling

Estrogens are steroid hormones synthesized from cholesterol. The most potent estrogen is 17β-estradiol (E_2), but lower levels of two other estrogens, estrone (E_1) and estriol (E_3), are also present. The relative concentrations of the three estrogens change after menopause E_1 becoming the predominant one (Gruber et al. 2002, Nelson 2008). In the production of estrogens, cholesterol is transferred from the cytosol into the inner membrane of the mitochondrion, a location in which cytochrome P450 enzymes catalyze the cleavage of the side chain of cholesterol. The process is the rate-limiting step in steroid production. The last step in the formation of estrogens from their obligatory precursors, androstenedione and testosterone (T), is aromatization that is catalyzed by the P450 aromatase monooxygenase enzyme complex, which is located in the smooth endoplasmic reticulum (Gruber et al. 2002). In the reproductive era, the primary sources of estrogen are the theca and granulosa cells of the ovaries (Hillier et al. 1994, Lieberman 1996). In the postmenopausal period estradiol is mainly produced via extragonadal conversion of testosterone (Gruber et al. 2002). Aside from the gonadal tissues, the activity of aromatase has also been reported in skeletal muscle (Matsumine et al. 1986), nervous tissue (Naftolin et

al. 1975), fat (Miller 1991), and testes (Brodie & Inkster 1993). The primary receptors mediating the effects of estrogen and the following signaling pathways are approached below via generalized models.

2.2.1 Receptors for estrogen

The scientific expedition to understanding the detailed mechanisms of estrogen action was launched in the 1950s by the pioneering work carried out in the Jensen's laboratory leading to identification of the classical ER referred to as ERα (or ESR1) (Jensen & DeSombre 1973). The following decades with huge developments in biochemical techniques allowed more precise investigation concerning the structure and action of ERα as a transcriptional regulator. In the 1990s, the area became more complicated as another ER, namely ERβ (or ESR2), was discovered and a wealth amount of data concerning the interaction of ERs with a plethora of co-regulatory proteins started to accumulate (Kuiper et al. 1996, Chambon 2005, O'Malley 2005). As a response to estradiol, ERβ is known to activate the same genes as ERα, albeit generally with less efficiency (McDonnell & Norris 2002). Moreover, ERβ is a dominant inhibitor of ERα transcriptional activity in cells expressing both of the receptors (Hall & McDonnell 1999).

Each of the two classical ERs is encoded by a unique gene. Both belong to the superfamily of nuclear receptors and share a hallmark modular structure with five distinguishable domains. The DNA- and ligand-binding domains exhibit a high degree of homology between the ERs (97% and 60%, respectively), thus declaring similar affinity of these two for identical DNA sequences and for an array of endogenous, synthetic and naturally occurring estrogens (Kuiper et al. 1997). The majority of ERα and ERβ are typically found in the cytoplasm or alternatively in the nucleus. A small proportion (2-10%) is thought to associate with the plasma membrane (Luconi et al. 1999, Norfleet et al. 2000, Monje & Boland 2001, Monje et al. 2001, Ropero et al. 2002, Li et al. 2003), while a minute amount of ERs are also found in mitochondria (Zheng & Ramirez 1999, Horvat et al. 2001, Monje & Boland 2002, Yang et al. 2004, Solakidi et al. 2005) and in the intracellular membranes (Parikh et al. 1980, Watson & Muldoon 1985, Muldoon et al. 1988, Monje & Boland 1999, Watson et al. 1999) (Figure 2A). The tissue expression patterns of ERα and ERβ are somewhat distinct. ERα predominates in the uterus and the mammary gland, while ERβ is established in the central nervous, cardiovascular and immune systems; urogenital tract, bone, kidney, and lungs (Couse et al. 1997, Shughrue et al. 1997, Gustafsson 2000, Taylor & Al-Azzawi 2000).

Moreover, careful investigation of ER knockout mice suggests differential biological functions for this duo; the phenotypes of αERKO and βERKO mice are contrasting, but both survive to adulthood, albeit exhibit retarded growth. The most striking phenotypes in female αERKO mice can be listed as estrogen insensitivity in the reproductive track, hypergonadotropic hypergonadism, lack of pubertal mammary gland development, and excess adipose tissue, while the

major factors in male mice are testicular degeneration and epididymal dysfunction (Couse & Korach 1999). Furthermore, both sexes of αERKO mice are infertile. On the contrary, βERKO male mice are fertile and thus far show no obvious phenotypes, whereas βERKO females have inefficient ovarian function and are subfertile. The combined knockout model, αβERKO, heavily resembles the phenotypes of αERKO (Couse et al. 1999, Dupont et al. 2000). In αERKO males the release of endothelium-derived nitric oxide (NO) is decreased (Rubanyi et al. 1997) and estrogen-mediated production of NO dismantled (Pendaries et al. 2002). Confirmatory data on ERs in cardiovascular health come from Zhu and colleagues documenting that βERKO mice develop hypertension with age in both genders (Zhu et al. 2002). Moreover, αERKO mice of both sexes have insulin resistance and impaired glucose tolerance (Heine et al. 2000). Supporting data come from an extreme case-study with a man exhibiting a premature stop codon in the 2nd exon of the ERα gene. This male patient had no functional receptors and significant glucose intolerance (Smith et al. 1994). In addition to the above-mentioned data on the differential biological functions between ERα and ERβ, both are expressed as several diverse splice variants (Poola et al. 2002a, Poola et al. 2002b, Poola 2003), whose detailed function is only poorly described, complicating the area even more.

In addition to the obvious complexity of estradiol signaling even in the context of ERα and ERβ, the discovery of a third receptor mediating the action of estrogen opened a true Pandora's box for the scientific community captivated by estrogen signaling. Several recent articles (Prossnitz et al. 2008, Prossnitz & Maggiolini 2009, Maggiolini & Picard 2010) carefully review the current knowledge concerning this 7-transmembrane, G-protein coupled receptor, entitled GPR30 or GPER (G-protein coupled ER1) originally identified simultaneously by several groups in the late 1990s (Owman et al. 1996, Carmeci et al. 1997, Takada et al. 1997, O'Dowd et al. 1998). GPR30 is nowadays accepted as a mediator of some of the rapid signaling events, such as calcium mobilization and kinase activation, in response to estrogen, while also regulation of rapid transcriptional activation of oncogene *c-fos*, used as an early molecular sensor for estrogen action, have been described (Kanda & Watanabe 2003, Maggiolini et al. 2004, Albanito et al. 2007). Importantly, GPR30 is reported to likely bind estrogen (Revankar et al. 2005, Thomas et al. 2005). GPR30 knockout mice show hardly any changes in phenotype and no reproductive or mammary gland disruption of structure or function (Levin 2009).

2.2.2 Pathways mediating the effects of estrogen

Estrogen is able to induce the desired physiological response within hours, or more rapidly, in the time frame of seconds or minutes (e.g. Kelly & Levin 2001, Segars & Driggers 2002, Björnström & Sjöberg 2005, Hewitt et al. 2005b). The ultimate outcome of the complex signaling events depends on several conditions such as age, gender, duration of exposure and the amount of the

ligand. A simplified view covering estrogen signaling is presented in Figure 2B. To start with, estrogen signaling is a topic with constant development and far from being a simplified, clearly delineated arena. For the clarity of the present thesis, the various types of signaling events are described separately, although it is extremely important to conceptualize estrogen action *in vivo* as collateral, possibly divergent network of pathways instead of a linear signal.

Along the "classical" or "genomic" pathway of estrogen action, exerting its effects typically with a time lag of hours, this uncharged steroid molecule passively diffuses into the cell and binds to ER located in the cytoplasm or in the nucleus. The estrogen-ER complex further sits on a particular section of DNA either directly on the estrogen response element (ERE) sequences or indirectly through highly specific protein-protein interactions (non-ERE-dependent signaling) with for instance AP1, SP1 or NF-κB (Porter et al. 1997, Björnström & Sjöberg 2005). Co-regulatory proteins are recruited into the gene expression machinery in the promoter region and the expression of the given mRNA increased or decreased leading to the physiological response initially on order. This mechanism is also referred to as ligand-dependent stating that in the absence of the hormone, the receptor is sequestered in a multiprotein inhibitory complex located in the nucleus of the target cell (Hall et al. 2001, McDonnell & Norris 2002, Edwards 2005, O'Malley 2005, Deroo & Korach 2006). The cell specific conditions such as relative balance of receptors, coactivators and corepressors is critical in determining, whether this classical pathway initiates responses or not.

The "rapid response" models of estrogen action work within seconds or minutes. These processes are sometimes also designated as "non-genomic", but this misleading term is avoided here, as only some portion of the cytoplasmic signaling cascades involving estrogen affect via gene expression and others are totally independent of transcription. The rapid signaling events emerging from the plasma membrane were first described forty years ago in Clara Szego's laboratory (Szego & Davis 1967, Pietras & Szego 1977). The mechanisms can be divided into two types; the first depends on the ability of estrogen to interact with ERs located in the plasma membrane, while the second emerges from the binding of estrogen to another membrane-associated estrogen-binding protein such as GPR30. Both of these systems lead to the activation of several kinases and finally a specific cellular response (Driggers & Segars 2002, Segars & Driggers 2002, Lorenzo 2003, Hewitt et al. 2005b).

When it comes to signaling via ERs, also ligand-independent activation is known to occur, i.e. activation of the receptors by for instance epidermal growth factor (EGF) or insulin-like growth factor I (IGF-1) instead of estrogen. These events typically depend on various phosphorylation cascades. For instance, the activation of EGF receptor by EGF leads to phosphorylation of ERα at Ser118 by p44/42 mitogen-activated protein kinase (MAPK) (Kato et al. 1995, Bunone et al. 1996). Similar activation of ERβ has also been reported (Tremblay et al. 1999). However, aside from the activation of ERs by MAPK, E_2 is also known to activate MAPK and phosphatidylinositol 3-kinase (PI3K) further inducing

mitogenesis in MCF-7 cells (Lobenhofer et al. 2000). Intriguingly, a "feed-forward" system in the E_2-MAPK-ER-axis involving also Ca^{2+} as a second messenger has been suggested. More precisely, E_2 have the capacity to activate MAPK, which in turn possesses the ability to phosphorylate ER (Improta-Brears et al. 1999).

The pathway along which E_2 phosphorylates PI3K can proceed into phosphorylation of a renowned serine-threonine kinase Akt (v-Akt Murine Thymoma Viral Oncogene also known as protein kinase B), a lynchpin of several signaling cascades in a range of mammalian cells and activated by several growth factors, cytokines, mitogens and other hormones as well (Fernando & Wimalasena 2004, Guo et al. 2006). Along this PI3K/Akt route, PI3K is responsible for the generation of phosphatidylinositol-3,4,5-triphosphate (PIP_3), which again results in the activation of Akt. Phospho-Akt, in turn, modulates the activation of a portfolio of substrates involved in the regulation of numerous biological responses all sharing the same outcome; inhibition of apoptosis and stimulation of cell proliferation (Burgering & Coffer 1995, Franke et al. 1997, Franke et al. 1997, Coffer et al. 1998, Bodine et al. 2001b, Xu et al. 2003). Rapid signaling of estrogen via MAPK or PI3K/Akt axis have also been shown to contribute to the generation of endothelial nitric oxide synthase (eNOS) in endothelial cells in response to estrogen treatment (Chen et al. 1999, Haynes et al. 2000, Simoncini et al. 2000). Enhancing the bioavailability of eNOS represents one manner by which estrogen exerts its rapid vasodilatory effects (Reis et al. 1994, Farhat et al. 1996, Guetta et al. 1997)

When it comes to typically distinct functions of GPR30 and classical ERs, the interrelationship between the receptors is evident in a study by Revankar and colleagues who reported that GPR30, but not ERα, stimulates PI3K activity in response to tamoxifen, an antagonist for estrogen, and that activation of PI3K by estrogen depends on the presence of GPR30. Furthermore, ERα was shown to mediate the activation of PI3K in response to estrogen, but this mechanism did not involve EGF receptor transactivation, which was required in the case of GPR30. To conclude, both ERα and GPR30 are capable of activating PI3K as a response to estrogen, but the respective receptors utilize separate signaling pathways and respond dissimilarly to tamoxifen (Revankar et al. 2005).
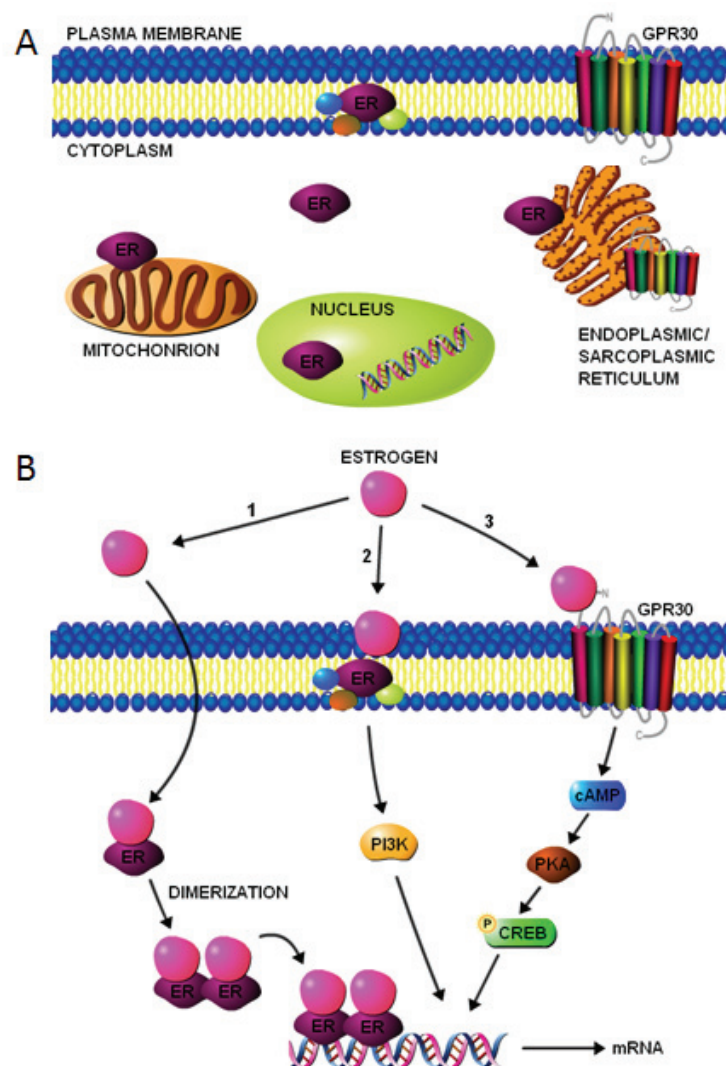
FIGURE 2   Figure 2A presents the suggested localization of estrogen receptors in mammalian cells. ERs are suggested to localize in the cytoplasm, nucleus, plasma membrane, mitochondria and intracellular membranes such as endoplasmic reticulum, while GPR30 is suggested to be found in the plasma membrane and also in the sarcoplasmic reticulum i.e. the endoplasmic reticulum of muscle fibers. Figure 2B presents a simplified diagram of estrogen signaling. 1) Along the classical signaling pathway E_2 binds to the estrogen receptor (ER). The resulting complex further sits directly to estrogen response elements, or alternatively indirectly to other response elements, together with multiple coregulatory proteins further leading to up- or down-regulated gene expression. 2) E_2 can induce rapid response via membrane-bound ERs acting coordinately with other membrane proteins, which further activate signaling cascades involving a spectrum of kinases such as PI3K, 3) or via non-ER membrane-associated proteins, such as GPR30. Figure 2A is adapted from the review by Du and colleagues (Du et al. 2006). The

schematic figures are generated utilizing the Pathway Builder of ProteinLounge[BETA] (www. proteinlounge.com). ER=estrogen receptor, PI3K=phosphatidylinositol 3-kinase, GPR30=G-protein coupled ER1, cAMP=cyclic adenomonophosphate, PKA=protein kinase A, CREB=cAMP response element-binding.

2.2.3 Postmenopausal period with and without hormone replacement: a model to study estrogen

One of the most important landmarks in female lifespan, confronted by all women, is the menopause resulting largely from reduced secretion of the ovarian hormones, namely estrogens and progesterone, as the finite store of ovarian follicles become depleted (Gruber et al. 2002, Nelson 2008). The decreased serum concentration of these hormones is orchestrated by a significant rise in the level of follicle-stimulating hormone (FSH) indicating that menopause has been launched. Twelve consecutive months of amenorrhea between 45 and 55 years of age not associated with any pathological cause leads to diagnosis of natural menopause (McKinlay et al. 1992). Since the mesenchymal cells of adipose tissue serve as the primary source of estrogen in the postmenopausal period (Simpson et al. 1997), the extent of adiposity largely determines the degree of estrogenization after menopausal transition (Sheffield-Moore & Urban 2004). Besides the above-mentioned hormonal changes, also the systemic concentrations of dehydroepiandrosterone (DHEA), DHEA sulphate, sex hormone-binding globulin (SHBG), growth hormone and T confront a clear decline in the postmenopause (Maltais et al. 2009).

The period just before or instantly after the onset of the menopausal era include several immediate symptoms such as hot flushes (Stearns et al. 2002), while the postmenopausal period is accompanied by a number of long-term consequences, for instance genital atrophy, osteoporosis, cardiovascular diseases, and decline in cognitive function (Kanis et al. 1994, Castelo-Branco et al. 2005, Atsma et al. 2006, Lokkegaard et al. 2006, Burns & Iliffe 2009), which all are associated with advancing age per se, but start to appear especially in the postmenopause. Comparing postmenopausal women with HRT to non-users provides a useful study model to assess the association between estrogen status and a given phenotype; the users serve as participants with high estrogen levels, while the non-users represent postmenopausal women without any counteractive treatment and loss of intricate production of estrogen from the ovaries.

2.3 Human skeletal muscle: a non-classical target of estrogen

2.3.1 Estrogen and muscle phenotype

Despite the quite ample research on sarcopenia and the related mechanisms, the contribution of estrogen to the condition of skeletal muscle is frequently neglected and studies mostly focus on other hormones such as testosterone. However, aside from the primary role of this ovarian steroid hormone in the establishment and maintenance of the reproductive function, it also targets a variety of other tissues, such as skeletal, immune, nervous and cardiovascular systems, as a regulator of growth and differentiation.

Intriguingly, there is a noteworthy amount of direct and indirect evidence indicating that the decline in circulating estradiol may affect skeletal muscle, one of its non-classical target tissues. In terms of muscle size, estrogen has been reported to affect the growth of murine myoblasts *in vitro* (Kahlert et al. 1997) and to associate with the development of muscle size *in vivo* in female mice (Sciote et al. 2001). In human subjects, the connection between estrogen and muscle size is more ambiguous as both positive associations and no significant links have been published. More precisely, a year-long double-blind randomized controlled trial (RCT) on 50-57-year-old women resulted in improved muscle size in the HRT users compared to placebo (Sipilä et al. 2001, Taaffe et al. 2005b), while a shorter RCT of 12 weeks documented a significant positive effect of HRT on lean body mass in women aged on average 55 years (Sorensen et al. 2001). A secondary analysis of a one-year exercise intervention supported this view by showing a gain of lean soft tissue in women in the non-exercising control group voluntary using HRT already before the actual intervention (Teixeira et al. 2003). Two cross-sectional studies have as well reported either positive association between serum estradiol and muscle mass (Iannuzzi-Sucich et al. 2002) or better muscle mass and composition within HRT users than the non-users in older women (Taaffe et al. 2005a). On the contrary, randomized controlled (Tanko et al. 2002), randomized open (Skelton et al. 1999) and non-randomized (Brown et al. 1997, Maddalozzo et al. 2004) interventions with the duration between six months and three years on participants ranging from 45 to 72 years of age have reported no effect of HRT on lean tissue mass or exact muscle mass, with some support from cross-sectional data (Taaffe et al. 1995, Bemben & Langdon 2002). Importantly, however, women have been proposed to experience accelerated loss of mass and strength at an earlier age than men, more precisely around the time of menopause (Petrofsky et al. 1975, Phillips et al. 1993, Stanley & Taylor 1993, Calmels et al. 1995, Lindle et al. 1997) with a speed of mass decline proposed to equate to 0.6 % per year after menopause (Rolland et al. 2007). Also muscle quality has been suggested to be affected in that the amount of intramuscular fat would increase after menopause (Forsberg et al. 1991, Brown 2008).

With respect to muscle strength, it is also lost concurrently with menopause. For instance a study by Greeves et al. showed that women on HRT preserved their muscle strength along the 39-week follow-up period in comparison with the women having no counteractive treatment, who lost on average ten percent of their strength (Greeves et al. 1999). Moreover, a recent systematic review gathering data on twenty-three relevant studies concerning the association between estrogen-based HRT concluded that although individual studies are inconsistent the treatment had overall beneficial effect on muscle strength that equated to on average five percent advantage in strength parameters (Greising et al. 2009). In another review, also tibolone was found to have positive effects on muscle strength in addition to beneficial effects on body composition (Jacobsen et al. 2007). When it comes to muscle power, an important element of overall muscle function, and the influence of estrogen the findings are ambiguous. While some cross-sectional studies (Seeley et al. 1995, Uusi-Rasi et al. 2003) and a 48-week long RCT (Armstrong et al. 1996) report non-existing connection between these two, other studies including data from RCT (Sipilä et al. 2001, Taaffe et al. 2005b) or cross-sectional design (Carville et al. 2006) suggest that HRT would actually result in better muscle power. The same applies to studies investigating muscle strength during the menstrual cycle: increases in strength during the follicular and mid-cycle phases of the menstrual cycle, i.e. as the levels of estrogen are rising or are at their highest, have been documented (Phillips et al. 1996, Sarwar et al. 1996), as against also no significant changes have been discerned in other studies (Bassey et al. 1996, Elliott et al. 2003). A possible link between mobility and the use of HRT is rather poorly studied. Taaffe and colleagues have reported improved running speed over 20 meters after year-long HRT in 50-57–year-old women, while two cross-sectional studies with participants aged over 65 years (Seeley et al. 1995) or 70-79 years (Taaffe et al. 2005a) found no differences between HRT users or non-users in walking speed. Taking into account all the reports available, the possible connection between the use of HRT and muscle properties is still open to dispute.

2.3.2 Indications of estrogen signaling in human skeletal muscle

Despite the somewhat confusing evidence concerning the link between estrogen and the condition of human skeletal muscle at the phenotype level, a decent amount of molecular data suggest that these two are not that insignificant to each other. In addition to fairly scarce data from human studies, a series of animal and cell culture studies gives some insights into the putative mechanisms by which estrogen may affect human muscle tissue as well. To start with, both ERα and ERβ are expressed in human skeletal muscle tissue of both genders (Lemoine et al. 2003, Wiik et al. 2003, Wiik et al. 2005a, Wiik et al. 2009), thus believably proposing muscle as a target tissue for estrogen signaling. The most recent advance in identifying the expression of ERα in about two-thirds of the nuclei in human muscle at protein level (Wiik et al. 2009) was

published in 2009. In the respective study, generally both ERα and ERβ were shown to be co-expressed in the same nuclei. This may be a momentous notion since ERβ is able to repress the transcriptional activity of ERα (Hall & McDonnell 1999). In some series of circumstances the ERs may even oppose each other's effects resulting in a minor net effect of estrogen. For instance mouse mammary epithelial cells fail to respond to estrogen, although blocking the expression of ERα by RNA interference results in increased apoptosis, while blocking the expression of ERβ leads to proliferation (Helguero et al. 2005). No data are thus far available, which would explain the mechanisms behind the co-expression of ERs in skeletal muscle.

The exact localization of ERs in muscle fibers in human skeletal muscle is not completely understood, but it may reflect the observations partially found in other contexts (see Figure 2A). Besides the expression within muscle fibers, twenty-five percent of ERα are localized in the capillaries (Wiik et al. 2009), while the corresponding proportion for ERβ has been reported to equate to 24% (Wiik et al. 2005a). This vasculature-related expression may suggest a physiologic role for estrogen in the response of skeletal muscle to strain. In fact in 2005 Wiik and co-authors showed that the mRNA levels of ERs and vascular endothelial growth factor (VEGF) are higher within the skeletal muscle of highly endurance trained men in comparison with the moderately active male subjects suggesting improved adaptation to endurance training (Wiik et al. 2005b). A connection between the signaling concerning ERs and VEGF is supported by *in vivo* data reporting that estrogen regulates the mRNA levels of VEGF in the human endometrium (Shifren et al. 1996), rat uterus (Cullinan-Bove & Koos 1993) as well as in endometrial carcinoma cell line (Charnock-Jones et al. 1993) and that functional EREs are evident in the gene encoding VEGF (Hyder et al. 2000, Mueller et al. 2000). The reported results were also suggested to emerge through ligand-independent mechanisms via the MAPK signaling cascade, which is activated upon physical exercise (Widegren et al. 1998) and increases the transcriptional activity of ERs (see chapter 2.2.2). Aside from the increase of steady-state mRNA levels Wiik and colleagues also reported that the mRNA levels of both ERs and citrate synthase are positively correlated and suggested an involvement of ERs in the regulation of mitochondrial biogenesis (Wiik et al. 2005b). Supporting data concerning muscle-related circumstances come from studies with murine myoblasts proposing that a subpopulation of ERα localizes outside the nucleus, more precisely in the mitochondria and the perinuclear compartments (Milanesi et al. 2008), while ERβ has been shown to localize in the mitochondria in human heart (Yang et al. 2004). Also sequences resembling EREs have been identified in the mouse mitochondrial genome (Demonacos et al. 1996).

The mitochondrial expression of ERs may suggest a role for estrogen in processes related to energy metabolism. E_2 treatment is reported to decrease the expression of lipogenic genes in skeletal muscle of mice thereby possibly partitioning free fatty acids towards oxidation instead of storage as triglycerides. In addition, E_2 has been shown to activate AMP-activated protein

kinase in a dose- and time-dependent manner (D'Eon et al. 2005) through 2-hydroxyestradiol, a metabolite of E_2 but not a ligand for ERs, in myotubes (D'Eon et al. 2008). Estrogen is also known to modulate insulin sensitivity and thus play a role in glucose homeostasis and in the development of diabetes mellitus (Barros et al. 2006a, Ropero et al. 2008). Although both αERKO and aromatase knockout mice are shown to be insulin-resistant (Heine et al. 2000, Takeda et al. 2003, Ropero et al. 2008), the underlying mechanisms are poorly understood. They may, however, be related to proper function of skeletal muscle, since insulin resistance is connected with decreased glucose uptake by muscle and adipose tissue (Bell & Polonsky 2001). Glucose transporter 4 (GLUT4) is the protein maintaining the proper glucose uptake of skeletal muscle traditionally in response to insulin (Gould & Holman 1993, Ryder et al. 2001). A link between estrogen and glucose uptake comes from data showing that ERs modulate the expression of GLUT4 in skeletal muscle of mice (Barros et al. 2006b).

2.3.2.1 PI3K/Akt pathway, IGF-1, and estrogen in skeletal muscle milieu

At least a part of the positive effect of various agents on the condition of skeletal muscle comes through the PI3K/Akt signaling pathway along which Akt activates mammalian target of rapamycin (mTOR), which further activates a pathway leading to increase of protein synthesis (Coleman et al. 1995, Nave et al. 1999, Rommel et al. 2001, Shavlakadze & Grounds 2006, Velloso 2008) (Figure 3). In addition, Akt is reported to attenuate the activity of forkhead box O (FOXO) transcription factors (Sandri et al. 2004, Stitt et al. 2004), which induce the transcription of atrophy genes (Bodine et al. 2001a, Bodine et al. 2001b). The phosphorylation of FOXO by Akt results in its sequestration in the cytoplasm away from the target genes (Brunet et al. 1999).

One of the important signals activating PI3K/Akt pathway in skeletal muscle cells is IGF-1 (Rommel et al. 2001). Together with insulin and IGF-II, IGF-1 is the only extracellular growth factor known to promote the terminal differentiation of myoblasts thus enhancing muscle growth (Sheffield-Moore & Urban 2004, Lluis et al. 2006). The possible connection between IGF-1 exerted signaling, PI3K/Akt pathway and E_2 holds relevance, since E_2 has the capacity to increase the expression and the activation of IGF-1 receptor (Mendez et al. 2006). A clue concerning the functionality of the link between E_2 and Akt in muscle setting comes from a previous study, in which the signaling cascade including PI3K and Akt was rapidly activated by E_2 in an ER-dependent manner in undifferentiated, mononucleated C2C12 myoblasts (Vasconsuelo et al. 2008). The modulation of the PI3K/Akt signaling pathway by E_2 has also been reported in other cell types (Simoncini et al. 2000, Castoria et al. 2001, Marino et al. 2003, Lee et al. 2005, Guo et al. 2006). In the study with C2C12 myoblasts of mice origin, the researchers concluded that E_2 has the capacity to exert anti-apoptotic effects through inactivation of a pro-apoptotic protein Bcl-2 associated death agonist (BAD) following activation of Akt by PI3K (Vasconsuelo et al. 2008). Similar results are available in other cell types, at least

in cardiomyocytes (Patten et al. 2004) and in MCF-7 breast cancer cells (Fernando & Wimalasena 2004) as well.

The present publications do not include other reports covering the effects of E_2 on PI3K/Akt route in the milieu of human skeletal muscle. Although the PI3K/Akt route is activated by a series of growth factors, cytokines, mitogens and other hormones (Fernando & Wimalasena 2004, Guo et al. 2006) as already stated above, the present thesis focuses on investigating the PI3K/Akt pathway as a downstream pathway from IGF-1 and the role of HRT or its effective agents, estrogen and progestogen, in this context. Thus the remaining part of this thesis refers to the pathway as IGF-1 pathway.
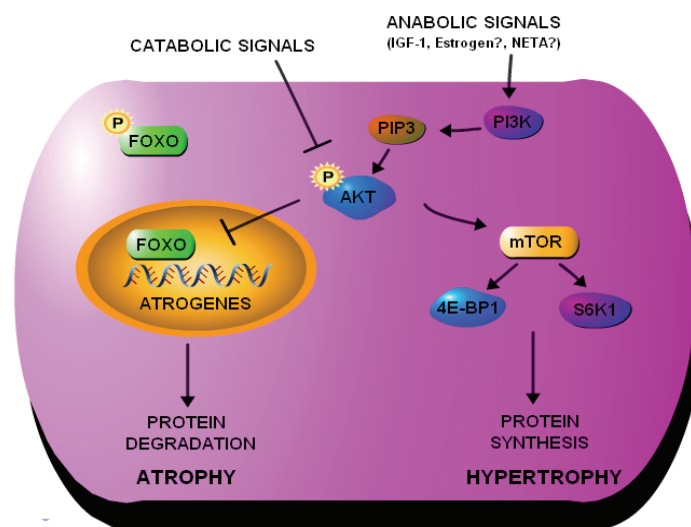
FIGURE 3   Akt-related pathways leading to atrophy or hypertrophy of a muscle fiber. The activation of Akt by anabolic signals, such as IGF-1, or potentially by 17β-estradiol or norethisterone acetate (NETA) the focus of the present study, leads to regulation of protein synthetic machinery, while its inactivation by catabolic signals is followed by increased expression of atrophy-associated genes. Several proteins have to be activated or inactivated in order to hypertrophy or atrophy signaling to be executed. ($\rightarrow$ activation, $\perp$ inhibition). PI3K=phosphatidylinositol 3 kinase, PIP$_3$=phosphatidylinositol-3,4,5-triphosphate, 4E-BP1=eukaryotic initiation factor 4E binding protein, S6K1=ribosomal S6 protein kinase. The schematic figure is adapted from Nader (2005) and generated utilizing the Pathway Builder of ProteinLounge[BETA] (www. proteinlounge.com). FOXO=forkhead box O, Akt=protein kinase B, mTOR=mammalian target of rapamycin, 4E binding protein, S6K1=ribosomal protein S6 kinase.

2.4 Methods to study the association between HRT and skeletal muscle

As the title states, this thesis was originally launched with a specific goal to assess the association between HRT and human skeletal muscle with a special focus on identifying the so far poorly understood molecular and cell biological mechanisms in this context. Aside from phenotype data from human participants, the aim was approached via a set of molecular techniques at the level of gene expression, protein activation and genetic variance.

2.4.1 Gene expression

The response of a target tissue to extracellular signals generally results in constructing a messenger RNA (mRNA) molecule, a template for proteins (Figure 4A). The level of a given mRNA within the cell is always a matter of the rate of mRNA synthesis and the rate of mRNA decay. About four decades ago transcriptional events were reported to be involved in loading-induced muscle hypertrophy (Goldberg & Goodman 1969, Sobel & Kaufman 1970). That skeletal muscle represents a transcriptionally active organ is nowadays considered as a solid fact and the advent of novel methodology to examine various pools of RNA have revolutionized this field of genetic research. For instance advances in microarray technology, originated from Southern blotting, have evolved with outstanding speed; the use of DNAs in arrays was introduced in 1987 (Kulesh et al. 1987), miniaturized arrays were first described in 1995 (Schena et al. 1995) and ground-breaking arrays carrying the entire eukaryotic genome presented in 1997 (Lashkari et al. 1997). The modern high-throughput microarrays provide an opportunity to investigate thousands of transcripts simultaneously and thus allow an explorative approach in which transcriptional regulation of gene clusters or biological processes can be studied. The research in this area has indeed exploded and microarrays have also been utilized in an attempt to identify changes in human skeletal muscle tissue as a response to aging and exercise (Chen et al. 2003, Welle et al. 2003, Zambon et al. 2003, Welle et al. 2004, Giresi et al. 2005, Mahoney et al. 2005, Teran-Garcia et al. 2005, Timmons et al. 2005, Zahn et al. 2006). The article by Zahn and colleagues reported that a so-called common aging signature of skeletal muscle is characterized by up-regulation of pathways including genes encoding factors involved in extracellular matrix, cell growth, complement activation and cytosolic ribosome, while pathways related to chloride transport and mitochondrial electron transport chain were down-regulated (Zahn et al. 2006). When the current work was launched, no studies exploring the enriched processes, which would underlie the response of skeletal muscle to HRT in human participants, were available.

2.4.2 Protein activation

A general rule has been presented suggesting that the direction of the change of a given mRNA and the corresponding protein is typically the same when it comes to the molecular responses of skeletal muscle to changes in contractile activity (Booth & Baldwin 1995, Fluck & Hoppeler 2003). In some specific cases the observations that RNA and protein translation have diverging changes in muscle as a response to a certain stimuli have been explained by high turnover or basal concentrations of the protein in question (Andersen & Schiaffino 1997). The classical assumption that up-regulated transcription of mRNA is translated into protein followed by micro-adaptation in protein concentration is still typically made (Day & Tuite 1998). Even so, whatever the study question, the protein level support for mRNA data is often pursued. In this context, the concentration, post-translational modifications or activity of the protein of interest are frequently the outcomes under investigation. One of the areas with enormous progress is the utilization of two-dimensional gel electrophoresis in assessing the status of the whole proteome. The most widespread field of signaling research with vast amount of publications is the investigation of protein activation by reversible phosphorylation. A phosphate group is added by a protein kinase usually on a serine, threonine or tyrosine residue to activate or inactivate the protein. Consecutive phosphorylation cascades deliver signal quickly through several proteins finally resulting in an appropriate cellular response (Hunter 2000, Olsen et al. 2006). Phosphorylation can in fact be regarded as the basis for cell signaling networks and also the size of the kinome, a set of 518 known kinases phophorylating a spectra of substrates, reflects the importance of the issue in cell biology (Johnson & Hunter 2005).

2.4.3 Genetic variance: single nucleotide polymorphisms

Inherited differences in DNA sequence contribute to individual phenotypic variation and at least partly govern the response to extracellular signals. Mutations in the genetic code vary from a substitution of a single nucleotide (Figure 4B) to the large scale mutations such as loss or gain of entire chromosomes or large chromosomal regions. Small scale mutations are traditionally considered functionally significant only if they are evident as a change in the sequence of amino acids in the corresponding protein, but more recently also silent substitutions in exons and mutations in the non-coding area are acknowledged to potentially have strong effects on gene transcription and on the final gene product. The most common type of human genetic variation is single nucleotide polymorphism (SNP), which affects only a single nucleotide within a gene and gives rise to separate alleles in a specific locus. In 2001, Sachidanandam and co-authors described a map of 1.42 million SNPs along the human genome (Sachidanandam et al. 2001). SNPs are widely used in association studies and allow the investigation of the relationships between genetic variation and a given trait such as muscle phenotype. While a significant focus has been paid for large scale analysis of SNPs during the last

couple of years (Ding & Jin 2009, Fisher et al. 2009, Franke & Jansen 2009), a smaller amount of them insist special attention based on existing literature and a well-reasoned null hypothesis when selecting the variants for further analysis.

The recent review summarizing studies concerning the human gene map for physical performance and health-related phenotypes, including several muscle phenotypes, documented that the respective map now includes 214 autosomal gene entries and quantitative trait loci and also seven others on the X chromosome and 18 in the mitochondrial gene set (Bray et al. 2009). These data clearly point out that the genetic background is of importance, when the condition of the musculoskeletal system is assessed and investigated. Upon launching the current study, a careful literature search was carried out and a SNP in gene encoding catechol-*O*-methyltransferase (COMT) and another SNP in *ERα* gene were chosen for analysis.

NON-SYNONYMOUS VALINE TO METHIONINE SNP WITHIN COMT. COMT catalyzes the transfer of a methyl group from S-adenosylmethionine to catecholamines, such as the neurotransmitters dopamine, epinephrine, and norepinephrine (Zhu & Conney 1998, Zhu et al. 2002). *O*-methylation serves also as one route through which estrogenic hormones are converted hormonally less active or inactive metabolites. On their way to conversion to methoxyestrogens, estrogens are first hydroxylated by isoenzymes belonging to the cytochrome P450 family followed by methylation by COMT (Zhu & Conney 1998). Intriguingly, a non-synonymous, functional G to A polymorphism in the fourth exon of *COMT* gene results in a valine to methionine amino acid substitution at codon 158 leading to thermolability and lower activity of the enzyme (Scanlon et al. 1979, Lotta et al. 1995, Männisto & Kaakkola 1999). Albeit the respective polymorphism has typically been recognized as a contributor related to various neurophysiologic and psychological traits (Heinz & Smolka 2006, Hosak 2007, Dickinson & Elvevag 2009), its role also, for instance in the responses to HRT (Worda et al. 2003) as well as in pubertal development and bone mass (Eriksson et al. 2005), have been assessed. Previous studies with men (Lorentzon et al. 2004, Stolk et al. 2007), early pubertal girls (Eriksson et al. 2005), as well as premenopausal (Lurie et al. 2005) or postmenopausal women (Worda et al. 2003, Dunning et al. 2004, Tworoger et al. 2004) have reported contradictory results, whether this polymorphism is associated with serum estrogen levels or not. A hint of the link between this polymorphism and muscle properties comes from a study by Eriksson and colleagues reporting that prepubertal girls homozygous for the low activity allele have larger muscle area compared to the other genotypes (Eriksson et al. 2005). The polymorphism has also been reported to affect the responses to HRT (Herrington et al. 2002a, Herrington et al. 2002b). The question concerning the potential of the respective site with respect to sarcopenic phenotype has so far been left unanswered.

T TO C SUBSTITUTION WITHIN THE INTRONIC SEQUENCE OF ERα. A T to C transition in the first intron of the gene encoding ERα results in the loss of PvuII restriction site (referred to as PvuII polymorphism). The mutated site has been shown to result in a functional binding site for the B-myb transcription factor (Herrington et al. 2002a), which is activated by estrogen (Jeng et al. 1998). The expression of luciferase reporter gene within the construct spanning the respective polymorphic region and including the C allele was augmented >10-fold with cotransfection of a vector carrying B-myb (Herrington et al. 2002a). With respect to the musculoskeletal system, most studies have focused on investigating a possible link between PvuII polymorphism and bone properties. A careful review by Gennari and colleagues concluded that studies investigating the association between PvuII polymorphism within *ERα* gene and osteoporosis have yielded fairly inconsistent results (Gennari et al. 2005). Owing to musculature, no link between this polymorphism and hand grip strength (Vandevyver et al. 1999, Salmen et al. 2002) or quadriceps isometric strength has been verified (Vandevyver et al. 1999).

Theoretically, polymorphisms residing in genes related to estradiol metabolism and action, in this case *COMT* and *ERα*, respectively, may modulate the association between HRT and skeletal muscle. More precisely, a polymorphism affecting the activity of COMT may directly or indirectly modulate the amount of estradiol available to be bound by membrane-bound or intracellular ERs, whereas a polymorphism potentially modulating the amount of ERα transcript may further affect the availability of these receptors. On the other hand, the two estrogen-related polymorphisms may act in conjunction with physical activity resulting in a specific muscle phenotype.
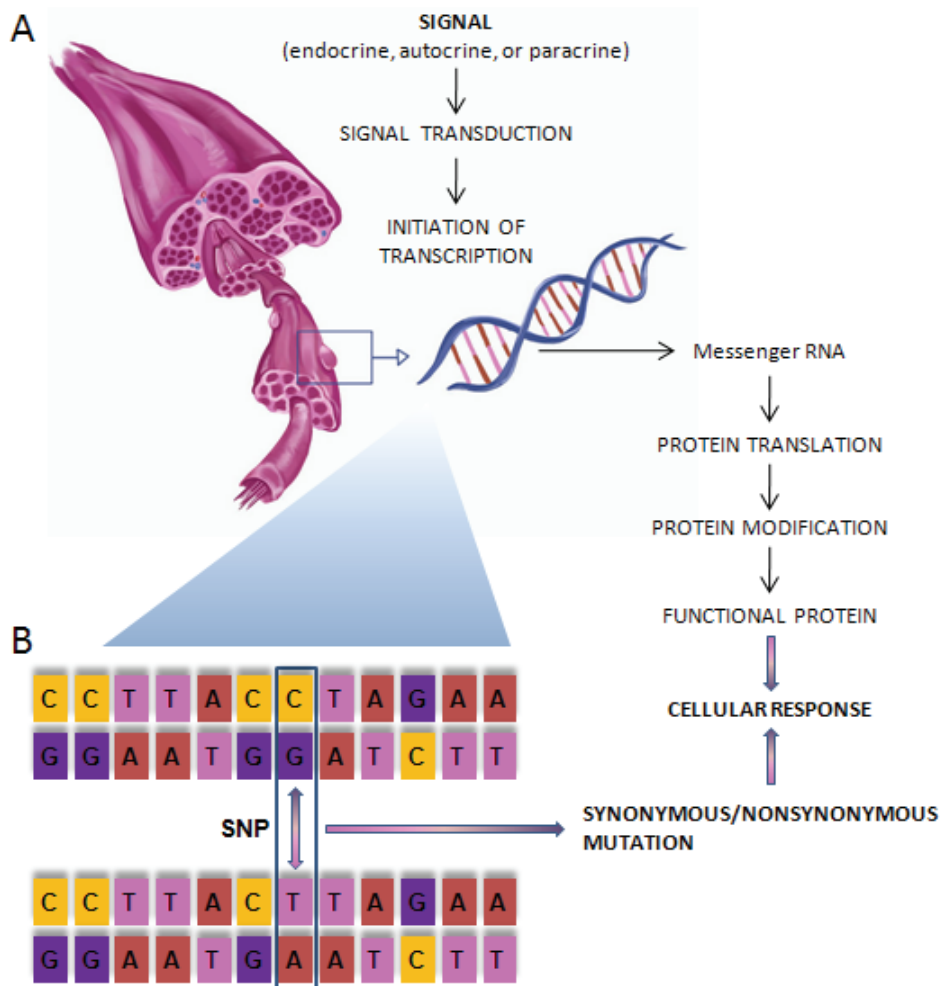
FIGURE 4   In a classical signaling cascade a given extracellular signal is transferred into the cell, which leads to ultimate cellular response via generation of mRNA molecule and protein translation (A). One form of single nucleotide polymorphisms (SNP) giving rise to distinct DNA variants is a substitution of a single nucleotide within the coding or non-coding area within a gene or intergenic regions between genes. A SNP is called synonymous or silent if it does not change the amino acid sequence of the resulting protein or nonsynonymous if it does. A functional SNP produces a protein, which have distinct properties of those of the most common protein. A set of individually inherited SNPs contribute to cellular responses to variable signals.

3   AIM OF THE STUDY

The main objectives of the present study were to extend current knowledge concerning the associations between postmenopausal HRT and adequate amount, quality, and function of skeletal muscle, identify the molecular pathways responsible for the observed association, and assess the role of two genetic single nucleotide polymorphisms in estrogen-related candidate genes in muscle properties.

The specific aims of the study were:

1. To investigate whether long-term postmenopausal HRT is associated with skeletal muscle mass, composition, strength, power, and mobility among 54-62-year-old women.

2. To identify the cellular processes, which underlie the association between estrogen-based HRT and the phenotype of human skeletal muscle and assess the importance of IGF-1 signaling pathway both in human muscle samples and in cell culture setting.

3. To dissect if genetic variation in estrogen-related candidate genes explains the variation observed in muscle phenotypes in women aged 63 to 74 years and whether physical activity modulates this association.

4   STUDY DESIGNS, PARTICIPANTS AND METHODS

4.1   Study designs and participants

In a traditional case-control study design the involvement of the genetic effects in determining the trait under investigation cannot be controlled for. The variation in participants' genetic background can harmfully interfere with the results obtained. One of the study designs utilized in this thesis is based on natural twinning. An idea for a classical twin study was first suggested by Galton in 1875 and it relies on the fact that if identical (monozygotic, MZ) twin pairs sharing 100 % of their genes are more similar for the given trait than non-identical twins (dizygotic, DZ) sharing on average 50 % of their genes, the trait is under genetic influence (Martin et al. 1997, Posthuma et al. 2003). In addition to this classical mode of twin study, an example of utilizing natural twinning in science is so-called discordance design, in which one co-twin exhibits a given trait or is exposed to a specific agent, while the other does not or is not. Discordance design controls over the genetic background as well as early life events and family environment. The design is useful if a trait, potentially affected by variation in genetic sequences, is studied. Furthermore, the discordance design enables the dissection of the long-term effects of a given exposure and therefore represents a real-life example compared to RCT, which has an advantage of higher controllability.

This study utilizes sample resources and data from three larger studies and complements the observations with a cell culture model. The recruitment processes for each of the three studies are shown in Figure 5. The participants for cross-sectional studies titled SAWEs (*S*arcopenia - Skeletal Muscle *A*daptation to Postmenopausal Hypogonadism and Effects of Hormone Replacement Therapy and Physical Activity in Older *W*omen: a Genetic and Molecular Biological Study on *Es*trogen-related Pathways, I, II) and FITSA (Finnish Twin Study on Aging, IV) were recruited from the Finnish Twin Cohort (Kaprio et al. 1978, Kaprio & Koskenvuo 2002), while 50-57-year-old

women living in the city of Jyväskylä (Finland) were approached to set up the HRT/Exercise study, a placebo-controlled RCT (III).

The SAWEs (I, II) was launched in 2007 with the aim of identifying MZ twin pairs discordant for the use of HRT (one co-twin is a current user, and her sister has never used HRT). Totally 537 female twin pairs born between 1943 and 1952 were approached. A total of 16 pairs from those who responded to the invitation met the inclusion criteria and were further invited to the laboratory examinations (Figure 5A). After determining zygosity by a battery of ten highly polymorphic gene markers fifteen MZ twin pairs discordant for the use of HRT formed the SAWEs study group. Of the users five women used estrogen-only preparations, whereas six were taking a combined treatment including estrogenic and progestogenic effective agents and four women used tibolone, which embodies estrogenic, progestogenic and androgenic properties (Kloosterboer 2001, Notelovitz 2007). Thirteen women were taking preparations as pills, one used a hormonal patch (estrogen-only), and one used a gel preparation (estrogen-only). All the fifteen twin pairs were included in Study I, while a subgroup of eleven pairs using estrogen-based HRT (estrogen-only or combined preparation, E users) was included in the microarray study (II). Each participant took part in the laboratory measurements during two consecutive days and in the biopsy sampling on the third day, approximately one week to one month after the laboratory measurements.

The data from randomized, double-blind, placebo-controlled HRT/Exercise study (III) was originally collected in 1996-1997 to investigate the structure of bone and muscle in relation to HRT and physical exercise. The recruitment process is described in detail by Sipilä and co-authors (Sipilä et al. 2001). Shortly, altogether 80 women eligible to the study according to extensive medical and physical examination were randomized into HRT (n=20), exercise (n=20), HRT+exercise (n=20) and control (n=20) groups for one-year intervention (Figure 5B). All the participants used either continuous, combined HRT preparation (2 mg of estradiol, 1 mg of NETA, Kliogest, Novo Nordisk, Copenhagen, Denmark) or placebo (composed of lactosemonohydrate, cornstarch, gelatin, talc and magnesiumstearate, which were auxiliary substances in the Kliogest tablet) one tablet every day. In the present thesis only samples from HRT and CO groups are utilized. In total of 10 participants from the HRT and nine participants from the CO group completed the trial and had eligible muscle biopsies for the purposes of this thesis.

The FITSA (IV) was conducted during 2000-2001 in order to investigate the genetic and environmental effects on disablement process. An invitation was sent to 414 pairs followed by measuring altogether 217 pairs in the laboratory (Figure 5C). Detailed recruitment process is described by Tiainen et al. (Tiainen et al. 2004). In the present thesis, the twin pairs of FITSA are treated as individuals.

40

A

Finnish Twin Cohort: female
twin pairs born in 1943-1952
Invitation to SAWEs
(including a pre-questionnaire)
n = 537 pairs

Reasons for exclusion (pairs):
Unsuitable hormonal status   62
Only one co-twin responded   19
Medication for heamorrhaging   5
Refused   5
Diseases   2

Pre-questionnaire received
n = 114 pairs

Reasons for exclusion (pairs):
Mode of HRT: vagitorium   3
Haemorrhagic disease   1
Unsuitable hormone status   1

Contacted by telephone
n = 21 pairs

Reasons for exclusion (pairs):
dizygotic   1

Laboratory measurements
n = 16 pairs

Mode of HRT:
estrogen- only therapy   5
combined tharepy   6
tibolone therapy 4

Final sample size
n = 15 MZ twin pairs
discordant for long-term HRT

B

Pre-questionnaire
Random sample, n = 1298
Volunteers, n = 35
Pre-questionnaire received
n = 912

Reasons for exclusion:
Use of hormones   288
Menstruating   176
Menopause > 5 years   176
Refused   122
Contraindications   32

Invited to the laboratory
measurements
n = 118

Excluded   27
Cancelled   11

Randomization
Random sample, n = 52
Volunteers, n = 28

Control
n = 20

HRT
n = 20

Exercise
n = 20

Exercise+HRT
n = 20

Samples available from
baseline and 12 months
n = 9   n = 10

FIGURE 5   Recruitment of the participants for the A) SAWEs (I, II), B) HRT/Ex (III), and C) FITSA (IV) studies.

4.2   Ethical aspects

The Ethics Committee of the Central Finland Health Care District approved the studies and they were conducted according to the guidelines as laid down by the World Medical Association in the Declaration of Helsinki (I, II, IV 2000; III, 1989). Written informed consent was provided by the participants before the measurements.

4.3   Methods

Table 1 summarizes the main methods and tissue samples utilized in assessing body anthropometry and muscle phenotype as well as the regulation of these two in SAWEs, HRT/Ex and FITSA studies.

TABLE 1   Summary of the main methods and samples utilized in substudies I-IV. The cell culture part of the Study III is not included in this summation.

Outcome	SAWEs (I, II)	HRT/Ex (III)	FITSA (IV)
BACKGROUND			
Medication	medical examination	medical examination	medical examination
Physical activity	modified scale of Grimby*		modified scale of Grimby*
Energy intake	5-day food record		
ANTHROPOMETRY AND BODY COMPOSITION			
Body height	beam scale	beam scale	beam scale
Body weight	stadiogram	stadiogram	stadiogram
Body fat	BIA	BIA	BIA
LBM	BIA	BIA	BIA
Muscle CSA	QCT, thigh	QCT, thigh	pQCT, lower leg
Fat CSA	QCT, thigh		
MUSCLE STRENGTH AND MOBILITY			
Hand grip	dynamometer chair		dynamometer chair
KES	dynamometer chair		dynamometer chair
Muscle power	vertical jumping height		leg extension power rig
Mobility	HWS, MWS		
HORMONE MEASUREMENTS, SERUM			
Estradiol	extraction radioimmunoassay	time-resolved fluorometric assay	competitive immunoenzymatic colorimetric assay
Testosterone	LC-MS/MS	time-resolved fluorometric assay	
Estrone	LC-MS/MS		
SHBG	chemiluminescent immunometric assay	time-resolved fluorometric assay	chemiluminescent immunometric assay
FSH	chemiluminescent immunometric assay		
IGF-1		chemiluminescent immunometric assay	
SNP, GENE EXPRESSION AND PROTEIN LEVEL ANALYSIS, MUSCLE			
Gene expression	microarray (enrichment analysis)	microarray (analysis on single genes)	
Protein activity	histochemistry		protein phosphorylation
Genotyping			RFLP

BIA=bioelectrical impedance, CSA=cross-sectional area, QCT=quantitative computed tomography, KES=knee extension strength, HWS=habitual walking speed, MWS=maximal walking speed, pQCT=peripheral quantitative computed tomography, LC-MS/MS=liquid chromatography-tandem mass spectrometry, RFLP=restriction fragment length polymorphism. * Grimby, 1986.

4.3.1 Phenotyping of the participants

HORMONE ANALYSIS. Fasting (I, II) or non-fasting (III, IV) blood samples were collected between 7.30 and 9.30 AM (I, II) or just before the muscle sampling (III). The sera were stored at -70°C immediately after sampling for later analysis. Serum FSH (I, II), SHBG (I, II, IV) and IGF-1 (III) concentrations were measured using solid-phase, chemiluminescent immunometric assays (Immulite 1000, Diagnostic Products Corporation, Los Angeles, CA). In Study III, FSH, SHBG, E_2, and testosterone (T) were measured by time-resolved fluorometric assay (DELFIA, Wallac, Turku, Finland). In the other studies serum E_2 levels were determined in duplicate by extraction RIA, which has been validated especially for measuring low serum E_2 concentrations, as previously described (Ankarberg-Lindgren & Norjavaara 2008) (I, II), or by competitive immunoenzymatic colorimetric assay (NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany, IV). Serum T was measured as previously described (Turpeinen et al. 2008) (I, II). E_2 and T levels were utilized together with SHBG in calculating the respective free hormone levels according to previously presented methods (Vermeulen et al. 1999, Bjornerem et al. 2004). Estrone (E_1) was determined as a dansyl derivative by LC-MS/MS with an API 4000 mass spectrometer as previously described (Nelson et al. 2004) (I).

ANTHROPOMETRY AND BODY COMPOSITION. Body weight and height (I-IV), as well as waist and hip circumference (I) were measured using standard procedures. Body fat and LBM were assessed with a bioelectrical impedance analyzer [InBody (720), Biospace Co. Ltd., Seoul, Korea, I, II; Spectrum II, RJL Systems, Detroit, MI, III, IV].

MUSCLE MASS AND COMPOSITION. Quantitative computed tomography (QCT) scans (I, II: Siemens Somatom Emotion scanner, Siemens AG, Erlangen, Germany; III, Siemens AG, Erlangen, Germany) were obtained from the midpoint between the greater trochanter and the lateral joint line of the knee to assess thigh muscle mass and composition. Total thigh muscle (I, III) and fat CSAs (I), the relative proportions of muscle and fat within the whole thigh CSA (I, II), fat area within the muscle compartment (infiltrated fat, I), and skeletal muscle attenuation (Hounsfield units, HU, I) were determined. Lower leg muscle cross-sectional area (mCSA) was assessed by peripheral quantitative CT (pQCT, XCT-2000, Stratec Medizintechnik, Pforzheim, Germany, IV). Tomography slices were obtained at 55% upwards from the joint surface of the distal tibia. The scans were analyzed using Geanie (version 2.1, Commit Ltd, Espoo, Finland, I, II), or Bonalyse software (IV: version 1.3; III: version 1.0, Commit Ltd).

MUSCLE STRENGTH AND POWER. Maximal voluntary isometric strengths were measured in a sitting position using an adjustable dynamometer chair (Good Strength, Metitur, Palokka, Finland). Knee extension strength was measured on

both legs (I) or on dominant side (IV) at a knee angle of 60° from full extension, while hand grip strength was measured on the dominant side by fixing the arm to the armrest of the chair with the elbow flexed in an angle of 90° (I, IV). The participants were instructed to lift their leg/squeeze the handle with as much force as possible and make the best they could each time. The contraction was maintained for 2-3 seconds. After two to three practice trials, the measurement was performed at least three times until no further improvement occurred.

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 the participant was able to elevate her body's centre of gravity during a vertical jump on a contact mat (I, II). Flight time was measured, and jumping height calculated as follows: vertical jumping height (cm) = $(g \times t^2) : 8 \times 100$ (Bosco et al. 1983). Three maximal efforts were conducted. Leg extensor power of single leg was assessed using the Leg Extensor Power Rig (Nottingham, UK, IV). Five to nine maximal efforts were conducted. In all measurements, the best performance with the highest value was accepted as the result.

MOBILITY. Mobility was assessed as habitual (I) and maximal walking speed (I, II) along 10 meters in a laboratory corridor. Five meters were allowed for acceleration and the time taken to walk ten meters was measured using photocells. Two trials were conducted for each test and the faster performance was documented as the result.

GENERAL HEALTH AND MEDICATION. A medical examination was carried out to assess participant's general health status, possible chronic conditions and contraindications for the measurements requiring physical effort. During this session a physician assessed also the participant's gynecological history and/or history of HRT (in particular for I-III). Questionnaires concerning general living habits, such as alcohol use and smoking, as well as socioeconomical status were completed by the participants. Self-report data on weight, height, and general living habits were available from prior questionnaires completed by the HRT-discordant twin pairs in 1975, 1981 and 1990 (I, II).

PHYSICAL ACTIVITY AND ENERGY INTAKE. Physical activity was assessed using the scale of Grimby (Grimby 1986) with slight modifications (I, II, IV). The participants were categorized on the basis of their self-reported physical activity into groups labeled sedentary (no other activities, but at the most light walking ≤ 2 times/week), moderately active (walking or other light exercise at least 3 times/week, but no other more intensive activities) and active (moderate or vigorous exercise at least 3 times/week). Daily energy intake was assessed by a five-day food record encompassing three weekdays and two weekend days (I, II). The records were analyzed using Micro Nutrica software (version 2.5, the Social Insurance Institution of Finland, Helsinki, Finland).

4.3.2 Cells (III)

CELL LINE AND MAINTENANCE. C2C12 murine (*Mus Musculus*) skeletal muscle cells (ATCC, LGC Standards AB, American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX™ (Gibco, InVitrogen, Carlsbad, CA). The medium was supplemented with 10% inactivated fetal bovine serum and 1% penicillin-streptomycin (both from InVitrogen). Cells were grown in monolayer cultures in a humidified 5% CO_2 atmosphere at +37ºC and subcultured during the maintenance period after 70-80% confluence was reached. The myoblasts were differentiated into myotubes with DMEM+GlutaMAX™ supplemented with 2% horse serum and 1% penicillin-streptomycin (all from InVitrogen).

TREATMENT OF MYOTUBES. In order to identify the effects of the separate effective agents of combined HRT, C2C12 myotubes were treated with either E_2 (1 nM/10 nM) or NETA (1 nM/10 nM). The treatments were started as the myoblasts exhibited high degree of differentiation into multinucleated myotubes and only a minute amount mononucleated cells were present. The appropriate confluence of myotubes was achieved between the 5th and the 6th day of differentiation. Myotubes were first serum-starved for one hour followed by the respective treatments. The cells for the time point of 0 h served as a basal control. In addition to this baseline control, control cells without E_2 or NETA in the treatment medium were collected at each time point. Cells were collected at 0 h, 2 h, 6 h, and 24 h for RNA analysis and at 0 h, 5 min, 20 min, 40 min, and 2 h for the analysis of protein phosphorylation. At least three independent experiments were carried out.

4.3.3 Analysis of gene expression (II, III)

PRE-PROCESSING RNA AND DNA SAMPLES FOR GENE EXPRESSION ANALYSES. Muscle biopsies were obtained from the mid-part of the *vastus lateralis* defined as a midpoint between the greater trochanter and the lateral joint line of the knee (II, III). Following the removal of all visible residues of fat and connective tissue the biopsy samples for RNA analysis (on average 40 mg) were snap frozen in liquid nitrogen and stored at -70°C until use. Frozen muscle biopsies were homogenized in Trizol-reagent (Invitrogen, Carlsbad, CA) utilizing FastPrep FP120 apparatus (MP Biomedicals, Illkrich, France) followed by extraction of total RNA (II, III) and DNA (II) according to the manufacturer's guidelines. Total RNA from C2C12 samples (III) was extracted utilizing RNeasy Mini kit (QIAGEN Corp., Gaithersburg, MD). RNA from muscle biopsies were used in microarray hybridizations (II, III), and RNA from cell samples in quantitative PCR (qPCR) analysis (III). DNA from muscle biopsies were utilized in determining mitochondrial copy number (II). Concentration and purity of the samples were measured with NanoDrop ND-1000 equipment (NanoDrop, Wilmington, DE).

MICROARRAY HYBRIDIZATION AND ANALYSIS OF THE MICROARRAY DATA.
Microarray technology was utilized in identifying significantly up- and down-regulated biological pathway compositions in muscle samples of identical twin pairs discordant for long-term HRT (II) and in analyzing the mRNA levels of individual genes from the IGF-1 pathway after year-long use of HRT in comparison with placebo (III). The cRNA concentration was assessed with Nanodrop and RNA/cRNA quality using Bio-Rad's Experion electrophoresis station (Bio-Rad Laboratories, Hercules, CA) both before and after amplifications. The amplification and biotinylation of total RNA (200 ng, II; 500 ng, III) was performed with Ambion's Illumina RNA TotalPrep Amplification kit (Ambion, Austin, TX). The samples were hybridized to Illumina's Sentrix® Human-WG6 V3 (II), HumanRef-8 v1.0 (III) or HumanWG-6 v1.0 BeadChips (III, all from Illumina, San Diego, CA). Generation of cRNA, hybridizations of the arrays and quality control of the raw data were performed by the Finnish DNA Microarray Center at Turku Center for Biotechnology.

With MZ discordance design initial data analyses for microarray data was performed utilizing R (http://www.R-project.org) together with Bioconductor development software (http://www.bioconductor.org). After quantile normalization hierarchical clustering was carried out in estimating the quality of the data. Pairwise analyses were conducted utilizing limma package with a model of Bayesian shrinkage (Smyth 2004). The data were ranked according to co-twin fold change (FC), which is a base-two logarithm resulting from dividing the expression value of each HRT user with the expression value of the respective sister (the non-user) to correct for the identical genetic background. Enrichment for functionally related genes across a spectrum of 825 gene sets of Gene Ontology biological processes (C5: BP, http://www.broadinstitute.org/ gsea/msigdb/ collections.jsp) was tested using Gene Set Enrichment analysis (GSEA; Version 2.0) (Subramanian et al. 2005). Pre-ranked gene lists including either the up-regulated or the down-regulated genes with each list sorted according to ascending p value were constructed. Five thousand permutation cycles were carried out and gene sets with at least seven and no more than 250 genes were taken into account in each analysis. For the genes with multiple probes on the chip the probe with the highest rank in the pre-ranked list of genes (i.e. the smallest p value) was chosen to represent a given gene. Each analysis was carried out at least three times. A gene set with false discovery rate (FDR) q value ≤ 0.150 was considered as significantly enriched.

The normalized mean expression values were calculated for the most important biological processes and correlated to the phenotype data. These mean expressions were calculated from quantile normalized data by including the data from genes in the leading edge subset (top-ranked gene subset) of each biological process in the analyses. "Standardized values" were calculated for these quantile normalized expression values of each gene set for each individual utilizing SPSS software. "The normalized mean expression value" for each biological process used in correlation analyses is the mean of these standardized values of the leading edge genes in a given biological process.

In identifying the possible effects of one-year combined HRT on gene expression along the IGF-1 pathway or steroid receptors, the expression levels of *IGF-1* and its splice variants, *IGF-1Ea*, *IGF-1Eb* and *mechano growth factor* (*MGF*), as well as *IGF-1 receptor, Akt1, mTOR, FOXO1, FOXO3, atrogin,* both *ERs* and *androgen receptor* (AR) were identified from microarray data (III). Data from ten samples from the HRT and nine from the CO group were utilized. The correspondence of the data from HRT group and five samples from the CO group produced by HumanRef-8 BeadChips with four samples from the CO group produced by HumanWG-6 BeadChips was carefully tested as described by Pöllänen et al. (Pöllänen et al. 2010). Shortly, hybridization of three samples from the CO group on both platforms followed by exposure of the data on correlation analysis revealed that the two separate arrays nicely resembled each other (Pearson correlation coefficient, r=0.88-0.94). Therefore, the data were combined and a clear batch effect, which was, however, evident, was controlled for in statistical analysis. Before further analysis of the expression of individual genes the microarray data were quantile normalized with the same manner as in microarray study on identical twins (II). General statistical methods (see chapter 4.8) were utilized in further analysis.

QUANTITATIVE PCR ANALYSES. One microgram of RNA from cell cultures (III, High Capacity cDNA Reverse Transkription Kit, Applied Biosystems, CA) was reverse transcribed into cDNA for qPCR analysis (III). Mitochondrial DNA (mtDNA) copy number was determined from DNA extracted from muscle biopsies utilizing qPCR essentially as previously described (Pietiläinen et al. 2008) (II). Briefly, mtDNA copy number was calculated based on simultaneous amplification of the mitochondrial cytochrome b (CytB) and the nuclear amyloid protein beta precursor protein (APP) and expressed as the ratio of mtDNA to nuclear DNA converted to percentage in each specimen. Cloned plasmids containing the human APP or CytB gene (kind gifts from Professor Anu Wartiovaara, University of Helsinki, Helsinki, Finland) were utilized in constructing the standard curves. Aside from the determination of mtDNA copy number in relation to long-term use of HRT (II), qPCR was utilized to analyze the expression of genes related to IGF-1 signaling pathway in C2C12 myotubes after treatment with E_2 or NETA (III). All qPCR assays were run with an Applied Biosystems' ABI 7300 unit using standard PCR conditions recommended by the manufacturer. Each sample was run in triplicate and the reference sample was included in all plates in order to control for inter-assay variation. The dilution series of the reference sample was used as a standard curve. Table 2 specifies the gene expression assays utilized in this thesis.

TABLE 2   Gene expression assays (Applied Biosystems) utilized in qPCR analysis.

Gene	Assay type	Exon-intron boundary	Article
(h)APP	Forward: TGT GTG CTC TCC CAG GTC TA Reverse: CAG TTC TGG ATG GTC ACT GG Probe: CCC TGA ACT GCA GAT CAC CAA TGT GGT AG	–	II
(h)CytB	Forward: GCC TGC CTG ATC CTC CAA AT Reverse: AAG GTA GCG GAT GAT TCA GCC Probe: CAC CAG ACG CCT CAA CCG CCT T	–	II
(h)GAPDH	Hs 99999905_m1	–	II, III
(h)IGF-1Ea	Forward: AGCGCCACACCGACATG Reverse: TCCCTCTACTTGCGTTCTTCAAA Probe: CAAGACCCAGAAGGAAGTA	3-5	III
(h)IGF-1Eb	Forward: GAGGAGCAGACAGCAAGAATGA Reverse: CCAGCAGGCCTACTTTTCTTCA Probe: AAGCAGAAAATACAATAGAGG	–	III
(h)IGF-1Ec /MGF	Forward: ACGAAGTCTCAGAGAAGGAAAGG Reverse: CTTGTTTCCTGCACTCCCTCTAC Probe: AAGTACATTTGAAGAACGCA	4-5	III
(m)Akt1	Mm 01331624_m1	12-13	III
(m)atrogin-1	Mm 01207879_m1	6-7	III
(m)AR	Mm 01238475_m1	7-8	III
(m)ESR1	Mm 00433149_m1	4-5	III
(m)ESR2	Mm 01281854_m1	2-3	III
(m)Foxo1	Mm 00490672_m1	2-3	III
(m)Foxo3	Mm 00490673_m1	1-2	III
(m)GAPDH	Mm 99999915_g1	2-3	III
(m)IGF-1	Mm 01233960_m1	1-2	III
(m)mTOR	Mm 00444968_m1	6-7	III

h=human, m=mouse

4.3.4 Protein level analysis (II, III)

OXIDATIVE CAPACITY PER CROSS-SECTION. The oxidative capacity of muscle was assessed by histochemical staining of the components of the succinate dehydrogenase (SDH) complex from muscle cryosections. The biopsies were oriented vertically and mounted in O.C.T. embedding medium (Tissue-Tek®, Sakura Finetek Europe B.V.) followed by snap freezing in isopentane (–160°C) pre-cooled in liquid nitrogen. Adjacent transverse cross-sections (10 µm) were cut with a cryomicrotome. SDH staining was done as previously described (Pette & Tyler 1983). The 8-bit images converted from the stained sections were processed and analyzed using ImageJ software (http://rsbweb.nih.gov/ij/). Intensity threshold separating areas with low and high oxidative capacity according to SDH activity was set manually and separately for all images. Finally, two intensity scaled fractions representing low and high level of oxidative capacities were expressed as the percentage of the total measured area studied. Average percentage area fractions from the images (1-3 from each specimen) were analyzed. Five samples from the non-users and seven from the

users were eligible for further analysis. These samples consisted of four complete twin pairs. The data analysis was performed both between the groups (Man Whitney U test) and within the four pairs (Wilcoxon's signed rank test).

PROTEIN PHOSPHORYLATION. Cells for the analysis of phosphorylation were homogenized with a 20G needle in fresh, ice-cold homogenization buffer (20 mM Hepes, pH 7.4, Sigma-Aldrich, St. Louis, MO; 1mM EDTA, IDRANL®III, Sigma; 5 mM EGTA, Sigma; 10 mM magnesium chloride, Merck & Co. Inc., Whitehouse Station, NJ; 100 mM β-glyserophosphate, Sigma; 1 mM natrium orthovanadate, Sigma; 2 mM DTT, Sigma; 1% Triton X-100, Fluka Chemie GmbH, Buchs, Switzerland; 40 μg/ml leupeptin, Fluka; 40 μg/ml aprotinin, Sigma, 80 μg/ml PMSF, Sigma; 1 μl/100 μl phosphatase inhibitor cocktail, Sigma) during 15 min incubation on ice followed by centrifugation (12 000 g, 10 min, +4ºC) and collection of the supernatant. Total protein concentration was assessed using BCA Protein Assay Kit (Pierce, Rockford, IL).

The homogenized samples (35 μg total protein/sample) were solubilized in 6x Laemmli buffer, heat-denatured (5 min, 95°C), separated by 4-15% gradient gels (Bio-Rad Laboratories, Richmond, CA), and analyzed using phosphospecific rabbit polyclonal primary antibodies recognizing Akt on Ser[473] and mTOR on Ser[2448] (both 1:1000, Cell Signaling Technology, Beverly, MA). After incubation with the secondary antibody (horse radish peroxidase-conjugated anti-rabbit IgG, 1:40 000, A9169, Sigma) the phosphorylated proteins were visualized by enhanced chemiluminescence utilizing Amersham™ ECL Advance™ Westernblotting Detection Kit (GE Healthcare) according to manufacturer's protocol. Quantification of specific protein bands was performed using a ChemiDoc XRS together with Quantity One software (version 4.6.3, Bio-Rad Laboratories).

4.3.5 Genotyping (IV)

Genomic DNA was extracted from EDTA-anticoagulated whole blood according to standard procedures (PUREGENE® Kit, Gentra Systems Inc., Minneapolis, MN). Restriction fragment length polymorphism (RFLP) identification of the genotypes under investigation was carried out by two independent investigators from whom data on phenotypes was concealed.

The G to A transition at the 158th codon in the *COMT* gene (COMT Val158Met polymorphism) was successfully determined from all the participants in FITSA (n=424) by copying a 109-bp fragment as previously described (Al-Hendy & Salama 2006) with slight modifications. The 109-bp fragment was digested by NlaIII restriction endonuclease (New England Biolabs, Ipswich, MA) followed by separation of the resulting fragments in agarose gel and determination of the genotypes. Genotypes were coded as HH, HL and LL, in which capital H denotes the presence of valine and thus high activity allele, whereas L refers to the presence of methionine and the low activity allele.

ESR1 PvuII genotype was successfully determined for 421 participants from FITSA. A 373-bp PCR fragment was produced using a primer pair consisting of forward (5′-GATATC CAGGGT TATGTGGCA) and reverse primers (5′-TTACCT CTTGCC GTCTGTTGC, Oligomer Oy, Helsinki, Finland). The resulting PCR product was digested by PvuII restriction endonuclease (InvitrogenTM, Carlsbad, CA) before separation of the digested products in agarose gel. Genotypes were determined due to resulting fragments and coded as PP, Pp and pp. Uppercase letters indicate the absence (nucleotide C) and lowercase letters the presence (nucleotide T) of a restriction site.

4.3.6 Statistical methods

The differences between the means in phenotypes of the co-twins discordant for the use of HRT were tested using Wilcoxon's signed rank test (I, II). Intra-pair differences are expressed as percentages (IPD%) and calculated as follows: (HRT user – non-user) : (non-user) x 100. In addition, the 95% confidence interval (95% CI) was calculated for each IPD%. For group comparison of outcomes from SDH analysis non-parametric Man Whitney U test was utilized (II). Univariate analysis of variance was utilized in comparing the gene expression between the HRT and CO women of the year-long RCT with the baseline measurement and array type as covariates (III). Analysis of variance for repeated measures was applied for cell culture data to investigate, whether the treatments affect the expression of given genes or protein phosphorylation (III). The level of significance was set at p≤0.05 in all analysis. SPSS software was utilized in these basic analyses (Version 14.0, SPSS Inc., Chicago, IL).

Statistical models for detecting associations between genetic variance and given phenotypes as well as the possible contribution of physical activity in this context (IV) were constructed in SAS (SAS Institute INC., version 9.1) using the generalized estimating equations approach (GEE), which allows taking into account the twinning of the individuals. In this analysis, the twins were treated as individuals. Single genotype models, one including the unadjusted main effects of the genotypes, and another adjusted for age and height, were constructed. To assess genotype-genotype and genotype-physical activity interactions a reference category was selected for the categorical predictor variables of physical activity (sedentary level), COMT (the HH genotype) and ESR1 Pvull (the pp genotype). Planned contrasts were used in comparing mean levels of each outcome variable between the predictor variable levels and their interactions against the reference category. Partial correlation coefficients from the GEE model contrasts (Natarajan et al. 2007) were computed as estimates of effect size. The reference groups were chosen according to the initial hypothesis; subjects with potential low amount of circulating estradiol (HH genotype) or suggested low amount of ESR1 transcript (pp genotype) combined with sedentary life-style, were assumed to be weaker and have smaller muscles than other combinations. Mean values of other groups were compared to that of the reference groups. The main effects of the two components of interest are always presented in contrast to the reference group.

5 RESULTS

5.1 Participants' physical characteristics, anthropometry, and body composition

Mean duration of HRT use was 6.9 ± 4.1 years (2-16 years) for all the fifteen HRT users in the SAWEs (I) and 7.3 ± 3.7 years (2-16 years) for the users of estrogen-based HRT (I, II). There were no differences in physical activity, medication, smoking behavior or alcohol use between the HRT users and their non-using co-twins at the time of data collection or prior to the use of HRT (the latter according to questionnaires in 1975, 1981 and 1990, data not shown).

Physical characteristics of the participants in SAWEs (I), HRT/Ex (III) and FITSA (IV) studies are shown in Table 3. The data on users of long-term HRT and their sisters are shown for the entire study group (I). The sisters did not differ in the mean values for LBM, body mass index (BMI), body fat percent or waist or hip circumference (I, II). The users of long-term, especially estrogen-based HRT had, however, lower percentage of body fat (p=0.026; I, II) and lower, although not statistically significant, BMI (p=0.091; I, II) in comparison with the non-users. Aside from these observations concerning long-term HRT, year-long HRT including both the estrogenic and the progestogenic effective agent resulted in significantly increased LBM compared to the controls (change in the HRT users vs. controls: 2.1% vs. -0.7%, p=0.028) (III). LBM, BMI, or body fat percent did not differ between the subjects with different COMT or ESR1 genotypes (IV).

As regards genotyping, 18% of the subjects were homozygous for the high activity allele (HH) with respect to COMT Val158Met polymorphic site, 48% heterozygotes (HL) and 32% homozygous for the low active allele (LL, IV). The genotype distribution of the entire cohort was in Hardy-Weinberg equilibrium (x^2=0.004, p=0.95). There again, the most common genotype concerning ESR1 PvuII polymorphism was Pp (43%), whereas pp genotype was more frequent (33%) than PP (21%). The genotypes were slightly out of Hardy-

Weinberg equilibrium (x^2=3.943, p=0.047) suggesting that the study sample may not be representative of the target population.

TABLE 3   Physical characteristics of the participants according to the use of HRT (SAWEs and HRT/Ex) or ESR1PvuII and COMTVal158Met genotypes (FITSA).

Variable		Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)	LBM (kg)	Body fat (%)
SAWEs (I)							
HRT user (n=15)		57±2	164±5	69±9	26±3	47±4	31±6
non-user (n=15)		57±2	163±4	71±14	27±6	46±4	35±9
HRT/Ex (III)							
HRT (n=10)	0 month	53±1	161±7	73±12	28±3	48±4	34±8
	12 month		161±7	72±10	28±3	48±4	33±6
Control (n=9)	0 month	53±2	162±5	68±7	26±2	47±5	31±4
	12 month		162±5	68±8	26±2	47±3	31±5
FITSA (IV)							
COMT	HH (n=79)	68±3	157±6	70±12	28±5	46±5	52±14
	HL (n=208)	69±3	158±6	70±12	28±5	46±5	53±18
	LL (n=137)	69±3	160±6	70±12	28±5	47±5	51±16
ESR1	PP (n=90)	69±4	159±7	70±11	28±5	46±4	52±16
	Pp (n=187)	69±3	158±6	70±12	28±5	46±5	51±15
	pp (n=144)	69±3	159±6	71±13	28±5	46±5	53±19

Data are expressed as mean±SD. HRT=hormone replacement therapy, BMI=body mass index, LBM=lean body mass.

Serum hormone concentrations reflected the use of long-term HRT as expected; the concentrations of E_2 and E_1 were on average five times higher in the HRT users than in their sisters (I, II, Table 4). A similar trend was observed for free E_2 as well. No differences were evident in the levels of total or free T or in SHBG. The users of estrogen-based HRT had similar results. When it comes to genetic polymorphisms, the serum hormone concentrations were similar across different COMT Val158Met and ESR1 PvuII genotypes (IV, data not shown).

TABLE 4   Hormone profiles of identical female twin pairs discordant for the long-term use of HRT. The data are presented for the entire group (n=15 pairs, I) and for a subgroup including only the users of estrogen-based HRT (E users, n=11 pairs, I, II).

Variable	HRT user	non-user	Intra-pair difference (95% CI)	P value
E_2 (pmol/l)	133 ± 185	30 ± 24	504 (-67 to 1074)	0.002
E users	1723 ± 203	33 ± 27	696 (-147 to 1540)	0.003
Free E_2 (pmol/l)	2.6 ± 3.0	0.7 ± 0.5	378 (-12 to 768)	0.003
E users	3.3 ± 3.3	0.8 ± 0.6	501 (-33 to 1035)	0.006
E_1 (pmol/l)	691 ± 1280	97 ± 26	562 (-101 to 1226)	0.001
E users	900 ± 1455	98 ± 27	760 (-153 to 1673)	0.003
T (pmol/l)	701 ± 273	763 ± 360	-0.1 (-16 to 15)	0.87
E users	715 ± 306	639 ± 269	14 (2.1 to 13)	0.061
Free T (pmol/l)	9.7 ± 5.3	10.6 ± 5.0	-5.9 (-21 to 9.3)	0.23
E users	8.4 ± 4.7	9.9 ± 4.7	-12 (-29 to 4.7)	0.075

E_2=17β-estradiol, E_1=estrone, T=testosterone

5.2   Muscle phenotype

5.2.1 Association of HRT with muscle phenotype (I, II, III)

Muscle CSA (mCSA) was measured to give an estimate of muscle mass in the measured site. Relative muscle area of the thigh was on average eight percent (p=0.047) larger and relative fat area five percent lower (p=0.047) among the fifteen users of long-term HRT than their sisters with no history of HRT (I, Figure 6). Muscle power assessed as vertical jumping height was on average 16% greater in the HRT users compared to their co-twins (p=0.023, I). No significant difference in maximal isometric strength between the HRT users and the non-users was, however, documented. The maximal walking speed of the HRT users was on average seven percent greater compared to that of the non-users (I). Habitual walking speed did not differ between the HRT users and the non-users (I).

The users of long-term estrogen-based HRT had 11% (p=0.013) higher proportion of muscle within the thigh area (Figure 6, I, II) and seven percent lower proportion of fat in the same site (p=0.013). They also had 21% greater lower leg muscle power than their twin sisters with no history of HRT (p=0.016, I, II).

Year-long intervention with combined HRT had a positive effect of 9% in magnitude on the mCSA within thigh, while only 1.5% increment was observed among controls with placebo treatment (p=0.003).

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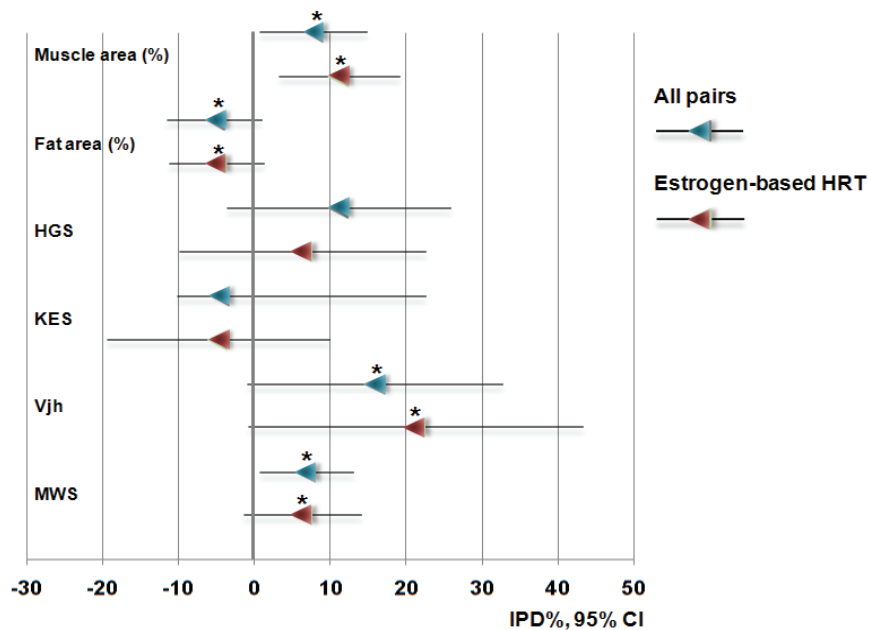
FIGURE 6   Forest plot presenting muscle composition and functional characteristics in SAWEs study with MZ twin pairs discordant for the long-term use of HRT (I, II). The results are presented for the entire study sample (n=15 pairs, dark grey triangles) and separately for the users of estrogen-based HRT (n=11 pairs, light grey triangles). Each bar represents the intrapair difference (IPD%) calculated as (HRT user – non-user) : (non-user) x 100, and the 95% confidence interval (CI) for IPD%. *p<0.05. HGS=hand grip strength, KES=knee extension strength, Vjh=vertical jumping height, MWS=maximal walking speed.

5.2.2 Estrogen-related polymorphisms and muscle phenotype (IV)

COMT Val158Met genotype was associated with mCSA in that individuals with LL genotype had larger mCSA than heterozygotes both in the unadjusted model (p=0.001) and after adjusting with age and height (p=0.004). COMT Val158Met polymorphism was not associated with other muscle variables and ESR1 PvuII polymorphism with neither of them. Further analysis aiming at discovering whether ESR1 modified the association of COMT with muscle phenotypes revealed that an interaction was present in knee extension strength between HH and LL subjects (p=0.031). In other words, an addition of two P alleles to LLpp genotype (LLpp →LLPP) was associated with a smaller difference in knee extension strength in comparison to the HH genotype (HHpp → HHPP). Other interaction effects between COMT Val158Met and ESR1PvuII polymorphisms were not significant.

In further analyses the possible modulation of the association between COMTVal158Met or ESR1PvuII polymorphism and muscle properties by physical activity level was examined. In the model with COMT genotype,

physical activity and age as explanatory variables the genotype had, again, a main effect on mCSA (p=0.021, Table 5, Figure 7). In the respective model physical activity had an expected main effect on all the muscle strength and power variables (sedentary subjects were weaker than moderately active or active individuals, p≤0.004 for all comparisons), but the association with mCSA was less clear (p≥0.078 for all comparisons). Significant interaction effects of the COMT genotype and physical activity were present in all muscle variables. In knee extension strength and leg extension power, all the interaction effects were statistically significant (p<0.05 for all comparisons). In these comparisons, an increase in physical activity from sedentary to moderate or from sedentary to active level within the HH genotype, creates a larger increase in both knee extension strength and leg extension power than among HL or LL individuals (p≤0.045). In hand grip strength, a significant interaction effect was observed only between HH and HL individuals, when sedentary subjects were compared to their moderate active counterparts (p=0.011). In general, the mean values of sedentary HH subjects in all the measured muscle outcomes were lower than subjects with other genotype and/or physical activity level. Moderately active or active subjects with HH genotype, however, had comparable values to those of other genotypes.

In the model including ESR1 genotype, physical activity, and age as explanatory variables, physical activity had a main effect on muscle strength and power (p≤0.004), but this association was not observed in mCSA. Neither main nor interaction effects of ESR1 genotype and physical activity were present in any of the studied muscle properties.

TABLE 5   Statistical significances for genetic effects in age-adjusted models including COMT Val158Met polymorphism and physical activity for mCSA, HGS, KES and LEP (IV).

Effect (ref group)		P value			
		mCSA	**HGS**	**KES**	**LEP**
Val158Met main effect (HH)	HL	0.699	0.538	0.646	0.997
	LL	**0.021**	0.157	0.649	0.701
Physical activity main effect	mod	0.199	**0.004**	**<0.001**	**<0.001**
(sed)	act	0.078	**0.001**	**<0.001**	**<0.001**
Val158Met*physical activity	HL-mod	**0.017**	**0.011**	**0.006**	**0.002**
interaction effect (HH-sed)	HL-act	0.411	0.122	**0.021**	**0.007**
	LL-mod	**0.001**	0.128	**0.045**	**0.045**
	LL-act	**0.051**	0.222	**0.026**	**0.024**

mCSA=muscle cross-sectional area, HGS=hand grip strength, KES=knee extension strength, LEP=leg extension power, sed=sedentary, mod=moderately active, act=active
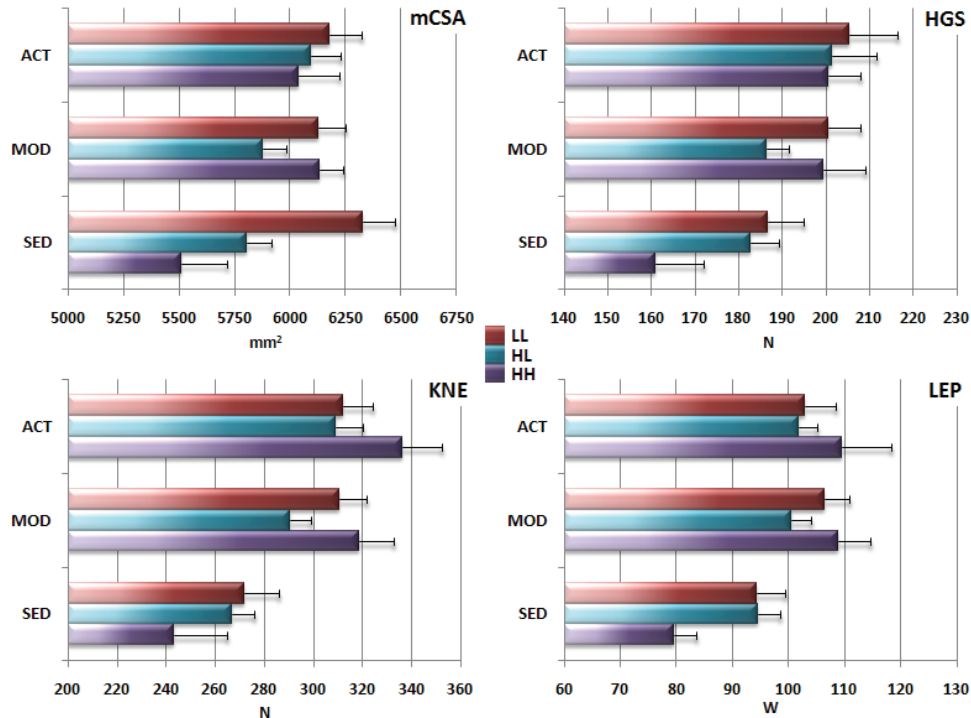
FIGURE 7   Muscle cross-sectional area (mCSA), hand grip strength (HGS), knee extension strength (KES) and leg extension power (LEP) according to COMT genotype and physical activity level (IV). The diagram presents the mean values (+SE) from GEE model according to COMT genotypes (HH, HL and LL) and physical activity (sed for sedentary, mod for moderately active and act for active). The model is adjusted with age. The main results from statistical testing are shown in Table 5.

5.3 Candidate pathways underlying the association between HRT and muscle phenotype

5.3.1 Pathways affected by long-term use of estrogen-based HRT (II)

The biological processes underlying the positive association between long-term use of postmenopausal estrogen-based HRT and muscle phenotype were explored with the aid of microarray technology. The use of HRT was associated with the up-regulation of 22 sequences and down-regulation of 33 sequences ($p<0.001$) in skeletal muscle according to pairwise analysis carried out for a list of individual probes. Microarray data mining with enrichment analysis was performed separately for the list of up-regulated and for the list of down-regulated genes each sorted according to p value, and resulted in identification of one significantly up-regulated biological process and eight down-regulated

ones in the co-twins using HRT. The complete list of genes responsible for the enrichment is presented in Table 6. The list involves some genes encoding proteins, whose function is only poorly known. Although those genes may be biologically important, this thesis focused on the genes, which are more widely described in the literature. Examples of the main results are introduced below. The results are presented from the HRT use point of view, i.e. up-/down-regulated pathways in co-twins using HRT.

TABLE 6   Genes responsible for the enrichment in the up-regulated and down-regulated pathways in the co-twins using HRT (II). The exhaustive list of abbreviations denoting each gene is not presented in this thesis.

GO Biological process	FDR q value	Genes responsible for the enrichment
UP-REGULATED GENE SETS		
Regulation of anatomical structure	0.061	ROBO1, FGD1, MAPT, FGD2, TAOK2, CENTD2, CDC42EP4, CDC42EP1, AMIGO1
DOWN-REGULATED GENE SETS		
Cell matrix adhesion	0.053	SGCE, RASA1, COL17A1, ADAM15, FBLN5, THY1, TAOK2, LYVE1, CDK6, ITGB1BP1, FXC1, PTEN, CDH13, ECM2, ACTN1, PKD2, PPFIA1, NF2, ITGA11, SORBS1
Cofactor catabolic process	0.091	SDHC, NNT, SDHB, SDHA, SDHD, PDHB
G1 phase	0.101	CDK2, CDK6, PRUNE2, RB1, E2F1, CDC25C
Maintenance of localization	0.103	NFKBIL1, MDFI, SRGN, THY1, SRI, TMSB4Y, LCK, TOPORS, PDIA2
Regulation of homeostatic process	0.107	BCL2, SELS, DDIT3, THY1, IFI6, GPX1, GLRX2, TXNDC4
Respiratory gaseous exchange	0.119	EDNRA, TMPRSS11D, HNMT, SFTPB, COX15, COX5B
Vitamin metabolic process	0.132	RDH11, TKTL1, BBOX1, ALDH8A1
Response to nutrient	0.146	PPARG, ENSA, CDKN2B, SSTR1, CDKN2D, ENPP1, STC1, GIPR, SST

FDR=false discovery rate

5.3.1.1 Up-regulated biological process in co-twins using long-term HRT

Gene set enrichment analysis (GSEA) on a pre-ranked gene list with genes carrying positive fold change (FC) ranked according to p value revealed three significantly up-regulated pathways in the co-twins using HRT; "regulation of anatomical structure morphogenesis" with nine genes responsible for the enrichment (FDR q value=0.061), "regulation of cell shape" (FDR q value=0.015)

and "regulation of cell morphogenesis" (FDR q value=0.018). Because the genes in the two latter were included in the first one, only "regulation of anatomical structure morphogenesis" is discussed further. One of the genes in the leading edge subset encodes a protein entitled roundabout, axon guidance receptor, homolog 1 (*ROBO1*), a member of the neural cell adhesion molecule subfamily, characterized as a single-pass transmembrane receptor and regarded as a guide for neuronal migration (Kidd et al. 1998). Another gene responsible for the enrichment encoded microtubule-associated protein tau (*MAPT*), a cytosolic phosphoprotein, which functions in stimulating and stabilizing the assembly of microtubules from tubulin (Alonso et al. 1996). Also two genes encoding CDC42 effector proteins (Rho GTPase binding) 1 and 4, which mediate the organization of actin cytoskeleton, were found in this up-regulated pathway.

Correlation analysis corrected for clustered sampling and including all the participants revealed that the mean expression of "regulation of anatomical structure morphogenesis" explained 19% of the variation observed in the relative proportion of muscle within thigh (p=0.001, Figure 8A).

5.3.1.2 Down-regulated biological processes in co-twins using long-term HRT

A total of ten gene sets were significantly down-regulated in the co-twins using HRT. The most significantly down-regulated biological process was "cell matrix adhesion" (FDR q value=0.053) with 20 genes included in the top-ranked gene subset. Also nine other down-regulated gene sets were found, of which "maintenance of localization" and "maintenance of protein localization", as well as "cell matrix adhesion" and "cell substrate adhesion" were identical. The latter ones of both pairs were included in the first gene sets shown in Table 6.

CELL MATRIX ADHESION. Pathways related to interactions between cells and their environment were down-regulated in the co-twins using HRT. "Cell matrix adhesion" included for example a gene encoding sarcoglycan, epsilon (*SGCE*), a membrane-associated glycoprotein, expressed in a variety of tissues and representing an important component mediating membrane-matrix interactions also in skeletal muscle (Ettinger et al. 1997), and a gene encoding fibulin 5 (*FBLN5*), essential in elastogenesis (Nakamura et al. 2002, Yanagisawa et al. 2002). Other important players in the adhesion of cells to its environment included genes encoding proteins such as RAS p21 protein activator 1 (*RASA1*), collagen type XVII alpha 1 (*COL17A1*), lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*), integrin beta 1 binding protein 1 (*ITGB1BP1*) and actinin alpha 1 (*ACTN1*). Also cyclin-dependent protein kinase 6 (*CDK6*), a regulator of cell cycle progression (Meyerson & Harlow 1994), and phosphatase and tensin homolog (*PTEN*), a negative regulator of the Akt signaling pathway (Li & Sun 1998), were listed among the genes responsible for the enrichment.

Correlation analysis revealed a significant negative correlation in that 20% of the variation observed in vertical jumping height was explained by the mean expression of "cell matrix adhesion" (p=0.008, Figure 9A).

COFACTOR CATABOLIC PROCESS. Mitochondria-related genes were down-regulated in the co-twins using HRT. "Cofactor catabolic process" with for example genes encoding four subunits – A, B, C and D – of the SDH complex was down-regulated in the co-twins using HRT (Table 6). Nicotinamide nucleotide transhydrogenase (NNT), also a component of the energy transfer system in the inner mitochondrial membrane, was included in the top-ranked gene subset of this biological process.

The mean expression value of "cofactor catabolic process" explained 26% of the relative proportion of muscle (p=0.004, Figure 8B) and 20% of jumping height (p=0.016, Figure 9B). This observation appeared not to be due to differences in the number of mitochondria, since the mitochondrial copy number was similar within the muscle tissue of both the users and the non-users (user vs. non-user; 331 ± 32% vs. 377 ± 107%, respectively, p=0.21). Moreover, histochemical staining of the SDH complex from muscle cryosections revealed no significant difference in the oxidative capacity per muscle cross-section between the users and the non-users (fibers with low oxidative capacity: 50.9 ± 9.5% vs. 47.4 ± 13.4% and high oxidative capacity: 37.3 ± 9.6% vs. 40.2 ± 12.4%, respectively).

RESPONSE TO NUTRIENT. Long-term use of HRT was associated with down-regulation of a gene set entitled "response to nutrient". One of the genes in the top-ranked gene subset was a gene encoding peroxisome proliferator-activated receptor gamma (*PPARG*), a nuclear receptor known to play a requisite and sufficient role in the regulation of adipocyte differentiation and to have significant contribution to the whole-body glucose homeostasis and insulin sensitivity (Tontonoz et al. 1994, Barroso et al. 1999). Also endosulfine alpha (*ENSA*), a gene encoding a protein suggested to regulate ATP-sensitive potassium (K_{ATP}) channels, which possess a key role in the control of insulin release (Heron et al. 1998), was identified in this category. Moreover, genes for somatostatin (*SST*), which inhibits the release of a number of secondary hormones (Luque et al. 2008), for SST receptor 1 (*SSTR1*), a G-protein coupled receptor for SST, and for stanniocalcin 1 (*STC1*) a secreted glycoprotein hormone (Madsen et al. 1998) were included in the top-ranked gene set.

The mean expression of this pathway was inversely correlated with both muscle mass and power explaining 18% of the variation in relative proportion of muscle within thigh (p=0.007, Figure 8C) and 19% in jumping height (p=0.023, Figure 9C).
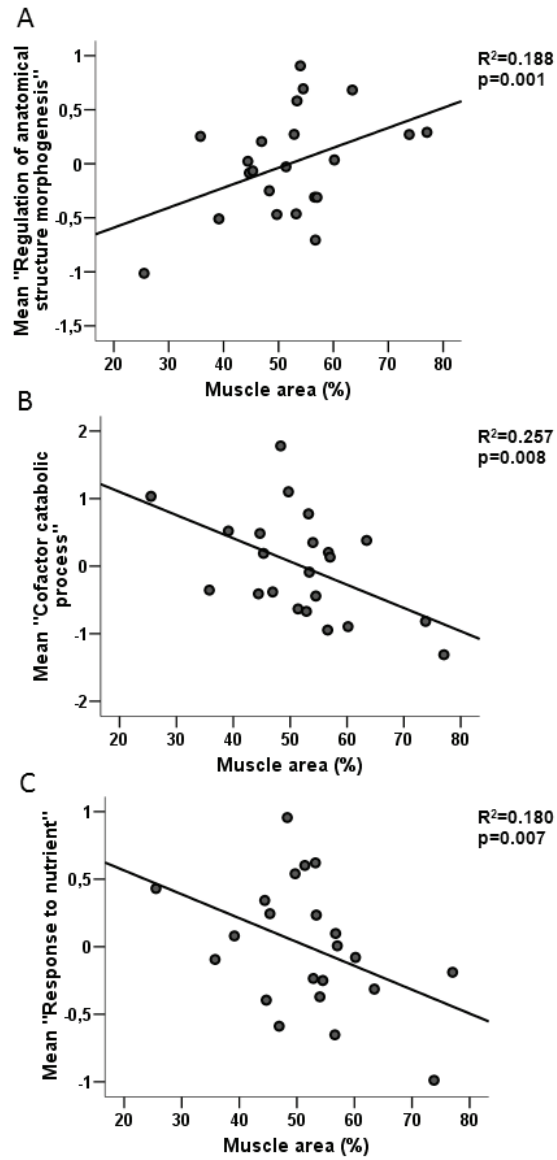
FIGURE 8   The relationships between the relative proportion of muscle within thigh and the normalized mean expression values of enriched biological processes titled "regulation of anatomical structure morphogenesis" (A), "cofactor catabolic process" (B) and "response to nutrient" (C) in the co-twins discordant for estrogen-based HRT.
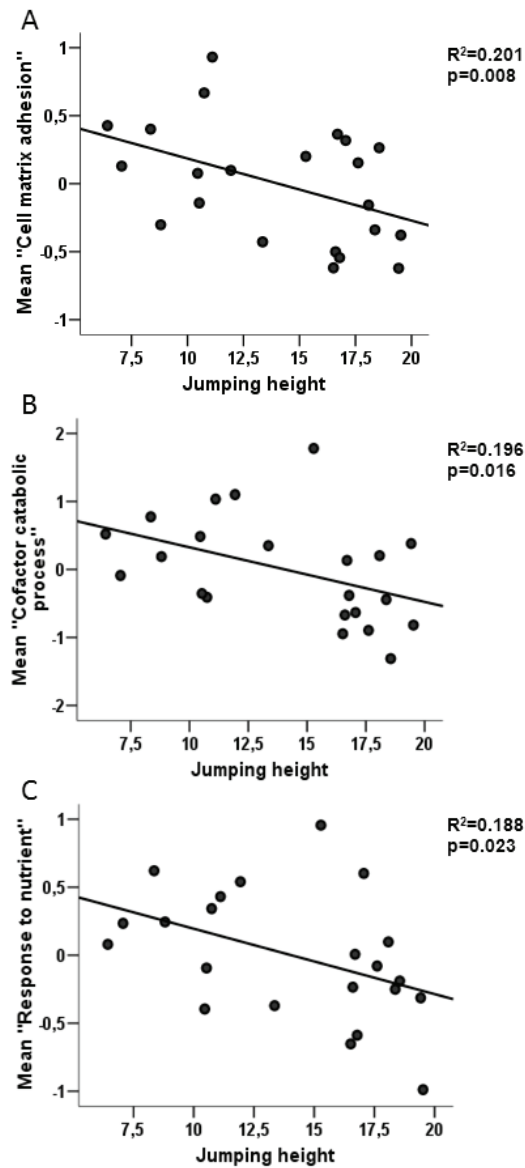
FIGURE 9   The relationships between muscle power measured as jumping height (cm) and the normalized mean expression values of enriched biological processes titled "cell matrix adhesion" (A), "cofactor catabolic process" (B) and "response to nutrient" (C) in the co-twins discordant for estrogen-based HRT.

5.3.2 IGF-1 signaling pathway and year-long combined HRT (III)

The gene expression of *IGF-1* in skeletal muscle was up-regulated after one-year use of HRT (13%) compared to the CO (-16%, p=0.014, Figure 10). All three

splice variants of *IGF-1*, i.e., *IGF-1Ea*, *IGF-1Eb* and *MGF* (*IGF-1Ec*) were up-regulated in the HRT group compared to the CO group, in which their expression was down-regulated (change in the HRT users vs. controls: *IGF-1Ea*: 62% vs. -30%, p=0.10; *IGF-1Eb*: 10% vs. -61%, p=0.31; *MGF*: 58% vs. -31%, p=0.003). Intriguingly, the change in the muscle expression of MGF was associated with muscle mass measured at post-intervention (r=0.50, p=0.035), but no such significant correlation with muscle mass was seen for any other splice variant of IGF-1 or other genes investigated. The level of *IGF-1 receptor* in the microarrays was too low for reliable analysis.

The expression of *Akt1* was down-regulated after year-long HRT (-8%) compared to placebo (0.7%, p=0.036). The expression of *mTOR* was slightly, but significantly up-regulated among the HRT group (2%) compared to the CO participants, who exhibited down-regulated expression (-13%, p=0.043). On the other hand, the gene expression of *Foxo3*, representing the key molecule for atrophy signaling, was also up-regulated among the HRT (2%) compared to the CO women (-9 %, p=0.021). The expression of *Foxo1* had a similar trend (change in the HRT group vs. controls: 2% vs. 9%, p=0.06), but was not statistically significant even though the absolute expression level of *Foxo1* was much higher than that of *Foxo3*. The downstream target of FOXO proteins, *atrogin*, was expressed similarly between the HRT and CO groups (change in the HRT users vs. non-users: 23% vs. 15%, p=0.36). The expression of *AR* was clearly up-regulated among the HRT users (12%) compared to the CO (-8%, p=0.001). The level of *ESR1* did not differ significantly between the HRT and CO groups (change in the HRT users vs. controls: 6% vs. 11%, p=0.36). The transcript level of *ESR2* was too low to be reliably detected.
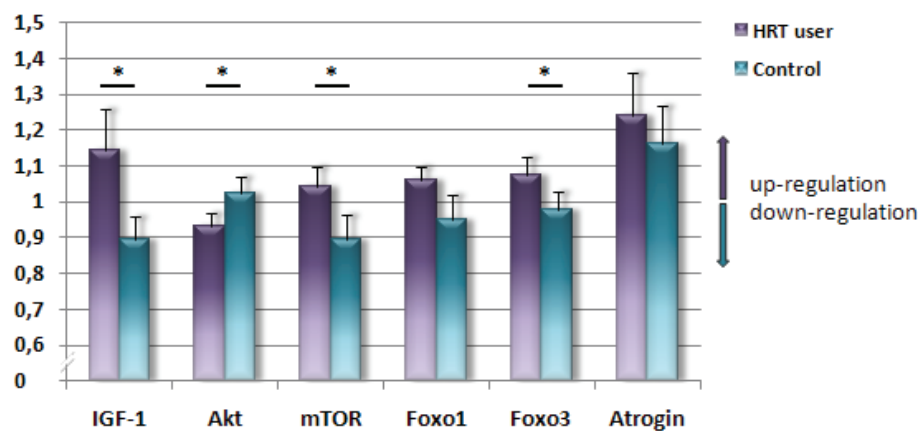
FIGURE 10   The steady-state muscular mRNA levels of genes related to IGF-1 signaling pathway after year-long use of HRT (violet) in comparison with the controls (blue, HRT/Ex intervention) analyzed from the microarray data (III). The change in the expression is expressed as fold change values. The mRNA levels of *IGF-1*, *Akt*, *mTOR* and *Foxo3* during year-long intervention was significantly different between the users of combined HRT (dark violet bars) and the control group having placebo (light violet bars). The expression of IGF-1, mTOR and Foxo3 were up-regulated in the HRT users, while the transcript level of Akt was down-regulated. Each bar represents the mean fold change value and error bars standard errors. *p<0.05.

5.3.2.1   Activation of IGF-1 signaling pathway in C2C12 myotubes

To dissect the contribution of the separate effective agents of HRT on the modulation of the expression of genes or on the activation of proteins along the IGF-1 signaling pathway, an *in vitro* muscle cell culture model, in which either E_2 or NETA were fed to differentiated C2C12 myotubes, was utilized. Neither of these compounds was able to induce statistically significant changes on the levels of transcripts investigated (P>0.05, data not shown). The same cell model was used to investigate the possible role of E_2 and NETA on the activation of IGF-1 signaling at protein level (Figure 11). Even though a clear indication of the effect, even a 5-fold, of 10 nM NETA on p-Akt was seen (Figure 11C), it did not reach statistical significance.
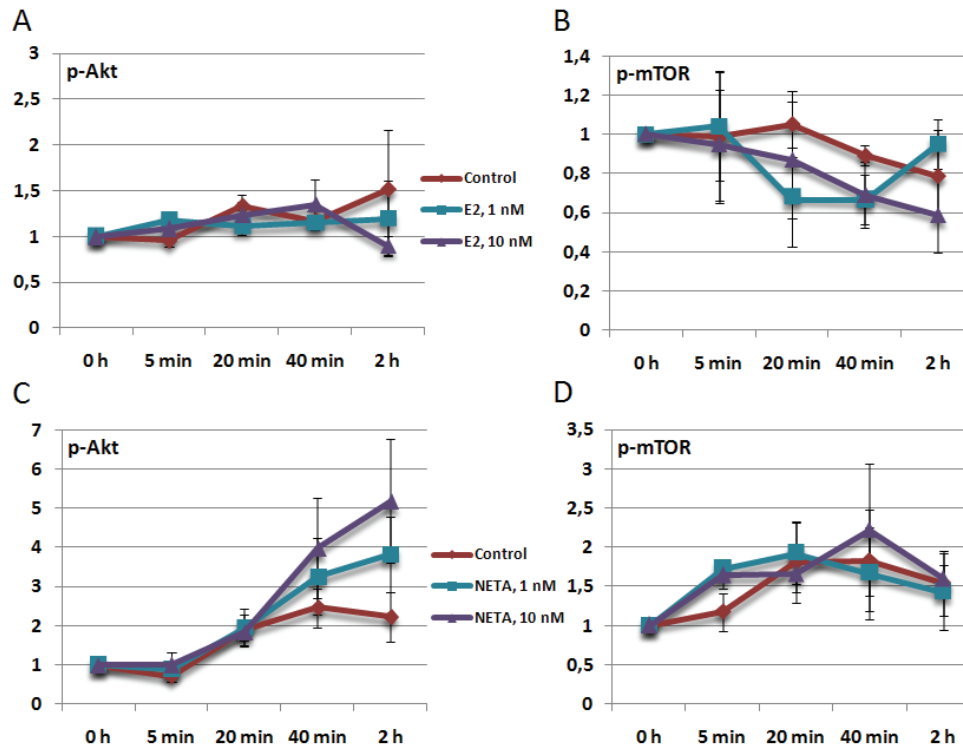
FIGURE 11   The amount of phosphorylated Akt and mTOR (arbitrary units) in C2C12 myotubes in response to E_2 or NETA (III). The figure shows the amount of p-Akt (A) and p-mTOR (B) in arbitrary units after treatment with E_2, as well as p-Akt (C) and p-mTOR (D) after treatment with NETA. No statistically significant effects due to the treatments were observed. Red line with diamonds represent the control samples, blue line with squares the 1 nM treatment and violet line with triangles the 10 nM treatment. Each value represents the mean value of three independent experiments and error bars standard errors.

6 DISCUSSION

The goal of this thesis was to assess the association between HRT and skeletal muscle tissue in the context of aging. The results showed that the use of postmenopausal HRT was positively associated with better muscle mass, composition, muscle power and mobility. Studies on gene expression revealed that the better muscle phenotype within the users of long-term, estrogen-containing HRT can be at least partly explained by regulatory actions of HRT on cellular cytoskeleton, on intramuscular extracellular matrix, and on energy metabolism. Furthermore, IGF-1 signaling pathway was recognized to represent a possible route mediating the positive effects of year-long HRT use on muscle mass. Moreover, the sedentary participants homozygous for the high activity allele in the COMTVal158Met polymorphic site had the poorest muscle properties when compared to women with other genotypes regardless of their activity level, or to the participants with the same genotype but more active lifestyle.

6.1 Association between HRT and muscle phenotype

6.1.1 Association of HRT with mobility and muscle power

In pursuing an authentic example from every-day practice to investigate the association between HRT and muscle phenotype, a rare and robust design with genetically identical female twin pairs discordant for postmenopausal HRT was set up. The fifteen pairs forming the study group were analyzed both as one group and separately after division into users of estrogen-based HRT and tibolone. HRT users, irrespective of the preparation, were faster walkers compared to their sisters without any counteractive treatment (I, II). A concomitant advantage, even up to 21 % in magnitude, of HRT on muscle power was observed (I, II). While walking speed is widely used as a key indicator of mobility limitation and disability in old age (Guralnik et al. 1995, Rantanen et al. 2001), muscle power – the ability of the neuromuscular system to produce

the greatest possible force as fast as possible – have been suggested to be more sensitive to the aging process and more important in mobility than muscle strength alone (Skelton et al. 1994, Bean et al. 2003). The finding concerning the link between muscle power and mobility is supported by a previous RCT with one-year HRT, which report a significant 2% net increase in 20-m running speed (Taaffe et al. 2005b) with a parallel increase of 7% in jumping height compared to the 5% decrease in the placebo group (Sipilä et al. 2001) in 50 to 57-year-old women. The beneficial effects of HRT on muscle power may indeed be suggested to be translated into better mobility, thereby putting flesh on the notion implying an overall positive effect of HRT in the context of sarcopenia.

6.1.2 Association of HRT with muscle mass and composition

The key phenotype results of this thesis showed a trend towards an association between long-term use of HRT and muscle mass (I). The effect of HRT on muscle mass was clearer after year-long use of combined HRT (III). The lack of statistically significant association within the study design addressing long-term HRT may be due to presence of various preparations and to on average lower dose of estrogen compared to the one-year RCT with a fixed, high-dose preparation. Study by Kenny and co-authors support the view that the dose is an important determinant in this context by reporting no effect of low-dose estrogen therapy on appendicular muscle mass in women aged over 65 years (Kenny et al. 2005). In addition to the low dose, the rather high age of the participants in the respective study may have affected the results. However, despite the high age (70 to 79 years) of the women in another study based on case-control analysis, a three percent higher thigh muscle mass was observed in women using estrogen-containing HRT in comparison with the controls (Taaffe et al. 2005a). In that study the duration of HRT was on average seventeen years, which is even greater than what is reported in this thesis as long-term use. Thereby, the use of HRT may be connected to higher muscle mass, but the dose, duration and timing of the treatment may represent important factors determining the association.

Aside from muscle mass, the use of long-term HRT was found to be associated with higher relative proportion of muscle and lower relative proportion of fat within the thigh area (I, II). With respect to the long-term estrogen-based HRT, also lower percentage of body fat as well as lower thigh fat area and subcutaneous fat area within thigh due to HRT were observed (I, II). The results and the possible interrelationships between these phenotypes and mobility are supported by previous data reporting that high body fat mass and BMI, and possibly also low muscle strength or mass, are significantly associated with mobility limitation and substantially increase the prevalence of and the risk for physical disability (Visser et al. 1998, Zoico et al. 2004, Stenholm et al. 2007a, Stenholm et al. 2007b, Stenholm et al. 2009). The clinical relevance of this finding is evident also since the amount of adipose tissue, especially within the fascia surrounding skeletal muscle, has been documented to be related to insulin resistance (Albu et al. 2005) and, at the level of the whole

body, adiposity has further been strongly associated with adverse health outcomes such as cardiovascular disease, diabetes, and cancer (de Ferranti & Mozaffarian 2008). The notion is supported by previous studies reporting decreased abdominal fat percent or prevention of weight gain with HRT compared to control (Haarbo et al. 1991, Kristensen et al. 1999, Jensen et al. 2003).

6.2 Molecular mechanisms behind the improvement or preservation of muscle phenotype by HRT

Despite the rather reasonable amount of studies focusing on the association between estrogen and the condition of skeletal muscle, the knowledge on the mechanisms through which the possible link is created has been scarce. During the preparation of this thesis, a handful of reports touching upon this issue were published (Buitrago et al. 2006, Vasconsuelo et al. 2008, Dieli-Conwright et al. 2009a, Dieli-Conwright et al. 2009b, Galluzzo et al. 2009, Muthusamy et al. 2009, Wiik et al. 2009, Dieli-Conwright et al. 2010, Pöllänen et al. 2010, Pöllänen et al. 2010, Ronda et al. 2010). Thus, the theme that this thesis addresses is topical and interests scientific communities worldwide.

6.2.1 Explorative pathway analysis reveals possible mechanisms mediating the association between HRT and muscle phenotype

A feasible and attractive strategy to identify the molecular changes owing to long-term exposure to postmenopausal HRT is to screen all genetic pathways in parallel with the HRT-related changes in muscle phenotype by utilizing full-genome oligonucleotide chips. Eleven identical twin pairs with the users of long-term, estrogen-based HRT were included in detailed pathway analysis (II).

The main results imply that the use of HRT is associated with somewhat subtle, but still pertinent changes in muscle transcriptome. The most notable changes were found in the expression of genes related to organization of cytoskeleton, cell-environment interactions, energy metabolism and responses to nutrition, which in their turn also explained a substantial fraction of the observed variation in muscle composition and power. Overall, the transcription profiles suggested that rather than up-regulating gene expression, HRT appeared to down-regulate transcriptional activity. On the other hand, due to cross-sectional nature of the data, the processes being "down-regulated" in the co-twins using HRT may actually be preserved near or at the premenopausal levels, while they would be "up-regulated" in the non-users as a result of aging and postmenopausal changes. Supporting findings come from a previous, longitudinal study from our laboratory suggesting that early postmenopausal years are characterized by vast amount of changes in gene expression in skeletal muscle, while HRT was reported to slow this phenomenon down (Pöllänen et al. 2007).

REGULATORY ACTION OF LONG-TERM HRT ON THE CYTOSKELETON. Only one biological process – "regulation of anatomical structure morphogenesis" – was significantly up-regulated within the co-twins using HRT. One of the genes responsible for the enrichment encodes MAPT, a stabilator of the microtubule network and a player important in the generation and maintenance of neurites. Overexpression of MAPT has been documented to inhibit kinesin-dependent transportation of peroxisomes into neurites predisposing the cells to oxidative stress (Stamer et al. 2002). A link between dysfunction of MAPT and deterioration of muscle properties has been reported as well. More specifically, transgenic mice expressing human MAPT with a common mutation, namely P301L, causing abnormal function of the respective protein exhibit skeletal muscle with neurogenic atrophy (Lewis et al. 2000). The link between the up-regulation of *MAPT* mRNA and the preserved muscle composition in the HRT users may thereby represent one hypothetical mechanism through which the use of HRT is associated with the positive condition of the neuromuscular system.

The top-ranked gene subset of this enriched process included also genes encoding proteins such as CDC42 effector proteins (CDC42EP) 1 and 4, interacting with CDC42 to regulate the organization and assembly of the actin cytoskeleton (Bishop & Hall 2000). Intriguingly, inhibition of CDC42 by exogenous expression of a dominant negative form has been documented to result in a block in myogenesis (Luo et al. 1994, Takano et al. 1998), although also contradictory results have been published (Gallo et al. 1999, Meriane et al. 2000). Nonetheless, CDC42 is undoubtedly an important agent in the milieu of skeletal muscle and, in the present study design, is included in the biological process, whose mean expression explains 19% of the variation observed in relative proportion of muscle within thigh. The result concerning the link between HRT and cell structure is supported by some studies such as that by Kublickiene and colleagues documenting that estrogen-based HRT may preserve the morphological integrity of endothelial cells by regulatory actions on cytoskeleton (Kublickiene et al. 2008). Moreover, exposure of endothelial cells to physiological levels of estradiol has been reported to result in rapid remodeling of actin cytoskeleton (Simoncini et al. 2006).

REGULATORY ACTION OF LONG-TERM HRT ON THE INTRAMUSCULAR EXTRACELLULAR MATRIX. Aside from being a major protein reservoir, skeletal muscle specializes in generating force and producing movement. The stabilizing structures of muscle tissue, located in contractile filaments, between sarcomeres and next to sarcolemma, hold the filaments together and enable appropriate function of this tissue and form an essential player in force transmission. With age, muscle stem cells are demonstrated to switch from myogenic fate into fibrotic one further resulting in impaired muscle regeneration and enhanced fibrotic response (Brack et al. 2007). The result implying that genes related to "cell matrix adhesion" are down-regulated in the

sisters using HRT may actually indicate that the respective process is up-regulated in the non-users and maintained close to premenopausal levels in the HRT users. This notion would explain the significant inverse association observed between the mean expression of "cell matrix adhesion" and jumping height. Support for this result is further brought by Zahn et al. by reporting that the up-regulation of genes of the extracellular matrix is involved in the common aging signature across three tissues; the skeletal muscle, the brain and the kidney, and suggesting that the observation reflects a well-known feature of aging, the overall, widespread fibrosis with advancing age. The higher expression of the genes regulating extracellular matrix and cell-matrix interactions, such as *SGCE*, *COL17A1*, *LYVE1*, and *ACTN1*, in skeletal muscle of the non-users may support at least two alternative theories. First, the observation may suggest improper force transmission through affecting the amount and quality of the matrix components in the interstitial space of muscle tissue, a process similar to fibrosis characterized by superfluous proliferation of the connective tissue. Second, the finding may reflect better cellular integrity. The negative correlation between the mean expression of this process and muscle mass and power suggest that the first view holds relevance. Furthermore, the scheme with impaired muscle composition is supported by a result that relative proportion of fat within thigh was on average 7% lower among the HRT users compared to the non-users in this same study sample (I, II).

Among the genes of leading edge was for example FBLN-5, an organizer of elastic fiber assembly. A truncated form of FBLN-5, unable to carry its role in elastogenesis, is reported to accumulate with age (Hirai et al. 2007). Thereupon, higher expression of this gene in the non-users may represent an indication to compensate for the aging-induced accumulation of the truncated form upon increased production of ECM components. Besides *FBLN-5*, the top-ranked gene subset included also a gene titled *PTEN*, which encodes a PIP_3 3-phosphatase, which negatively regulates the intracellular levels of PIP_3 and thereby antagonizes the activation of PI3K on Akt (Maehama & Dixon 1998, Pezzolesi et al. 2007). A connection within a trio of E_2, PTEN and PI3K/Akt in endometriosis has been postulated; E_2 promotes cell proliferation through the activation of PI3K/Akt pathway dependently on NFkappaB/PTEN pathway (Zhang et al. 2010). The data of this thesis support the view of the authors that high levels of E_2 coincides with low levels of PTEN and in the context of skeletal muscle, would lead to positive cell proliferative events. Thereby, this notion is in line with the observed positive association between estrogen-based HRT and muscle properties.

LONG-TERM HRT IS ASSOCIATED WITH CHANGES IN ENERGY METABOLISM. "Cofactor catabolic process", one of the down-regulated pathways among the co-twins using HRT, included for example the four subunits of the SDH complex functioning in the mitochondrial respiratory chain in the inner membrane of the mitochondria (Chen et al. 2009). The association between the

down-regulation of genes related to mitochondria and good muscle properties contrasts with a reported common aging signature, which suggest that an overall decrease in the expression of genes related to the mitochondrial electron transport chain is characteristics of aging (Zahn et al. 2006). If this notion supporting the traditional mitochondrial free radical theory of aging (Harman 1972, Balaban et al. 2005, Muller et al. 2007) holds true, the generation of free radicals by mitochondria would damage the electron transport chain protein complex, which would thereby be more "aged" in the HRT users with better muscle properties compared to their sisters in the present study. The results for example from the Dillin's laboratory with *Caenorhabditis elegans*, however, have implied that the overall down-regulation of the components of the electron transport chain slows down the physiology and thereby slows down the aging process (Dillin et al. 2002) thereby challenging the above-mentioned traditional theory. This novel explanation that mitochondrial dysfunction induces a physiological state that allows slow rate of aging is supported by other, quite recent studies (Yang et al. 2007, Lapointe et al. 2009, Van Raamsdonk & Hekimi 2009).

Thereby the results can have also alternative explanations. Pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), known to be expressed primarily in skeletal muscle and heart (Bowker-Kinley et al. 1998), had the second highest up-regulation of all the probes in the array (mean HRT user/non-user=2.4). PDK4 functions as an inactivator of the pyruvate dehydrogenase complex (PDC), a pivotal metabolic switch for fuel selection, thereby leading to utilization of fatty acids instead of glucose and contributing to the overall control of aerobic oxidation of carbohydrate fuels. Importantly, PDK4 has been suggested to contribute to the regulation of the adaptive response or long-term control of the activity of PDC (Randle 1986, Priestman et al. 1992). In human muscle cell culture, insulin reverses the up-regulation of *PDK4* caused by glucose deprivation and fatty acid supplementation (Abbot et al. 2005). The link between the down-regulation of the components of SDH complex and up-regulation of PDK4 could imply an overall decrease of glucose oxidation and possible preference for the utilization of fatty acids as an energy source after long-term use of HRT. In the present study population, this switch in metabolic status of muscle tissue may perhaps reflect the higher relative proportion of muscle tissue within thigh and better muscle power. The observation is supported by a previous study in which the decline in fat-free mass was the best single predictor of the decline in basal fat oxidation in humans (Calles-Escandon et al. 1995). Moreover, another study with rats reported that aging was associated with a decreased ability of muscle to oxidize fatty acids, a fact that was suggested to explain the accumulation of triglycerides in muscle, which again is a possible contributor to several metabolic disorders such as insulin resistance (Tucker & Turcotte 2002).

Intriguingly, complicating the area even more, one of the down-regulated genes in the co-twins using HRT in the pathway "response to nutrient" encoded PPARG, which plays a critical role as a transcriptional regulator of both

adipogenic and lipogenic programs (Spiegelman 1998). PPARG is also suggested to represent a molecular link between fatty acids and insulin sensitivity (Way et al. 2001, Evans et al. 2004, Tontonoz & Spiegelman 2008). The inhibition of the expression of PDK4 has been postulated to represent a mechanism by which PPARG agonists enhance glucose metabolism in muscle. In this context, the down-regulation of *PPARG* and up-regulation of *PDK4* with a parallel down-regulation of the components of mitochondrial respiratory chain may, again, pose an indication of lowered utilization of glucose in skeletal muscle after estrogen-based HRT, while exploitation of fatty acids as energy source would be enhanced. On the other hand, in another study with ovariectomized mice PPARG and its downstream targets were found to be up-regulated in response to E_2, and this was suggested to promote the partitioning of free fatty acids towards oxidation and away from triglyceride storage and thus also leanness of the ovariectomized animals fed with E_2 independently of reduced energy intake (D'Eon et al. 2005). Also contrasting the finding of this study concerning the down-regulation of PPARG and concomitantly observed better muscle properties are the results reporting reduced expression of PPARG coactivator 1α (PGC-1α) in obese persons (Mootha et al. 2003, Patti et al. 2003). Although the down-regulation of *PGC-1α* in the respective studies is associated with diabetes and somewhat contrasts the finding of this thesis i.e. down-regulation of *PPARG* with better muscle properties, the overall conclusion from the microarray data adheres to the above-depicted theory.

Interestingly, also *ENSA* was included in the top-ranked gene subset of this down-regulated pathway concerning responses to nutrients. Previously, ENSA has been shown to block K_{ATP} channels, possibly triggering insulin secretion via membrane depolarization, activating voltage-gated Ca^{2+} entry and finally increasing the levels of intracellular Ca^{2+} (Heron et al. 1998). Therefore, ENSA can be considered to play a key role in coupling cell metabolism to electrical activity. The high mean expression of this pathway together with poor relative proportion of muscle and muscle power in the non-users may indicate that calcium signaling is boosted at transcript level, but is not translated into improved function of this tissue, while the users would have a satisfactory calcium signaling and consequently better muscle properties.

6.2.2 Contribution of IGF-1 signaling pathway to the condition of musculature

Aside from the genome-wide analysis concerning the association between long-term, estrogen-based HRT and muscle transcriptome, the contribution of year-long HRT to the expression of genes along the IGF-1 signaling pathway in a randomized, double-blind, placebo-controlled design was investigated. The study was the first one to investigate the effects of combined HRT and the effective agents of the same HRT preparation (E_2 and NETA) on the IGF-1 signaling pathway. The results indicated that the intervention may perhaps affect the expression of several genes along the IGF-1 signaling cascade in comparison with the users of placebo (III). Noteworthy, the observed higher

level of gene expression along the IGF-1 signaling pathway coincided with improved muscle mass. The result of the induction of *IGF-1* gene expression by estrogen-based HRT is supported by a previous study reporting that E_2 treatment increases the level of IGF-1 in the rat uterus (Murphy et al. 1987) and natural progesterone in glial cells (Chesik & De Keyser 2010). Also testosterone has been shown to induce the expression of IGF-1 in human muscle samples (Ferrando et al. 2002).

In addition, the change in the expression of one splice variant of *IGF-1* gene, the *MGF (IGF-1Ec)*, was associated with post-intervention muscle mass. MGF, which was first identified as a factor responding to muscle contraction, is nowadays regarded as a major activator of muscle satellite cells and as a direct growth factor (Goldspink & Harridge 2004). Even though the synthetic MGF E-peptide has been shown to promote cellular proliferation and survival the actual role or even existence of the endogenous MGF E-peptide has recently confronted criticism (Matheny et al. 2010). However, there is no doubt that the *IGF-1Ec* mRNA exists and coincides with improvements in muscle mass. Hereby, greater muscle mass observed among the HRT users may hypothetically be a result from improved muscle repair systems, as well as, activation of growth promoting signaling cascades even though we do not know whether the effect is due to related (cleaved or not cleaved) E-peptide or the whole pro-MGF. In a very recent transcriptome-wide study the expression of several myogenesis-related genes was found to be up-regulated in muscles of both the HRT users and the controls suggesting an increased demand for muscle regeneration in the early stage of the postmenopause (Pöllänen et al. 2010). Also other studies suggest 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 (Kamanga-Sollo et al. 2004, Enns & Tiidus 2008, Enns et al. 2008, Kamanga-Sollo et al. 2008, Dieli-Conwright et al. 2009a). Since the amount of serum IGF-1 and T were similar between the users and the controls, the observed increment in muscle mass in the HRT users appears not to be due to endocrine effects of IGF-1 or T, but rather owes to the local activation of the IGF-1 pathway through autocrine or paracrine manner in response to HRT.

When it comes to the expression of other factors along the IGF-1 signaling pathway, the results were somewhat confusing. Of the "anabolic" genes *mTOR* was up-regulated and *Akt1* appeared to be down-regulated, while the expression of a "catabolic" factor, *Foxo3*, was up-regulated. Supporting an overall pro-anabolic microenvironment the transcript level of *AR* was also up-regulated. Interestingly, Hewitt and colleagues have proposed a model along which E_2 has the capacity to interact with AR in order to modulate gene transcription (Hewitt et al. 2005a). Thereby, the up-regulation of *AR, IGF-1* and *MGF* may be suggested to imply to possible cross-talk between the given sex steroids and growth factors. A cell culture setting with C2C12 myotubes fed with E_2 or NETA was utilized to discover the mechanism behind the

discrepancy of the expression data, but did not, however, extend the understanding in this context.

Since the steady-state level of a given gene transcript does not always necessarily reflect the true activation of signaling pathways, the effects of E_2 and NETA on the phosphorylation of Akt and mTOR were analyzed. The 10 nM NETA treatment was observed to induce the phosphorylation of Akt at Ser[473] even up to about five fold suggesting that NETA may exert an androgenic effect on the pathway under investigation, but the findings were not significant (III). The finding from these multinucleated myotubes contrasts with a recent study by Vasconsuelo and colleagues reporting that E_2 induces the phosphorylation of Akt at Ser[473] in single-nucleated, undifferentiated C2C12 myoblasts (Vasconsuelo et al. 2008). After the experimentation presented in this thesis was performed, also other studies within the area were published. Galluzzo and co-authors reported that E_2 increases the translocation of GLUT4 at membranes, while also augmenting the expression of the differentiation markers of myogenesis in rat L6 myoblasts. Furthermore, the effects were shown to involve ER-dependent activation of Akt and thereby suggested to participate in the regulation of the first step of myogenic differentiation (Galluzzo et al. 2009). Similar results were observed in a study with sex-steroid deficient adult male rats, whose glucose uptake mediated by Akt activation and GLUT4 expression was restored after E_2 and/or testosterone treatment (Muthusamy et al. 2009). The notion of this thesis concerning the lack of the effect of E_2 on the activation of Akt is reasoned by the fact that the activation of Akt is known to govern both the early and late steps of myogenic differentiation (Bodine et al. 2001b) and a recent study documenting that p-Akt is present between 24 and 120 hours during the differentiation of myoblasts into myotubes (Alisi et al. 2008). Thus, the high phosphorylation of Akt already present owing to ongoing myogenesis throws down the gauntlet on the experimentation. It may also have impeded the observation of the effects of each hormonal treatment, albeit several different concentrations of the hormones and time points of treatments were tested during optimization of the experimental procedure. Another point is that serum-starvation may have not been long enough to avoid background p-Akt. The too narrow time window may also have hindered the proper observation of phosphorylated mTOR, a key regulator of muscle protein synthesis (Glass 2005).

Although Vasconsuelo and co-authors investigated the activation of Akt in the context of apoptosis (Vasconsuelo et al. 2008) and Galluzzo in skeletal muscle differentiation (Galluzzo et al. 2009), their observation of activated Akt in response to E_2 treatment in myoblasts would also possibly be related to the generation of eNOS via the activation of the PI3K/Akt axis in response to estrogen treatment as has been shown in endothelial cells (Chen et al. 1999, Haynes et al. 2000, Simoncini et al. 2000). If true, the generation of eNOS via PI3K/Akt in response to E_2 in the context of skeletal muscle would to some extent explain the better muscle properties observed here within the HRT users in comparison with placebo. The hypothetical positive effect of HRT on the

musculature via the generation of eNOS would also be supported by an observation that estrogen-based HRT restores the vascular activity of NO to premenopausal levels (Majmudar et al. 2000) and a recent study with mice reporting that even a small decrease in the amount of eNOS suffices to impair exercise capacity (Lee-Young et al. 2010). Furthermore, the increase of the activity of eNOS through PI3K/Akt pathway has been reported to decrease the need for Ca^{2+} influx in human endothelial cells (Haynes et al. 2000). The link between estrogen and calcium signaling is supported by the previously discussed finding from the twin design that the expression of genes regulating calcium signaling are down-regulated in the co-twins using HRT, who also have favorable muscle phenotype. Any solid conclusions on if the activity of PI3K/Akt axis is affected by E_2 or NETA cannot, however, be drawn and the hypothesis remains speculative.

The IGF-1 signaling is so far mostly referred to as a potential hypertrophic pathway in this thesis. The scientific community dedicating a huge amount of research to this area has had inconsistent opinions whether the route is actually a major regulator of muscle mass or not. In fact, just in this ongoing year the Journal of Applied Physiology (volume 108, issue 6) devoted several pages to the discussion on this issue (Ameredes 2010, Baar et al. 2010, Barton & Philippou 2010, Esser et al. 2010, Flueck & Goldspink 2010, Harridge & Velloso 2010, Loughna 2010, Musaro 2010, Phillips 2010, Shavlakadze & Grounds 2010, Shenkman et al. 2010, Song 2010, Spangenburg 2010, Stewart & Pell 2010, Vinciguerra et al. 2010, Yang 2010). The alternative opinions supported the two views that IGF-1 represents the key regulator of muscle mass, or that it is just one of the factors affecting muscle size, or additional aspects in between the two extremes. Very recent data on transgenic mice generated to overexpress murine IGF-1 in skeletal myofibres underscore the importance of the exact experimentation utilized in studying the issue. More precisely, Shavlakadze and colleagues reported that elevated IGF-1 levels do not induce hypertrophy of adult non-growing skeletal muscles, while growing muscles were shown to respond to IGF-1 by activation of the downstream components from the IGF-1 receptor (Shavlakadze et al. 2010). The present experimentation may thus not represent the best possible set up as the mature myofibres on which the human data are based and the growing muscle cells may not have enough correspondence with regards to IGF-1 signaling. The discussion of the ultimate role of IGF-1 in the growth of skeletal muscle in the developmental period or in adult life is, however, beyond the focus of this thesis.

6.2.3 Contribution of estrogenic polymorphisms

Since individual differences in muscle phenotypes in old age are explained by both environmental and genetic factors (Loos et al. 1997, Thomis et al. 1997, Beunen & Thomis 2004, Tiainen et al. 2004, Tiainen et al. 2005, Tiainen et al. 2007, Tiainen et al. 2008, Tiainen et al. 2009), a thorough review of the literature was performed in order to build up a hypothesis of potential candidate genes, which may possibly mediate the effects of E_2 on muscle phenotype.

Intriguingly, a functional polymorphism in *COMT* gene putatively resulting in altered enzyme activity appeared to be associated with muscle phenotype in older Finnish women (IV). More precisely, participants with the LL genotype and thus the enzyme with low activity had significantly larger muscles than the heterozygotes, a notion that has some support from a previous study with early pubertal girls (Eriksson et al. 2005). No other studies investigating the association between this polymorphism and muscle phenotype were found. Furthermore, within the subjects with HH genotype – leading to the presumed higher COMT activity – and sedentary life-style, lower levels of muscle mass, strength and power were observed than within other sedentary subjects or subjects with more active life-style. This specific observation is totally novel and provides interesting evidence that a genetic predisposition into unfavorable muscle properties may be compensated for by physically active life-style. The contribution in this context was major as the adjusted mean value of knee extensor strength in moderately active subjects with the HH genotype were 37% higher in comparison with the respective value of the sedentary participants carrying the same genotype, while the difference was only 8% within the heterozygotes in favor of the more active individuals. Albeit a fairly interesting association was found in this study, it should always be stated that the result may also reflect some specific haplotype, a specific combination of closely located alleles.

With respect to ERα, a PvuII polymorphism was found not to be associated with muscle phenotype, supporting the results from previous studies concerning muscle strength (Vandevyver et al. 1999, Salmen et al. 2002). Here, it should be mentioned that the possible link between PvuII polymorphism and e.g. bone phenotypes recognized so far (for a review, see Gennari et al. 2005) may be due to some unknown polymorphism residing in close proximity of and being in linkage disequilibrium with PvuII locus within ERα. One suggestion for such a polymorphic site has been a TA repeat polymorphisms in the promoter region of ERα and thus upstream from PvuII site (Becherini et al. 2000). This polymorphic site or some other yet unidentified one may affect bone, but not muscle properties.

6.3   Methodological considerations

Some methodological aspects arise from this study. Although the results are based on a limited sample sizes and should be interpret with a breath of caution, the study designs are powerful. With the cross-sectional, but genetically controlled twin design with identical twins discordant for HRT the possible fundamental differences due to historic reasons in general lifestyle, including physical activity habits, present in a traditional case-control setting can be overcome. Twin study represents a strong controlled study design in humans owing to the complete or at least close match for genetic background,

age, and gender as well as for intrauterine and childhood environment. Although the HRT users employ different preparations, the discordance design with respect to long-term HRT represents a real-life example of HRT users and non-users who have made their decision on HRT in a clinical setting outside this study. Another study with HRT administration was a year-long, randomized, double-blind, placebo-controlled trial. The RCT holds an advantage with a fixed HRT preparation, since the study question addressed specific signaling pathway instead of explorative approach. Although the findings of this intervention on the IGF-1 pathway were not confirmed in the cell culture model, it should be noted that the systemic administration of the hormones in the one-year RCT trial is different model in comparison with the cell culture setting in which the effective agents of HRT are fed directly on the cells. The third study design with female participants utilized had rather small amount of subjects for testing an association between SNP and muscle phenotype. The results were, however, convincing and statistical significance is not that easily reached with this sample size making the findings not likely false positive ones.

The cell culture experimentation revealed a noteworthy methodological observation. Utilization of a "basal level" control sample in this kind of setting in which cells ongoing a differentiation process are studied is insufficient. In pursuing accurate and reliable findings the cells with supplement free medium were tracked along the entire time frame of the experiment. The expression of several transcripts or the level of protein phosphorylation changed in myotubes with ongoing differentiation as the time passed regardless of the samples; untreated or treated. If treated samples of each time point would have been compared to the control sample of 0 hours, some statistically significant effects due to hormonal treatments would most probably have been present. As an example, no statistically significant effect of NETA on the amount of p-Akt was identified with ANOVA testing, while the 10 nM treatment elicited a significant ~2-fold and 4-fold increase at 20 min and 40 min time points, respectively, when compared to 0 hours ($p < 0.05$, data from Figure 11).

The global gene expression profiling of skeletal muscle biopsies constitutes one central method of this thesis. The mRNA that the analysis is based on includes molecules not only from the nuclei of muscle cells, but also those of non-muscle cells such as fibroblasts, satellite cells, and endothelial cells of the capillaries (Goldberg et al. 1975). The myogenic nuclei, however, are readily the most abundant nuclei providing the RNA into the sample. The steady-state mRNA levels under investigation always reflect the net sum of RNA synthesis and decay and thereby give indirect evidence on the differences in actual gene transcription capacity. The expression profiles, however, rather nicely reflect the utilization of the genetic code at the particular moment of time, which is always controlled for as carefully as possible during the sampling.

6.4 Final evaluation of HRT as one lynchpin in powerful aging

The link between postmenopausal estrogen-related hormone replacement therapy and muscle properties has been complicated. A comprehensive review of the literature in this thesis clearly points out that no definitive consensus thus far exists, whether HRT exerts positive effects on the musculature or not. The fact that sarcopenia, however, entails huge tolls, such as morbidity (Sayer et al. 2005), disability (Janssen et al. 2002), increased costs of health care (Janssen et al. 2004) and even mortality (Gale et al. 2007), justifies the investigation concerning the underlying processes and the appropriate interventions, including postmenopausal HRT, to counteract the them.

The main results of this thesis are summarized in Figure 12. The findings indeed suggest that the elevation of the systemic concentration of estrogen, in the form of HRT, results in better muscle mass, composition and power, as well as mobility, but not as much in higher strength parameters (I). This result supports the view that loss or preservation of muscle mass and strength and even power possibly arise from separate processes (McDonagh et al. 1983, Young et al. 1985, Clark et al. 2006, Clark & Manini 2008, Clark & Manini 2010), although the transcriptional profiling suggest that muscle mass and power have at least some common denominators (II). More specifically, better mass and power are orchestrated by improved regulatory actions on cytoskeleton, preservation of muscle quality via regulation of intramuscular extracellular matrix and a switch from glucose-based metabolism into utilization of fatty acids (II). Also possible activation of IGF-1 signaling pathway appeared to underlie at least partially the positive association between HRT and muscle mass (III). The association between a selected genetic single nucleotide polymorphism within the *COMT* gene with muscle phenotype was evident and nicely depicts the importance of genetics in assessing the responses of the musculature to various interventions.

Previous molecular level data suggest that estrogen can sustain survival or, alternatively, induce apoptosis of various cell types depending on the biological context in case (Choi et al. 2001, Okasha et al. 2001, Seli et al. 2007, Florian & Magder 2008, Vasconsuelo et al. 2008). Recent studies have suggested that, in skeletal muscle cells, E_2 possesses anti-apoptotic effects and thus a survival action involving for example ERK and p38 (Buitrago et al. 2006, Vasconsuelo et al. 2008, Ronda et al. 2010). The results of this thesis support the given hypothesis and suggest that HRT creates a pro-anabolic microenvironment in skeletal muscle. Similar conclusions were drawn by Dieli-Conwright and colleagues, who only recently reported that HRT positively affects the expression of genes controlling myogenic growth and differentiation (Dieli-Conwright et al. 2009a). The answer for the positive link between HRT and muscle phenotype may also arise from the possible ability of estrogen to limit exercise-induced damage and inflammation as well as to promote the following repair processes (Stupka et al. 2000, Clarkson & Hubal 2001, Tiidus

2003, Tiidus 2005, Sewright et al. 2008, Tiidus & Enns 2009, Enns & Tiidus 2010). This study does not address the association between HRT and muscle damage in detail, but accepts that some of the mechanisms underlying the connection between high estrogen levels and good condition of the musculature may owe to the above-mentioned processes.
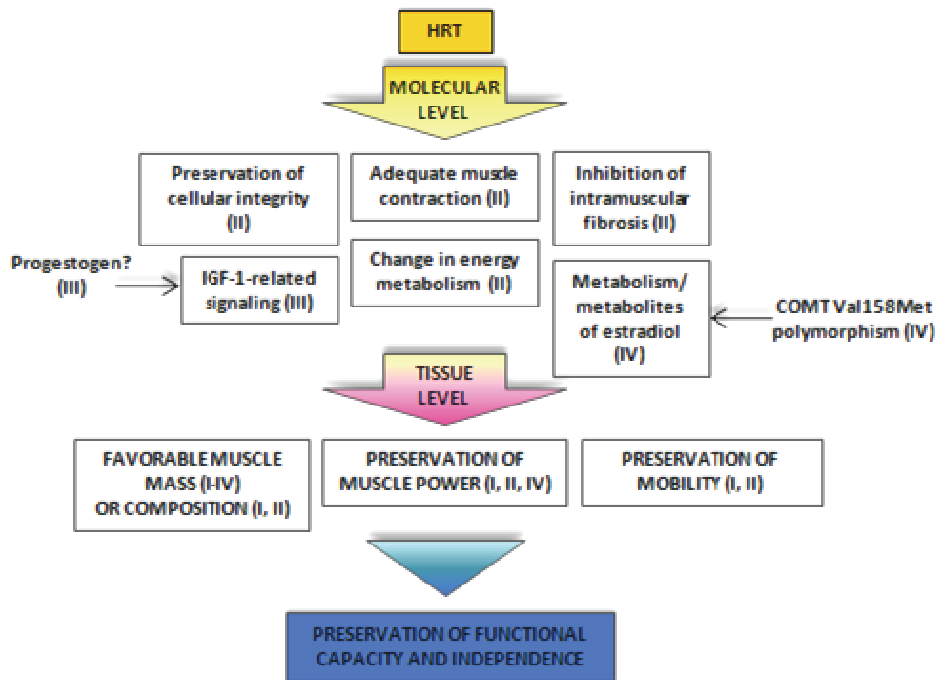
FIGURE 12   Summary of the main results from the four substudies of this thesis. Long-term, estrogen-based HRT is suggested to preserve cellular integrity via regulatory actions on microtubule network and actin cytoskeleton, aid in adequate muscle contraction through adequate Ca^{2+} signaling, result in favorable regulation of intramuscular extracellular matrix and thus inhibition of fibrosis, and change the energy metabolism through switch from glucose to fatty acid metabolism. Year-long intervention on HRT suggested that the treatment activates hypertrophy-related IGF-1 signaling (possibly together with progestogen) and results in better muscle mass in comparison to placebo. Moreover, a polymorphic site within the *COMT* gene (resulting in substitution of Valine to Methionine) is associated with muscle phenotype in older women. These molecular mechanisms together other coinciding events in muscles result in favorable muscle properties important in the preservation of functional capacity and independence.

The results of this thesis possibly tip the balance in the research on the area by reinforcing the conception that skeletal muscle can be considered to potentially be responsive to estradiol signaling. Consequently, estradiol should be recognized when the intricate pathways leading to deterioration of muscle properties are pondered on and finally constructed. The interventions should,

however, be carefully designed as the obvious interdependence of various hormones can lead to an undesirable synergistic effects, further creating a perfect storm, in which an intervention on a single hormone has a negligible effect. Also, the study designs, age of the participants, time since menopause, test conditions or the equipment used, muscles studied, sampling, duration of HRT and the amount and type of effective agent in the preparations should be carefully deliberated according to a specific study question in case.

This thesis mainly reports the effects of systemic or otherwise external estrogen on the musculature, while data on possible local production of estrogen in muscle are not available and await further research. This is, however, a notion to be recalled as systemic exposure to a given compound may even attenuate the production of the respective agent within target tissues creating a cellular response with negative feedback. Furthermore, meticulous search on the literature and cleverly made hypothesis concerning each intervention are of importance so that information on the precise causal relationships would accumulate. Ultimately, in the best-case scenario, future rehabilitation strategies would be more efficient if the practitioners knew whether to target muscle quality, muscle power, nervous system, cognitive ability or even some other factors. The most brilliant interventions would presumably include moderate strength training, optimization of nutrition, prevention of obesity-related complications, and ameliorated response to stress combined to cellular approach including pharmacological strategies, cellular therapies or even gene transfer (Doran et al. 2009). The processes behind sarcopenia and the related healthy aging remain complicated and obviously involve several physiological systems, but the contribution of estrogen to the underlying processes may perhaps now be considered to hold significant relevance.

6.4.1 Future perspectives

Future can be anticipated to most probably involve further studies on the direct or indirect association between estrogen and the condition of skeletal muscle. Aside from the investigation on human subjects, which are primarily emphasized in this thesis, also a spectrum of animal studies gives novel viewpoints in this context. As stated by Zahn and colleagues, however, a vast amount of regulation of gene expression in humans is specific to species (private) rather than universal to all animals (public) (Zahn et al. 2006). With respect to methodological point of view, a logical continuum for this study in humans would be to assess the influence of HRT on muscle at the level of the entire proteome by utilizing a proteomic approach, such as two-dimensional gel electrophoresis, a technique that has conquered a foothold in the arena of protein research and is utilized in the research on sarcopenia as well (Cai et al. 2000, Cai et al. 2001, Piec et al. 2005, Gelfi et al. 2006, O'Connell et al. 2007, Doran et al. 2008). This would offer an enormous progress for translating the results from this study closer into the level of cellular function. Detailed 2-D maps on the major soluble muscle proteome, including the players of the

actomyosin apparatus, the regulation of contraction, ion homeostasis, signal transduction, mitochondrial metabolism, cytosolic metabolism and stress response, of several mammals are available (Isfort 2002, Doran et al. 2007) and possess a prerequisite tool to draw rather versatile conclusions from the 2-D gel data (Doran et al. 2009). The biological processes that emerged from the microarray analysis and possibly modulate the effects of HRT on muscle would be intriguing to study in detail. The results of this thesis implying decreased adiposity following the use of HRT suggest that also examining the response of adipose tissue, nowadays frequently referred to as even the biggest endocrine organ of the human body (Trayhurn 2005, Gesta et al. 2007, Galic et al. 2010), would be highly important and interesting.

When it comes to the data setting, besides taking a blank, white canvas to design further research, a tantalizing opportunity would be to carry out follow-up measurements to the twin design that was utilized in this thesis. Longitudinal, genetically controlled study with identical twin pairs exhibiting long-term discordance for HRT combined with ample sample resources would drive the research in this area forward with huge leaps. On the other hand, following women from their pre-menopausal era through menopause and thereafter during the progression of the postmenopausal period both with and without HRT would offer a possibility to precisely assess the most beneficial timing and duration of HRT.

7 MAIN FINDINGS AND CONCLUSIONS

The main conclusions of this thesis are:

1. HRT is associated with better mobility, greater muscle power and muscle mass, as well as, favorable body and muscle composition among postmenopausal women.

2. Improved regulatory actions on cytoskeleton, preservation of muscle quality via regulation of intramuscular extracellular matrix and a switch from glucose-oriented metabolism into utilization of fatty acids constituted set of cellular processes at least partly responsible for the better mass and power emerging after long-term use of HRT compared to the non-users. In addition, possible activation of IGF-1 signaling pathway contributes to the positive association between the use of HRT and muscle mass.

3. Homozygocity with respect to the presumed high-activity allele in the functional polymorphism within the *COMT* gene is associated with lower muscle mass compared to the heterozygotes. Furthermore, sedentary individuals with the same genotype are the most prone to muscle weakness, but may also benefit the most from physically active lifestyle underscoring that a genetic predisposition to unfavorable muscle properties may be compensated for by favorable lifestyle.

The potential of postmenopausal HRT as an agent in improving or preserving the overall condition of the musculature and thereby preventing the onset of severe frailty upon aging in the light of the current knowledge encourage further studies in this field. It seems realistic to conclude that estrogen constitutes a significant player in the context of the processes related to sarcopenia. Despite the appealing potential of estrogen as a tool of powerful aging, the future only will tell how important this factor is in reversing the age-dependent muscle degeneration confronted by every individual and decreasing the standard of living for a remarkable portion of the society.

YHTEENVETO

Lihaksiston koko, koostumus ja voima heikentyvät ikääntymisen myötä. Ilmiön taustalla vaikuttavat lukuisat tekijät, joista yhtenä mainittakoon merkittävä muutos hormonien tuotannossa. Vaikka lihasten suorituskyvyn laskun tiedetään olevan yhteydessä naissukupuolihormoni estrogeenin tuotannon loppumiseen vaihdevuosi-ikäisillä naisilla, sen osuutta lihasheikkouden kehittymiseen ei ole juurikaan tutkittu. Eri tutkimuksissa on osoitettu osittain ristiriitaisia tuloksia estrogeeniä sisältävän hormonikorvaushoidon (HRT) käytön vaikutuksista lihasten kokoon, koostumukseen ja voimaan. Osassa tutkimuksista HRT ei ole todistettavasti parantanut tutkittavien lihasvoimaa, kun taas toisissa on raportoitu tuloksia HRT:n myönteisistä vaikutuksista mm. lihasvoimaan ja suorituskykyyn. HRT:n yhteyttä lihakseen massaan on tutkittu jonkin verran ja tulokset viittaavat, että käyttö voi joissain olosuhteissa parantaa lihaksen massaa. Tutkimuksia, joissa olisi tarkasteltu HRT:n käytön vaikutuksia lihaksen koostumukseen, ei ole saatavilla. Mahdollisten positiivisten vaikutusten taustalla toimivia solu- ja molekyylitason prosesseja tunnetaan huonosti.

Tämän tutkimuksen tarkoituksena oli selvittää vaihdevuosioireisiin käytettävän HRT:n yhteyttä luurankolihaksiston rakenteeseen ja toimintaan ja tunnistaa erityisesti niitä solussa tapahtuvia prosesseja, jotka välittävät mahdollisen yhteyden syntyä ja ylläpitoa. Tutkimuksessa hyödynnettiin kolmea eri tutkimusaineistoa. Ensimmäinen aineisto koostui 54–62-vuotiaista, identtisistä naiskaksospareista (n=15 paria), joista toinen oli pitkäaikainen HRT:n käyttäjä, kun taas toinen ei ollut koskaan käyttänyt HRT:a. Tämänkaltaisessa ns. diskordanssiasetelmassa sekä perimä että kaksosille yhteiset ympäristötekijät tulevat otetuiksi huomioon, mikä vähentää normaalia yksilöiden välistä vaihtelua. Toisen tutkimusaineiston muodosti randomisoitu, lumelääkekontrolloitu, kaksoissokkotutkimus, jossa 50–57-vuotiaat naiset satunnaistettiin vuoden ajaksi HRT- (n=10) ja kontrolliryhmään (n=9). Kolmannen aineiston muodostivat 63–76-vuotiaat kaksosnaiset (n=434), joita käsiteltiin tässä yhteydessä yksilöinä. Lisäksi hyödynnettiin hiiren C2C12-solulinjaa, jossa erilaistuneille myotuubeille syötettiin yhdistelmä-HRT:ssa käytettyjä estrogeenista ja progestogeenistä ainesosaa.

Tutkittavilta mitattiin asetelmasta riippuen kehonkoostumus, lihasten koko, koostumus, voima ja voimantuottoteho sekä kävelynopeus. Lihasnäytteistä (*vastus lateralis*) määritettiin merkitsevästi rikastuneita biologisia prosesseja HRT:n käyttäjillä verrattuna heidän siskoihinsa. Erityishuomiota kiinnitettiin PI3K/Akt-reitin, jonka yksi aktivaattori on insuliininkaltainen kasvutekijä 1 (IGF-1), aktiivisuuteen ja arvioitiin reitin vaikutusta lihasten ominaisuuksien kehittymisessä tai säilymisessä. HRT:n vaikuttavien ainesosien mahdollisia erillisvaikutuksia kyseisen reitin aktivoitumiseen tutkittiin tarkemmin hiiren lihassoluviljelmässä. Katekoli-*O*-metyylitransferaasi (COMT)- ja estrogeenireseptori-α-geeneissä sijaitsevien yhden nukleotidin polymorfioiden yhteyttä lihaksen

ominaisuuksiin. Lisäksi arvioitiin fyysisen aktiivisuuden vaikutusta näiden yhteyksien välittäjänä.

Tuloksemme antavat viitteitä siitä, että pitkäaikainen HRT:n käyttö on yhteydessä parempaan liikkumiskykyyn sekä lihaksen voimantuottotehoon. Lisäksi HRT:n käytön havaittiin olevan yhteydessä parempaan kehon ja lihasten koostumukseen sekä suurempaan lihasmassaan verrattuna ei-käyttäjiin. Koko perimän kattavien mikrosirutulosten mukaan HRT:n positiiviset vaikutukset lihaksen kuntoon näyttävät ainakin osittain olevan yhteydessä parantuneeseen solun tukirangan ja lihaksensisäisen soluväliaineen komponenttien säätelyyn sekä solun energiametaboliassa tapahtuviin muutoksiin verrattuna ei-käyttäjiin. HRT:lla havaittiin lisäksi olevan vaikutusta IGF-1-reitillä toimivia proteiineja koodaavien geenien ilmenemiseen, vaikka tuloksia ei saatu tarkennettua soluasetelmassa. Myös COMT-geenissä olevan polymorfisen kohdan, joka vaikuttaa vastaavan entsyymin aktiivisuuteen, havaittiin olevan yhteydessä lihaksen massaan. Fyysisesti inaktiivisimmat tutkittavat, joilla oli korkeaan entsyymiaktiivisuuteen mahdollisesti johtava genotyyppi, olivat kaikkein heikoimpia, mutta näyttivät toisaalta hyötyvän eniten fyysisesti aktiivisesta elämäntavasta. Tämä havainto on hyvä esimerkki siitä, että epäsuotuisaa, yksilöllistä muuntelua perimässä voi kompensoida hyvillä elintavoilla.

Tämän väitöskirjan tulosten perusteella voidaan todeta, että HRT:n käyttö on yhteydessä hyvään lihaksiston kuntoon ja estrogeeni voi mahdollisesti olla yksi tärkeä tekijä, jonka pitoisuuden väheneminen verenkierrossa edistää ikääntyessä tapahtuvien lihasten surkastumisen ja lihasheikkouden kehittymistä. HRT näyttää vaikuttavan useiden eri biologisten prosessien kautta, jotka liittyvät mm. solutukirangan eheyteen, soluväliaineen edulliseen koostumukseen ja määrään sekä energiametaboliaan. Tulosten mukaan myös estrogeenimetaboliaan osallistuvaa entsyymiä koodaavan geenin perinnöllinen muuntelu on yhteydessä lihaksiston ominaisuuksiin. Tämä tutkimus antaa myös aihetta uusille solu- ja molekyylitason tutkimuksille, jotta estrogeenin täsmällisiä vaikutusmekanismeja luurankolihaksistoon voitaisiin tarkentaa.

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