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EFFECT OF ENDURANCE EXERCISE ALONE AND IN COMBINATION WITH IGF-1 ADMINISTRATION ON CELLULAR MARKERS INVOLVED IN SARCOPENIA

PhD thesis

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List of Abbreviations

4E-BP1	4E-binding protein 1
AIF	Apoptosis-inducing factor
ALS	Acid labile subunit
AMPK	AMP-activated protein kinase
ANT	Adenine Nucleotide Translocase
Apaf-1	Apoptotic protease activating factor-1
ARC	Apoptotic repressor with a caspase recruitment domain
Atg	Autophagy-related protein
ATP	Adenosine three phosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
CAD	Caspase-activated DNase
Caspases	Cysteine-aspartic proteases
COX	Cytochrome c oxidase
CR	Caloric restriction
CREB	cAMP response element-binding protein
CS	Citrate synthases

CSA	Cross section area
CT	Computed tomography
CyPD	Cyclophilin D
Cyto C	Cytochrome C
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
DXA	Dual-energy X-ray absorptiometry
EAA	Essential amino acid
EDL	Extensor digitorum longus
eIF2B	Eukaryotic initiation factor 2 subunit B
EIM	Electrical impedance myography
EndoG	Endonuclease G
ERK	Extracellular signal-regulated kinase
ET	Endurance exercise training
ETC	Electron transport chain
ETS	Electron transport system
EWGSOP	European Working Group on Sarcopenia in Older People
Fis1	Mitochondrial fission protein 1
FLRG	Follistatin-related gene
FOXO	Forkhead box O
FSH	Follicle-stimulating hormone
GASP-1	GDF-associated serum protein-1
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	GH releasing hormone
H2O2	Hydrogen peroxide
HRT	Hormone replacement therapy
IGF-1	Insulin-like growth factor 1
IGFBPs	Insulin-like growth factor-binding proteins
IGFR1	Type 1 IGF receptor
IKK	IκB kinase

IL-6	Interleukin 6
IMM	Inner mitochondrial membrane
IR	Insulin receptor
IWGS	International Working Group on Sarcopenia
JNK	c-Jun N-terminal kinases
LC3	Microtubule-associated protein 1A/1B-light chain 3
MAC	Mitochondrial apoptosis-induced channel
MAFbx	Muscle atrophy F-box
MAPKs	Mitogen-activated protein kinases
Mfn1	Mitochondrial fusion 1
MGFs	Mechano growth factors
MHC	Myosin heavy chain
MKKs	MAP kinase kinases
MKPs	MAPK phosphatases
Mnk 1	MAPK-interacting kinase 1
MnSOD	Manganese superoxide dismutase
MOMP	Mitochondrial outer membrane permeabilization
MPS	Muscle protein synthesis
MPT	Mitochondrial permeability transition
mPTP	Mitochondrial permeability transition pore
MRFs	Muscle regulatory factors
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mtDNA	Mitochondrial deoxyribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MuRF1	Muscle RING-finger protein-1
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation 1 protein
NAMPT	Nicotinamide phosphoribosyltransferase
NF-κB	Nuclear factor κb
OC	Old control

OE	Old exercise training
OEI	Old exercise and IGF-1 treatment
OMM	Outer mitochondria membrane
p70s6k	70-kilodalton ribosomal S6 protein kinase
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1-α
PI-3K	Phosphatidylinositol-3-kinases
PNPase	Polynucleotide Phosphorylase
RDA	Recommended dietary allowance
ROS	Reactive oxygen species
RT	Resistance exercise training
SDH	Succinate dehydrogenase
SOCS-3	Suppressor of cytokine signaling-3
TCA	Tricarboxylic acid
Tfam	Transcription factor A, mitochondrial
TNF-R	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TSC2	Tuberous Sclerosis Complex 2
UCP3	Mitochondrial uncoupling protein 3
UPS	Ubiquitin-proteasomal system
VDAC	Voltage dependent anion channel
VO2max	Maximal oxygen consumption
YC	Young control
YE	Young exercise training
YEI	Young exercise training and IGF-1 treatment
YY1	Yin Yang 1

1. Introduction

1.1. Aging process and its consequences

Aging is an undeniable and complicated biological process that is indicated by a general time-dependent decline in the physiological and biochemical functions of the main systems [1, 2]. Aging is also a remarkable risk factor for the advancement of cardiovascular diseases [3] and is associated with a significant decline in neuromuscular function and performance [4]. The percentage of elderly people in the world is increasing steadily (Figure 1) [5] due to the impressive rise in average life expectancy in the past century [6].

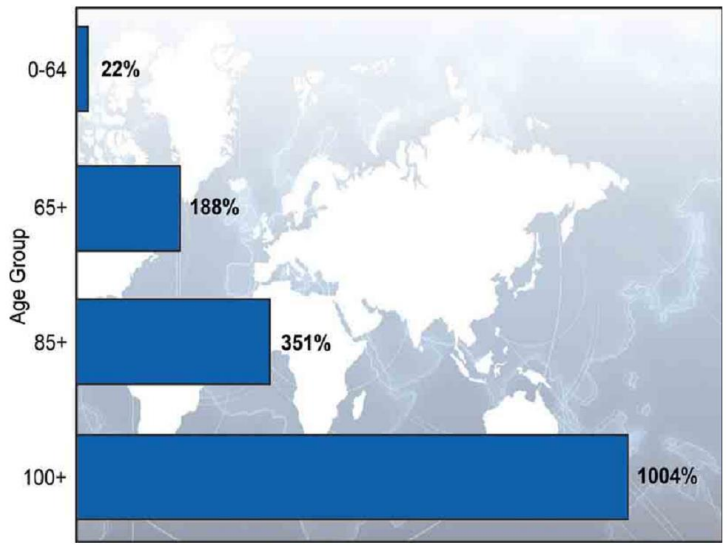

Figure 1. Percentage change in the world's population by age: 2010-2050 [1]

The rapid increase world's aging population has led to concerns about the general health of elderly [7]. It is estimated that until year 2050, the elderly population will increase from 600 million as it was in the year 2000 to more than two billion (Figure 2) [8]. Several changes are observable along with aging, including a reduced capacity oxygen consumption, an impairment in cardiorespiratory adaptation and degradation of the nervous system, and decadence in muscle mass which characterized by a reduction in muscle mass and by a qualitative and quantitative alteration in muscle fibers [2]. After age 30, a change in body composition occurs. However, a reduction of about 0.23 kg muscle mass per year is expected from ages of 30 to 60 which accelerates to 2% annually from the age of 60 [9]. Indeed, data from the most recent longitudinal aging study suggest that muscle strength decreases at an intermittent rate of ~ 3% yearly between the ages of 70–79 years [10].

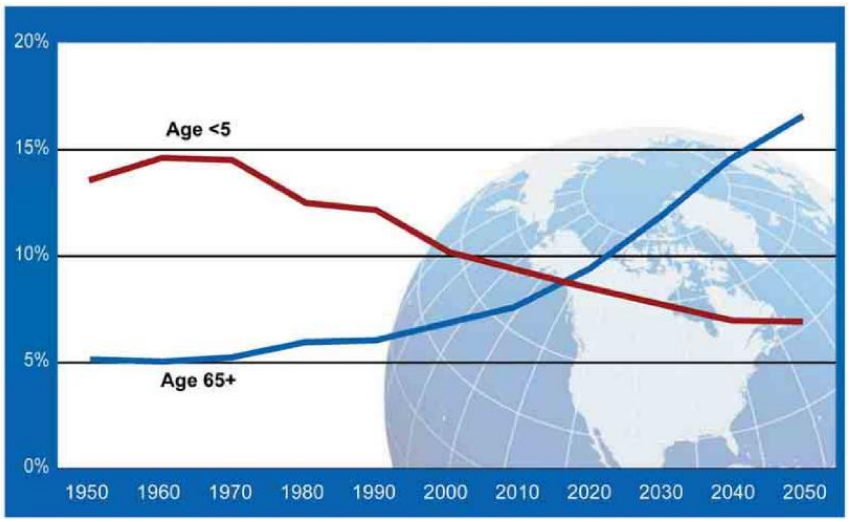

Figure 2. Young children and older people as a percentage of global population: 1950-2050 [1]

Due to increasing longevity and the fact that the elderly have twice as many disabilities and four times as many physical limitations as people less than 60 years of age [11], it is imperative to understand the aging process and the mechanisms associated with healthy aging and conservation of functional independence at the end of life [12].

1.2. Sarcopenia phenomenon as a natural consequence of the aging process

1.2.1. Definition

One of the most well-known features of aging is a change in body composition and decline in lean body mass [13]. Age related skeletal muscle wasting, also called sarcopenia, plays a significant role in reducing performance [14]. The contribution of lean muscle mass in whole body weight is approximately ~50% in young adults, which declines to 25% after age of 75 years. The reduction of muscle mass is generally counteracted by gains in fat mass. The lower extremity muscle groups are more at risk for losing muscle mass, with to 40% reduction in cross-sectional area (CSA) of the vastus lateralis between the age of 20 and 80 years [14]. Although sarcopenia has been widely recognized, however, its mechanisms remain poorly known and have not received proper attention until quite recently [13]. In the recent decades, research on sarcopenia and muscle wasting have grown considerably (Figure 3). The term sarcopenia has been derived from Greek sarx (flesh) and penia (loss),

letter by letter meaning deficiency of flesh [15, 16]. It has been suggested that up to 40% of muscle mass may be lost between the ages of 20 and 70 years. A reduction in skeletal muscle mass can start in the early 35 years of age and may speed up to 6% per decade after 30 year of age and 1.4% to 2.5% per year after age 60 [16].

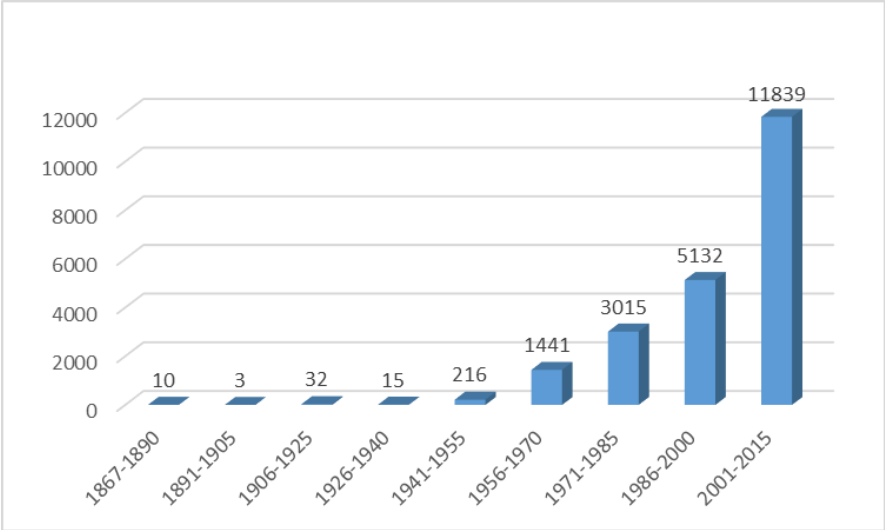

Figure 3. Number of PubMed entries retrieved after entering the search term "muscle wasting OR sarcopenia"

Assessed on 13 January 2015 from www.pubmed.gov

In parallel, leg strength is declined by 10–15 % per decade until 70 years of age, and then by 25–40 % per decade [17]. Fast twitch fiber are more affected and their size may be reduced by 20-50%, despite the fact that slow twitch fibers are less affected, they may be still have a reduction by 1-25% in size. The decrement in total muscle mass is more than loss of muscle fiber size because of an extra loss of fibers [18]. For example, a 40–45% reduction in CSA of the type II muscle fibers was observed in older males compared to young subjects [19].

Using the difference definitions of sarcopenia makes it inconceivable to compare studies for understanding the pathophysiological processes and developing the targeted therapies [20]. There are no clear clinical outcome parameter for sarcopenia yet and the evaluation of muscle quality based on physical performance and strength is undesirable, because other parameters such as the neural controller, cardiovascular fitness and joint function are also involved in physical performance and strength. Despite the correlation between the amount of muscle mass and muscle strength, it has been demonstrated that muscle mass and muscle strength are two different entities and therefore the loss of muscle strength occurring with age can be described by the term 'dynapenia' [20].

In 2009, The International Working Group on Sarcopenia (IWGS) provided an operational definition for sarcopenia. This definition includes people with functional decline, movement-related problems, history of repeated falls, recent inadvertent body weight loss, post hospitalization, and chronic conditions (such as type 2 diabetes, chronic heart failure, chronic obstructive pulmonary disease, chronic kidney disease, rheumatoid arthritis, and cancer), and was more suitable in clinical settings [16]. On the other side, in 2010 the European Working Group on Sarcopenia (EWGSOP) developed a new definition for sarcopenia [21]. EWGSOP recommendations cover both low muscle mass and low muscle function for assessment of sarcopenia in clinical and research tests. EWGSOP offers three levels for sarcopenia including: The pre-sarcopenia level is specified by low muscle mass but no change in muscle strength or performance. The second level of sarcopenia, is specified by low muscle mass along with low muscle performance and the third level, known as severe sarcopenia, is characterized by decrease of all of three components, muscle mass, strength, and performance [8]. A formula has been used for assessment of sarcopenia as appendicular lean mass (sum of lean mass of both arms and legs) divided by height squared. Values higher than two standard deviations below the mean of a young reference population were categorized as sarcopenia [20]. The first step in the management of sarcopenia is to diagnose the condition. Unfortunately, at the moment, there is no standard diagnostic criteria for detection low sarcopenia [22]. The most commonly used measurement techniques for muscle mass and body composition are Dual-energy X-ray absorptiometry (DXA), magnetic resonance imaging (MRI), computed tomography (CT) and electrical impedance myography (EIM) [15, 22, 23].

Due to practical problems in evaluating muscle mass, it is not easy to estimate the prevalence of sarcopenia. Many different methodologies have been used over the last 20 years, and new techniques are still being introduced. On average, it is estimated that 5–13 % of elderly people aged 60–70 years are impressed by sarcopenia, and the numbers raise to 11–50 % for those aged 80 or above [15]. In line with these data, other sources estimate the prevalence of sarcopenia in the range from 13% to 24% in adults over 60 years of age to more than 50% in persons aged 80 and older [24]. Current estimates suggest that ~200 million people worldwide will be affected by sarcopenia by the year 2050 [25]. Women show a higher decrease of muscle mass, especially after menopause. The reduction of muscle mass in aged people does not affect arms and legs the same way. Muscle wasting is higher in the lower limbs irrespective of the sex of the person. However, when Performance parameters, such as

muscle quality, are considered, it can be found a sex-dependent differences. Men undergo a greater loss in the upper limbs Compared to women whereas no differences in the decline of muscle mass in lower limbs have been observed [26].

1.2.2. Consequences

Sarcopenia is, therefore, a multifactorial consequence of aging and indicative a potent risk factor for the development of negative health-related conditions in the elderly [2]. Additionally, mobility disturbances resulting from muscle wasting is along with decreased quality of life and increased social and health care costs in elderly [27]. Sarcopenia is also related to acute and chronic disease states, increased insulin resistance, fatigue, falls, and mortality. Of the chronic disease states, sarcopenia has been especially associated with rheumatologic conditions, especially rheumatoid arthritis in women [28]. In addition, sarcopenia is a main risk factor of falls and disability among the elderly people. Dysfunction and physical inability in sarcopenic people are 2 to 3 times more likely [8]. Individuals with clinically sensible sarcopenia have 4 times higher risk of disability, three times more risk of balance impairment, and 3 times greater risk of falling. Therefore, sarcopenia is the single most prevalent etiology to falls and fall-related fractures in elderly people [13]. It has been estimated that direct healthcare costs related to sarcopenia in the United States of America in 2000 were $18.5 billion, which was around 1.5% of total healthcare expenses for that year [29]. It is estimated that a 10.5% reduction of the outbreak of sarcopenia could lead to a reduction of healthcare expenses by 1.1 billion US dollars per year in the United States [20].

1.2.3. Etiology of sarcopenia

Although the exact mechanisms involved in sarcopenia is still unclear, however, several factors have been proposed to be involved in the onset and progression of sarcopenia (Figure 4). Sarcopenia arises from several physiopathological factors including, but not limited to: sedentary lifestyle [9, 14, 30, 31], chronic inflammation [13, 17, 30, 32], impaired satellite cell function [12, 27, 31, 32], malnutrition [9, 12, 13, 30, 32], declines in neural function [12, 13, 18, 32, 33], hormonal changes [12, 18, 30, 34], mitochondrial dysfunction [18, 34]. It is also probable that certain underlying mechanisms are of greater influence than others when considering any specific age group, gender, or association with comorbid states [4].

1.2.3.1. Sedentary life style

Physical activity has been defined as any action generated by the contractive activity of skeletal muscles that increments energy expenditure. Physical activity is including of daily routines like standing up from a chair and taking stairs, as well as Voluntary movements for health benefits such as walking or biking. Inactive persons are characterized by doing only basic physical activities such as standing, walking slowly and lifting light objects. Lifestyle styles related to nutrition, physical activity, exercise, alcohol consumption, and smoking have a Significant influence on the advancement of sarcopenia and the ability to prevent and treat the loss of muscle mass and function in old age [9].

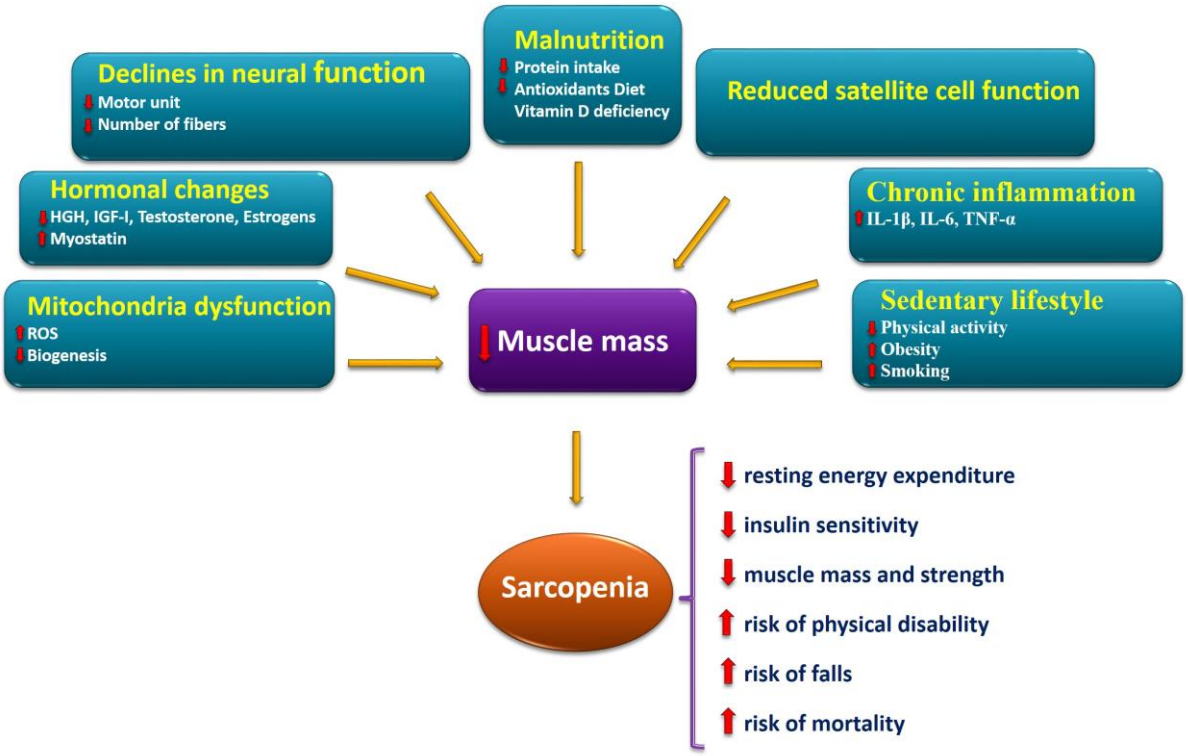

Figure 4. Schematic model of main factors involved in the onset and progression of the sarcopenia and its consequences

One of the main factor involved in sarcopenia is reduced physical activity among older people [35]. It has been suggested that at least some of the increasing prevalence of sarcopenia after the age of 65 is due to decrease physical activity and smoking [36].

The connection between lean body mass and degree of physical activity and exercise are complex. Reduction in physical activity can alter body composition in some ways. One study studied relation between body composition and physical activity in older women over a 10 years period and found that grater levels of physical activity reduced the advancement of sarcopenia. The level of muscle loss is increased even greater when an older person has to spending a period of bed rest because of illness [37]. In addition, inactive and smoker persons had a greater risk for a reduction in health status compared with active and non-smoker people. The net impact of a healthier lifestyle on the process of healthy aging is likely going together with a compressed cumulative morbidity [38].

1.2.3.2. Chronic inflammation

An age-related disruption in the intracellular redox balance plays an important role in generating a chronic state of low-grade inflammation. Chronic cellular inflammation is intended for fundamental mechanism of aging and age-related diseases, and it may consider as a link between normal aging and age-related pathological processes [39]. There are several lines of evidence suggestting that the inflammation being associated with loss of muscle strength and mass with aging [40]. Number of studies have implicated increased levels of two pro-inflammatory cytokines, Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), in the development of sarcopenia [41-44]. For example, it was observed a 2.8 fold increase in TNF-α expression in skeletal muscle of aged male compared to young male subjects [45]. An increased expression of TNF-α has also been reported in soleus and vastus lateralis of aged rats relative to young rats. Furthermore, it has been found that old animals have significantly higher plasma TNF-α levels than younger controls with corresponding elevations of IL-6 [45]. Animal studies have shown that the supplementation of IL-6 or TNF-α increases skeletal muscle breakdown, reduces the rate of protein synthesis, and decreases plasma concentrations of insulin-like growth factor 1 (IGF-1). The activation of the age related inflammatory process is thought to be the consequent of an up regulation of the transcription factor nuclear factor be (NF-κB), an important regulator of the innate immune response. This fundamental and increased activity of NF-κB that is associated with aging has been suggested to be one of the basic causes of sarcopenia [41]. Furthermore, reactive oxygen species (ROS) also seems to act as second messengers for TNF-α in skeletal muscle, activating NF-κB either directly or indirectly [39]. TNF-α is also linked to sarcopenia because

this pro-inflammatory cytokine is known to be associated with other factors that contribute to sarcopenia including protein degradation, ROS accumulation and apoptosis. In addition, TNF-α may be induced sarcopenia by promoting insulin resistance, impaired muscle repair, and amplifying the pro-inflammatory response by up-regulating IL-6 [45]. Notably, pro-inflammatory cytokines, in particular TNF-α, are potent stimulators of muscle proteolysis through activation of ubiquitin-proteasomal system (UPS). This pathway is thought to be responsible for the major part of muscle proteolysis and is stimulated by the repeated covalent binding of 76-amino acid ubiquitin monomers to proteins targeted for degradation [46]. Furthermore, there is evidence that due to hormone-cytokine receptor cross-talk, pro-inflammatory cytokines like TNF-α that cause muscle wasting may also operate by interfering directly with somatotrophic receptors, including insulin-like growth factor 1 (IGF-1) [47].

1.2.3.3. Mitochondrial dysfunction

Mitochondria are subcellular self-autonomous organelles primarily responsible for the generation of energy and adenosine three phosphate (ATP) synthesis. Besides this, mitochondria has a significant role in amino acid and lipid metabolism and regulation of apoptosis [48]. There are a number of evidence supporting the hypothesis that mitochondrial function and biogenesis appear to be altered in skeletal muscles of older adults [28, 49-51], which in turn may contribute to altered skeletal muscle mass and function [28]. Alterations in mitochondria have been considered in aging, such as reduced total volume, elevated oxidative damage, and decreased oxidative capacity. These alternations may resulted in not only a loss of muscle mass and function with age, but other diseases associated with aging such as ectopic lipid infiltration, systemic inflammation, and insulin resistance [52]. It is well established that the amount of synthesis of mitochondrial, myosin heavy chain (a key contractile protein) and mixed muscle protein are decreased with age, along with oxidative capacity of skeletal muscle [53]. Muscle biopsy samples have determined that certain measures of mitochondrial content decrease with age as measured by electron microscopy, mitochondrial deoxyribonucleic acid (mtDNA) copy number, proteomics or the activities of key tricarboxylic acid (TCA) cycle enzymes such as citrate synthase [53]. Several hypotheses have been proposed to explain the decline in mitochondrial function with aging. These theories are included increased ROS production, chronic inflammation and/or mtDNA

damage [54]. According to the widely-accepted mitochondrial free radical theory of aging, mitochondrial dysfunction arising from oxidative damage to mtDNA is the central mechanism driving the aging process [55]. Mitochondria have their own DNA (mtDNA); however, its role in mitochondrial protein encoding is only 1% of the approximately 1,000. The bulk of the mitochondrial proteins are encoded by nuclear DNA and are transported to mitochondria from the cytoplasm [48]. The mtDNA is especially unprotected to oxidative damage because of its proximity to the electron transport chain (ETC) (the main cellular source of oxidants) and the lack of protective histones. Moreover, because of the density of mitochondrial genome (i.e., lack of introns), each mutation is likely to affect gene integrity [27]. A defeat of replication of mtDNA may be the reason of a significant deletion in the mitochondrial genome; the shorter genome is replicated faster by inducing the formation of dysfunction or completely inactive mitochondria [2]. The relevance of mtDNA damage to sarcopenia is evidenced by reduced activity of complex I and IV of the ETC reported in aged skeletal muscles of various species. It should be noted that high levels of mtDNA deletions and ETC abnormalities in fibers often result in morphological distortions, including segmental atrophy, fiber splitting, and breakage [27]. Another important aspect to be considered is the motility of mitochondria which continuously undergo fusion (mitofusion) and fission (mitofission) events that actively alter their morphology [2]. The role of mitofusion and mitofission in aging human skeletal muscle still not fully understood, but are believed to be to be key components in regulating mitochondrial quality and function. By enhancing mitochondrial protein turnover, fusion and fission help the maintenance of mitochondrial and skeletal muscle health by avoiding the accumulation of protein damage that can evoke the stimulation of apoptotic and catabolic pathways. In order to remove damaged parts of mitochondria by exchanging and dilution, two mitochondrial membranes of separate mitochondria connect to each other by mitofusion process, whereas mitofission separates high damaged parts of mitochondria for removal by mitochondrial specific autophagy [56].

1.2.3.4. Hormonal changes

In humans, several hormonal systems appear a gradual reduction in activity during aging, as defined by their bioactive hormone concentrations [44]. Several studies have demonstrated age-related endocrine declines such as decreases in testosterone, estrogen, growth hormone

(GH) and IGF-1 [22, 40, 57, 58]. In addition to the well-documented decline in these anabolic hormones, other endocrine systems (including circulating levels of catecholamines) and paracrine/autocrine systems (including local IGF-1 production) may play an important role in sarcopenia [44]. These pathways also offer important potential opportunities for interventions [28]. We will discuss them in more detail in the following.

1.2.3.4.1. Growth hormone

Several hormones have been suggested for having effects on muscle mass, strength and function. Among them, GH has been one of the most studied [59]. GH is a single-chain peptide of 191 amino acids. The somatotrophs of the anterior pituitary gland are responsible for GH production and secretion. GH secretes in a pulsatory manner with a major boost at the onset of slow-wave sleep and less discernible secretory episodes a few hours after meals. GH production is controlled by the action of two hypothalamic factors, GH releasing hormone (GHRH), which stimulates and somatostatin, which inhibits GH secretion. During fasting, GH secretion is increased, whereas excess of glucose and lipids inhibits GH release [60]. The functions of GH are mediated through the growth hormone receptor protein (GHR), which subsequently activates the Janus kinase 2 (JAK2) signal transducer and activator of transcription pathway [44]. The main functions of GH are to increase the synthesis and releasing of systemic IGF-1 to stimulate local IGF-1 production in skeletal muscle [34]. IGF-1 derived from circulation and/or tissue then stimulates amino acid uptake and the synthesis of nucleic acids and proteins. In addition, GH reduces lipogenesis and promotes lipolysis [61]. However, GH also has a number of IGF-1 independent actions. GH can affects other cellular processes in skeletal muscle, which may be important during aging. For example, GH acutely regulates muscle mitochondrial function by increasing the transcript levels of several key mitochondrial proteins and shifting fuel utilization toward increased fat oxidation [44]. The secretion of GH is maximal at puberty accompanied by very high circulating IGF1 levels, with a gradual decline during adulthood [60]. The circulating (blood-borne) levels of GH declines progressively after ~30 years of age at an average rate greater than 1% per annum [44]. Indeed, in aged men, daily GH secretion is 5- to 20-fold lower than that in young adults [60]. It is therefore not surprising that the age-related decline in GH was believed initially to be indirectly responsible for age-related changes in skeletal muscle via IGF-1 [44].

1.2.3.4.2. Insulin and insulin-like growth factors

Several studies have been suggested that IGF-1 is an imperative modulator of muscle mass, strength and function, not only during development, but also across the entire life span [34]. Much of the anabolic effects of GH is mediated via IGF-1 [19]. Skeletal muscle contains a population of heterotetrameric transmembrane receptors that bind insulin, IGF-1 and/or IGF-II to regulate various stages of myogenesis, including proliferation, differentiation and fusion of muscle precursor cell [44]. In addition to the mature IGF-1 produced by the liver, skeletal muscle is an important source of this hormone. Studies have demonstrated there are at least two different types of IGF-1 which produced by skeletal muscle. They are derived from the IGF-1 gene by alternative splicing. One of the splice variants is expressed in response to mechanical stress like physical activity and is called 'mechano growth factor' or MGF and the other is similar to the systemic or liver type (IGF-1Ea) important for providing the mature IGF-1 required to up-regulate protein synthesis [44]. There are six forms of insulin-like growth factor-binding proteins (IGFBPs) and IGFBP-3, -4, -5 and -6 are found in skeletal muscle. Overexpression of any of these IGFBP isoforms suppresses IGF-1 function by inhibiting its binding to IGF-1R [62]. The mammalian target of rapamycin (mTOR) signaling pathway is important for translation initiation and is therefore critical for muscle protein synthesis. One mechanism that activates mTOR signaling is the IGF-1/PI3k/Akt pathway [45]. In general, tissue responsiveness to IGF1 is altered with aging. Aging is associated with reductions in IGF1R content and phosphorylation in skeletal muscle [60]. Cross-sectional studies have been shown, circulating levels of IGF-1 decrease with age in both men and women, as well as in rodents [63]. In the older compared to the young males subjects, GHR and IGF-1 messenger RNA (mRNA) were reduced by 45% [19]. In addition, muscle production of MGF is decreased in old rats in response to mechanical overload. Furthermore, both the density and affinity of the IGF type 1 receptor are reduced in the aged muscle [31]. Taken together, aging-related decline in IGF-1/Akt/mTOR signaling seems to significantly contribute to sarcopenia.

1.2.3.4.3. Testosterone and its precursors

During normal aging, the most notable and well characterized change in hormonal systems is the decrease in sex hormone production [64]. There is evidence that sex hormones such as testosterone, estrogens, and dehydroepiandrosterones (DHEAS), play an important role in the age-related onset of sarcopenia [58]. The available data suggest that low sex hormone concentrations are among the key mechanisms for sarcopenia and age-induced reduction in muscle strength and power. However, the underlying biological mechanisms by which age-induced sex hormone deprivation affects muscle mass and function are largely unknown [64]. It has been demonstrated that androgens, such as testosterone, regulate muscle mass in humans. Testosterone is secreted primarily by testicular Leydig cells in males and ovarian thecal cells in females, and directly binds to androgen receptors in skeletal muscle, resulting in transformation and dimerization of the receptor, followed by nuclear localization and subsequent DNA binding [44]. Sex steroids are found circulating in the bloodstream in three forms. The majority (~70 %) is strongly bound to sex hormone-binding globulin, with ~20 % bound to albumin and only 2–3 % circulating freely. The free and albumin-bound forms are considered biologically available components. Estradiol is produced from testosterone by the actions of aromatizing enzyme cytochrome P450 19A1. The effects of estradiol on gene expression are delivered through nuclear receptors (ESR1 and ESR2) [64]. Another hormone associated with muscle mass loss is DHEA, a pro-hormone that can transform into sex steroids, such as androgens and estrogens. DHEA plays important roles in the human body including increase in muscle mass, improvements in glucose and insulin levels, decline in fat mass and the reduction of breast cancer risk [65]. DHEAS may affect muscle function. In fact, skeletal muscle is capable to stimulate IGF-1 by converting DHEA into active androgens and estrogens and to stimulate IGF-1 which is important in muscle growth and recovery [58]. With aging, free testosterone levels are decreased in men and this decline parallels the decrease in muscle mass and strength [65]. Aging is associated with low testosterone which may lead to decreased muscle mass and bone strength, and thereby to more fractures and complications. Testosterone has proven effects to increase muscle mass and muscle function, but along with these benefits, there are also problematic side effects [57]. In males, levels of testosterone decrease by 1% per year, and those of bioavailable testosterone by 2% per year from age 30. In women, testosterone levels drop rapidly from 20 to 45 years of age [40]. A substantial number of older men are hypo gonadal. Hypogonadism has been defined as a total testosterone concentration of <9.26 nmol/L (2 SD below the mean for healthy young men).

As a result, approximately 20% of men >60 years and 50% men >80 years are categorized as hypogonadal [22]. In men, age-related decrease in serum testosterone levels has been linked with loss of skeletal muscle mass, strength, and physical performance [66]. However, it seems that the influence of sex hormones to maintain muscle mass and strength is greater in men than in older women [67]. Circulating levels of DHEA decline with age, especially at menopause in women. This decrease in DHEA has been shown to be associated with a decline in muscle mass and physical function [65]. It is suggested that the age-related decline in estrogen and testosterone are related to increases in levels of the pro-inflammatory cytokines IL-6 and TNF-α, which may promote the loss of muscle mass during sarcopenia [45].

1.2.3.4.4. Menopause

Menopause is defined as the permanent cessation of menstruation due to loss of ovarian follicular activity and is a sign of the end of a normal female fertility. Menopause is begun by a period of menstrual cycle irregularity, known as the menopause transition or peri-menopause, which usually begins in the mid-40s. The menopause transition is characterized by many hormonal changes that lead to a dramatic reduction in the ovarian follicle numbers. A remarkable reduction in inhibin B seems to be the first endocrine marker of the menopause transition with follicle-stimulating hormone (FSH) levels being marginally raised. Significant decline in estrogen and inhibin with marked increases in FSH occur only at the late stage of menopause transition. At the time of menopause, FSH levels elevate to 50% of final post-menopausal concentrations while estrogens levels have reduced to about 50% of the premenopausal concentrations [65]. The decreases in estradiol and estrone concentrations occur much faster, within a six month period around the menopause. The changes are more dramatic in estradiol. Both estradiol and estrone levels continue to decrease further during the first three postmenopausal years. The postmenopausal status is characterized by the presence of a very low constant systemic estradiol level. This is in contrast to the cyclic estradiol production during premenopause. Circulating estrone, synthesized from the adrenal steroids in peripheral tissue, becomes the most abundant estrogen in the circulation [64]. There is a correlation between menopause due to decreased estrogen levels and sarcopenia in women. A reduction of estrogen can be resulted in body composition changes, including a loss of muscle mass, but an increase in adipose tissue as well as a redistribution of body fat to the

visceral region. It was suggested that estrogen may reduce fat accumulation within skeletal muscle and may have a direct connection with lipoprotein lipase (which catalyzes triglyceride utilization). Therefore age-associated decline in estrogen levels can be related at least in part to increased intramuscular fat in postmenopausal and decreased muscle strength [45]. One mechanism by which the loss of estrogen may be contributing towards age-related sarcopenia may be due to the increase of pro-inflammatory cytokines, such as TNF-α or IL-6 [24]. Furthermore, estrogen is able to effect directly on muscle mass through estrogen beta-receptors on the skeletal muscle cell membrane. Therefore, it should be a close potential mechanistic relation between decrease in estrogen levels and an impaired in protein synthesis [40]. Both of menopause and sarcopenia are associated with decline in muscle mass, however, menopause lead to a rapid while sarcopenia refers to the loss of muscle mass with age. Because the muscle mass is affected by many factors, and these factors related to aging and menopause, Therefore It is difficult to determine the relative contribution of menopause on the initiation and progression of sarcopenia [65].

1.2.3.5. Neural degeneration

Muscle contraction is initiated and sustained through the successive recruitment of motor units; a motor unit defined as an alpha-motor neuron and all the muscle fibers it innervates [44]. All skeletal muscles are composed of motor units and each motor unit contains a motor neuron and muscle fibers. Motor units can be differentiated to two main types based on the fiber type present in the motor unit. Slow motor units are generally involved of type I fibers while fast motor units mainly composed of type II fibers [65]. One of the main endogenous factors of sarcopenia is likely related to the decrease in motor neuron function. It has been suggested that decline of muscle innervation play a crucial role in the sarcopenic process since innervation is important to the maintenance of muscle mass, as well as strength [2]. Fortunately, many fibers are re-innervated by other motor neurons thereby minimizing the loss of functional muscle fibers. However, the process is insufficient to fully compensate for denervation resulting in atrophy and progressive loss of muscle fibers. Fast motor neurons seem to be preferentially affected and over time the denervation/re-innervation process may result in loss and atrophy of type II fibers and fiber type grouping of particularly type I fibers [5]. Individuals over 65 years of age have a large volumetric decline in areas of the brain that are involved in producing voluntary muscle contraction [10]. Indeed, in man, a 25% decrease in the number of a-motor neurons occurs with aging [68]. The number of motor units is

almost steady until age of 60 years, but after that rapidly declines is occurred at a rate of 3% per year, as it would be expected 60% loss of motor units at age of 80 years [33]. However, the extent of these motor unit losses appears to vary considerably, and could be influenced by the neurotrophic effects of circulating growth factors (e.g. IGF-1) that can promote motor neuron survival [44].

1.2.3.6. Malnutrition

As a natural consequence of aging, it has been reported that food intake reduced by ~25% between 40 and 70 years of age, leading to an increased risk of having inadequate nutrient intakes among older people. The growing number of the existing data shows that nutrition can have an important moderating effect on sarcopenia particularly in relation to protein, vitamin D and antioxidant nutrients [69]. Malnutrition leads to loss of muscle mass. It has been shown that aging is associated with a gradual reduction in food intake, which prepares to energy-protein malnutrition. Furthermore the elderly may be due to the use of diet, weight loss and cholesterol control, inadvertently reduce protein intake [70]. Inadequate caloric intake, also known as anorexia of aging, can lead to the development of a reduction in the availability of amino acids and consequently reduction of protein synthesis [46]. Furthermore, the anorexia of aging may cause the malnutrition, which is related to modulation of different hormones including testosterone, leptin, growth hormone, and IGF-1 that contribute to muscle wasting [71]. The multiple complex mechanisms and interactions leading to reduced food intake with aging include early satiety secondary to decreased relaxation of the fundus, increased release of cholecystokinin in response to fat intake, increased leptin levels, which may in part be due to increase in fat mass with aging, and the effects of neurotransmitters such as opioids and neuropeptides [4]. Recent data indicate that lean mass in older adults is significantly and positively associated with dietary protein intake. Inadequate protein intake seems to be an important factor for sarcopenia progression in older adults [46]. The current recommended dietary allowance (RDA) of protein is 0.8 g/kg/day [9]. It has been shown that 15% of those over 60 years eat less than 75% of the RDA [4]. Furthermore, based on nitrogen balance studies, it has been recommended that aging population needs greater protein (1.14 g/kg/day) relative to the young (0.8 g/kg/day) [9]. One of the reasons that older people need more protein than the recommended amount, can be due to the phenomenon “anabolic resistance,” a blunted response of muscle protein synthesis

(MPS) following taking of dietary protein in the elderly compared to the young. Interestingly, this anabolic resistance is associated with decrease in IGF-1 levels in old age. IGF-1 activates mTOR which in turn regulates MPS by initiating translation. Thus, disruption in mTOR signaling leads to decreased capacity and efficiency of protein synthesis [9]. Nutrients, especially free amino acids, are sensed by the mTOR kinase, which then inhibits autophagy [72]. Vitamin D has recently received considerable attention as a potential factor involved in sarcopenia [69]. It is evident that vitamin D plays an important role in bone and muscle metabolism. Several mechanisms have been suggested for the role of vitamin D in muscle function [22]. Human skeletal muscle has a receptor for 1, 25-dihydroxyvitamin D. The change of muscle fibers as well as muscle differentiation-related genes (Myogenic factor 5 (Myf5), myogenin, E2A, and so on) occurs independently of calcium metabolism changes in vitamin D receptor-deleted mice [73]. Several studies have shown the beneficial effects of vitamin D on skeletal muscle and its ability to prevention of muscle damage [9].

1.2.3.7. Satellite cell dysfunction

While the underlying causes of sarcopenia have yet to be elucidated completely, one potential mechanism involves the age-related decline in muscle regenerative capacity, possibly as a consequence of a decreased number and/or function of quiescent skeletal muscle precursor cells (satellite cells) [43]. Myofibers are post-mitotic cells, and their nuclei do not proliferate. New myonuclei are provided by a population called satellite cells [74]. Satellite cells are a heterogeneous collection of adult muscle stem cells that are normally quiescent. They were first identified more than 50 years ago as a unique population of nuclei that were "sandwiched" between the sarcolemma and the basement membrane of the muscle fiber. While satellite cells might become activated to the changing cellular niche, they do not be exposed until a considerable injury or stress (e.g., exercise loading) occurs [32]. In response to injury, satellite cells are activated to form myoblasts create new fibers by fusion together. In response to an Injury the IGF synthesis increases and stimulates both satellite cell proliferation and differentiation into myoblasts [62]. After proliferation, satellite cells will fuse together with existing myofibers [75]. Satellite cells represent the endogenous source of muscle precursor cells which undergo activation, proliferation and differentiation to form 'new' muscle fibers, a process regulated by the muscle regulatory factors (MRFs) [44] such as myogenic differentiation 1 protein (MyoD), MRF4, Myf5, and myogenin. Specifically, MyoD and Myf5 are involved in stimulating myoblasts to enter differentiation and join the

muscle lineage, whereas MRF4 and myogenin are suggested to mediate terminal differentiation of myoblasts [76]. Pax7 regulates Myf5 and MyoD expression levels in satellite cells. The satellite cell pool is reproduced by the part of activated satellite cells that maintain a high level of Pax7 [32]. It is thus possible that alterations in MRF expression or activity may play a role in muscle wasting during aging [5]. The proliferation and fusion of the satellite cell is regulated by specific growth factors protein, (mainly IGF-1) but is also influenced by hormones such as GH, testosterone, and estrogen [2] and several signaling pathways, such as Wnt-ß-Catenin, DeltaL/Jagged1-Notch or Smad2/3- transforming growth factor ß (TGF-ß)/Activin/Myostatin [13]. With aging, Notch activation declines due to a fall in MAPK activity, thereby reducing satellite cell activation. This process is compounded by accumulation of cyclin-dependent kinase due to an elevation in the levels of TGF-ß. It can then deactivates satellite cells and suppresses their regenerative function to injury [33]. Reductions in Notch signaling is associated with reduced satellite cell proliferation and an inability to generate myoblasts in following muscle injury [32]. Wnt signaling is another mechanism witch has also been shown to be involved in satellite cell proliferation and differentiation in skeletal muscle regeneration [71]. By several studies, it has been demonstrated an age-related decrease in satellite cell number in rodents and humans. It has been reported that the mean number of satellite cells decreased in type II, but not type I fibers of the vastus lateralis muscle of healthy elderly men, which may help to explain the various responses of fast type II fibers compared with slow type I fibers with aging [44]. Although the mechanisms involved in satellite cell function changes associated with aging is unclear, however, the reduced efficiency of anabolic hormones such as IGF1 and testosterone is seems to be a key factor. In this regard, recent studies have been demonstrated that a spliced variant of IGF1, known as mechano-growth factor (MGF), may also play a critical role in satellite cell proliferation [46].

1.2.4. Cellular mechanisms and signaling pathways involved in sarcopenia

Approximately 50 percent of total body weight is composed of lean muscle mass in young adults, but declines with aging to 25% at 75–80 years of age. The loss of muscle mass is more obvious in in the lower limb muscle groups. For example, it has been reported about 40% reduction in the CSA of the vastus lateralis between the ages of 20 and 80 years [77]. Maintenance of skeletal muscle mass is mainly dependent on the balance between protein synthesis and breakdown. An increase in protein breakdown leads to the muscle atrophy, whereas an increase in protein synthesis leads to muscle hypertrophy [78]. Numbers of cellular mechanisms and signaling pathways are involved in age-related skeletal muscle wasting in mammals (Figure 5). These are discussed in more detail in the following section.

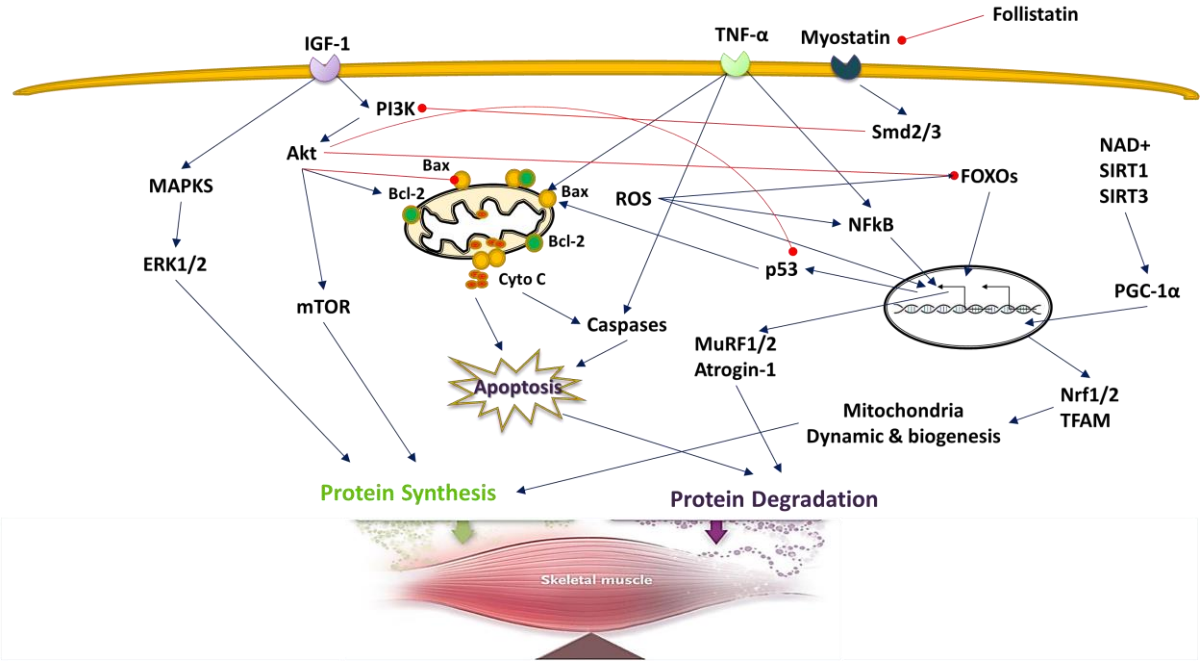

Figure 5. Molecular mechanisms involved in sarcopenia which can be viewed as the result of a protein synthesis/degradation imbalance

1.2.4.1. IGF-1/Akt/mTOR

One of the central pathways to muscle size control is the PI3K/Akt pathway, a pathway modulated by IGF-1 and insulin. Stimulation of protein synthesis and hypertrophy involves these hormones interacting with their respective tyrosine kinase receptors to phosphorylate

IRS-1 and activate PI3K/Akt signaling that activates mTOR, and in turn, phosphorylates the targets 70-kilodalton ribosomal S6 protein kinase (p70S6K) and 4E-binding protein 1 (4E-BP1) [79]. Compared with wild-type controls, transgenic mice that overexpress IGF-1 under the control of muscle-specific promoters has increased muscle mass, CSA, and maximum isometric force [80]. The effects of IGF-1 are mediated generally by the type 1 IGF receptor (IGFR1), which has tyrosine kinase activity and signals through the PI3K/AKT pathway. IGF-1 also binds to the Insulin receptor (IR) but with much lower (about 100-fold lower) affinity than to the IGF1R. There are six IGFBPs. Most serum IGF-1 is found in a tripartite complex with IGFBP3 and the acid labile subunit (ALS). IGF-IGFBP complexes are able to leave the circulation and effect on tissue unless they are bound to the ALS. In serum, they increase the circulating half-life and delivery of IGF-1 to tissues. In tissues, IGF function can be modulated due to a higher affinity for IGFs than the receptors. Releasing IGFs from IGFBPs Can occur by proteolysis of IGFBPs or binding of the IGFBPs to the extracellular matrix [74]. Akt/protein kinase B is a ser/thre kinase that has been shown to be a critical signaling component for the regulation of cellular metabolism, growth, and survival [81]. The Akt family is composed of three members: Akt1, Akt2 and Akt3. These three isoforms share over 80 % homology and are expressed in a tissue specific manner, thus the Akt1 and Akt2 isoforms are predominantly expressed in skeletal muscle, the brain, heart and lungs and Akt3 is more expressed in the brain and testicles [82]. Akt plays a number of roles that may be important in sarcopenia. These roles are included the inhibition of apoptosis and protein degradation in skeletal muscle by increasing phosphorylation and inactivation of the pro-apoptotic protein Bad and forkhead box O (FOXO) transcription factors, respectively [39]. The activation of Akt at same time reduced atrophy by phosphorylating FOXO transcription factors, preventing translocation to the nucleus where they would otherwise promote the transcription of atrophy-related genes muscle RING-finger protein-1 (MuRF1) and muscle atrophy F-box (MAFbx), both of which are ubiquitin ligases that degrade proteins [79]. In response to activation of Phosphatidylinositol-3-kinases (PI-3K), phospholipid generation increase inside the plasma membrane, which in turn recruit and activate AKT kinase, resulting in activation of mTOR and p70S6K [62]. The mTOR acts as a main integrator of a broad range of signals that regulate protein synthesis and cell growth. Moreover, mTOR indirectly inhibits the translation initiation factor elF4E through directly phosphorylation of the protein 4E-BPI. Other mTOR role in increased protein synthesis and muscle mass due to its impact on decreasing phosphorylation of S6K kinase, leading to the increase of skeletal muscle cross-sectional area [82]. Furthermore, Yin Yang 1 (YY1) physically interacts with

mammalian target of rapamycin complex 1 (mTORC1) and mediates mTOR-dependent regulation of mitochondrial gene expression via an YY1– Peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1a) complex [72]. An important negative regulatory element in this pathway is the protein Tuberous Sclerosis Complex 2 (TSC2). The TSC2 can negatively regulates p70S6K activation in response to IGF-1 by downregulating mTOR activation. Phosphorylation of TSC2 by AKT on critical residues, leads to reduce its negative regulation on mTOR and consequently increase protein translation. This ability of TSC2 to regulate translation is linked directly to cellular energy status [62]. Recent studies demonstrate an age-related decline in both systemic and locally derived IGF-1, which may be responsible, at least in part, for the age-related decrease in skeletal muscle mass and function because of decrease activity of the Akt signaling pathway. Numerous of studies have indicated cross-talk between ROS, the proinflammatory cytokine TNF-α, and IGF-1 [39]. Skeletal muscle biopsies from older male subjects show a reduction in the cross section area (CSA) of type II muscle fibers by 40–45%, in parallel with a 45% decreased GHR protein and IGF1 mRNA levels, as well as increased TNF-α and suppressor of cytokine signaling-3 (SOCS-3) mRNA levels, when compared with younger donors. Furthermore, total Akt, but not pAkt, proteins levels increased by 2.5-fold, resulting in a 30% decline in the efficiency of Akt phosphorylation in older subjects [60].

1.2.4.2. MAPKs

Mitogen-activated protein kinases (MAPKs) are protein Ser/Thr kinases that induct extracellular signals into a broad range of cellular processes. In the eukaryotic cells coordination of multiple MAPK pathways regulate several cellular processes such as gene expression, cell division, metabolism, motility, survival, apoptosis, and differentiation [83]. The MAPK family of proteins is composed of four distinct signaling modules in skeletal muscle: 1) extracellular signal-regulated kinase (ERK) 1/2; 2) p38 MAPK; 3) c-Jun N-terminal kinases (JNK); and 4) ERK5 or big MAPK. These MAPK subunits are activated by cytokines, growth factors, and cellular stress [84]. MAPKs are stimulated by phosphorylation on regulatory tyrosine and threonine residues by upstream MAP kinase kinases (MKKs), and are deactivated by dephosphorylation on by MAPK phosphatases (MKPs). Despite the important role of MAPKs in myogenesis, relatively little information have been known about the role of the MAPKs in fiber type establishment. One function for ERK1/2 has been

indicated in type I fiber expression by increases myosin heavy chain (MHC) type I expression via activation of Ras. The ERK1/2 signaling pathway can stimulates several substrates, such as p90RSK, leading to the initiation of transcription factors and the ribosomal subunit S6 [54]. ERK1/2 can also activate kinases associated with protein translation such as Mnk1 and its downstream substrate, eIF4E. One study recently found that the higher baseline levels of ERK1/2, p90RSK and Mnk1 in aged compared to young muscle, is possibly a compensatory mechanism by the skeletal muscle with increasing age, trying to increasing protein synthesis [39]. It has also been shown that p38 MAPK actives the MHC type IIx (intermediate) gene expression in myoblasts [54]. In this regard, It has been indicated exposing myotubes to either TNF-α or Hydrogen peroxide (H2O2) led to activation all the p38, ERK1/2, and JNK [39]. Although role of MAPK in the aging process in vertebrates is still not clearly understood, however, some studies in drosophila as well as c. elegans have reported a contribution for MAPKs for increase of longevity. Whereas the role of p38 MAPK in enhancement of longevity is controversial, JNK activity promote longevity by antagonizing insulin signaling. Additionally, decreased ERK1/2 activity throughout aging is known to promote senescence [54].

1.2.4.3. FOXOs

FOXOs transcription factors consist a large family of proteins Identified by a protected DNA-binding domain termed the FOXO [39]. The FOXO family members which play a considerable role in skeletal muscle include three isoforms: FOXO1, FOXO3 and FOXO4 [72]. The FOXO isoforms are predominantly located in the nucleus where they are activated. However, when they are phosphorylated, mainly by Akt protein, these FOXO proteins are displaced to cytosol, and they are not able to induce the transcription of genes involved in muscle atrophy [82]. Thus, when Akt is active, protein breakdown is suppressed, and when FOXO is induced, protein synthesis is blocked. The FOXOs activity is regulated by several post-translational modifications, such as phosphorylation, acetylation and mono- and polyubiquitination. Adding an additional level of complexity, the regulatory consequences of these changes appear to be specific for individual FOXO members [72]. Thus the FOXOs proteins may very well play a role in the loss of muscle mass or muscle nuclei with aging [85]. Recent studies have been provided evidences that FOXO1 suppresses the efficiency of anabolic pathways in skeletal muscle via increased expression and reduced phosphorylation

of the translational repressor protein 4E-BP1 and impaired signaling via reductions in mTOR and Raptor levels. Based on these observations, the possibility can be considered that, in mammalian skeletal muscle, FOXO1 may not only stimulates the catabolic processes through activation of ubiquitin ligases, but may also inhibits anabolic pathways. FOXO1 may be an important therapeutic target for human diseases in which anabolism is impaired [39]. FOXOs are among the age related transcription factors which are involved in the redox regulation. Increased FOXO1 mRNA has been reported in aged muscle using standard microarray analysis, while another study demonstrated increased atrogin-1 mRNA in aged rats [39]. Furthermore, It has been found FOXO1 expression in nuclei of aged muscle was higher than those of young muscle [85]. It has also been identified that the FOXO-regulated ubiquitin E3-ligases Atrogin-1/MAFbx and MuRF1 is common in muscle atrophy caused by a range of etiologies [86]. In this regard, it has recently been shown that under energy stress situation AMPK increased activation of FOXO3 in myofibers, inducing expression of atrogin-1 and MuRF1 [72]. In skeletal muscle, FOXO4 is believed to be the most common expressed member of the FOXOs [86]. FOXOs activity is also regulated by direct or indirect functions of co-factors and by interaction with other transcription factors. An interaction has been demonstrated between FOXOs with PGC-1a. Under catabolic conditions similar to the effect observed for expression of constitutively active FOXO3 during aging and sarcopenia, maintaining high levels of PGC-1a protects muscle mass.

1.2.4.4. TGFβ (Myostatin)

Other factors that have been shown to modulate muscle regeneration belong to the family of TGFßs, which are known to suppress myogenic differentiation [43]. Myostatin, a member of TGFß superfamily, is one of the main signaling pathway that regulates skeletal muscle growth. Myostatin is produced by skeletal muscle and negatively regulates muscle growth [72]. It is expressed in both embryonic and adult skeletal muscle, suggesting that myostatin acts as a regulator of both prenatal and postnatal myogenesis [87]. Myostatin, similar with other family members, after synthesis to a precursor protein then is cleaved by furin proteases to produce the active C-terminal dimer [40]. Studies indicate that myostatin regulates cell cycle progression and myogenic regulatory factor levels, thereby controlling myoblast proliferation and differentiation during developmental myogenesis. In addition, myostatin

also influences postnatal muscle growth. In support, it has been reported that myostatin is able to regulate myogenesis via the suppression of satellite cell activation in mice [87]. It has been demonstrated that adding purified myostatin to differentiated myotubes in culture led to an inhabitation of protein synthesis and decreased myotube size. Furthermore, systemically administration of myostatin has been shown for inducing muscle atrophy in mice [72].

Myostatin is believed to mediate its actions on skeletal muscle via activin type II receptors A (ActRIIA) and activin type II receptors B (ActRIIB), with more affinity for ActRIIB [44], Binding myostatin to ActRIIB resulted in forming a complex with a second surface type I receptor, either activin receptor-like kinase 4 (ALK4) or activin receptor-like kinase 5 (ALK5), leading to stimulation of the phosphorylation of receptor Smad (Rsmad), and Smad2/3 transcription factors in the cytoplasm. Then Smad2/3 modulate nuclear gene transcription such as MyoD by translocation from cytosol to nuclear through a TGF-ß-like mechanism [29]. The effect of myostatin, which mediated by the transcription factors Smad2 and Smad3, also interact with IGF1-Akt signaling. Myostatin has been reported to upregulate the ubiquitin ligases atrogin1 and MuRF1 via FOXO transcription factors. In this regard, myostatin administration has been shown to blocking the IGF1-PI3 K-Akt pathway and activation of FOXO1, allowing increased expression of atrogin-1. This connection between the two pathways is independent of NFκB [63]. In contrast, Smad2/3 inhibition increase muscle hypertrophy partially dependent on mTOR signaling [40].

The involvement of myostatin in loss of muscle mass during aging is still debatable. Significant increase in myostatin mRNA and protein levels (2- and 1.4 fold respectively) has been found in the older males compared to the young subjects [19]. In contrast, there was an increase in serum levels of TGF-ß in old humans, but circulating levels of myostatin were not different between young and older subjects. It has been indicated that despite any recognizable increase in circulating myostatin levels, local intramuscular content of myostatin are increased with aging in humans [80].

In order to examine the contribution of myostatin in age-related sarcopenia, Siriett et al. [87] studied muscle mass and regeneration in young and old myostatin-null mice. Their result demonstrated there was massive hypertrophy and hyperplasia and an increase in type IIB fibers in Young myostatin-null mice subject. Aging was associated with increasing oxidative and fiber atrophy in muscle of wild-type. In contrast, there was no fiber type switching and also lower atrophy in aged myostatin-null muscle. Aging did not lead to significant effect on

satellite cell number, however, satellite cell activation reduced significantly in both wild-type and myostatin-null muscles [87]. GH and IGF-1 may have an inhibitory effect on myostatin, therefore one potential cause of increased myostatin in aged skeletal muscle is the attrition of GH and IGF-1 expression. Myostatin may decreased muscle growth by inhibition satellite cell activation, as well as promoting an adipogenic cell fate over myogenicity [45]. Proteolysis effect of myostatin can be modulated by at least three interacting proteins, namely, GDF-associated serum protein-1 (GASP-1), follistatin, and follistatin-related gene (FLRG). It is accepted that the abundance of these endogenous inhibitors of myostatin and/or the degree to which they interact with myostatin is independently affected by aging [77].

Follistatin is expressed in different tissues and acts as an antagonist of different TGF-ß family members [82], Follistatin is expressed in different tissues and acts as an antagonist of different TGF-ß family members, which has been thought to be involved in the regulation of skeletal muscle mass. Follistatin prevents myostatin from binding to the activin IIb receptor, by binding and thereby neutralizing myostatin in the circulation. As a consequence, the expression of intramyocellular Smad is prevented, thereby blocking gene transcription. One study investigated knockout mice for the gene of follistatin and found excessive loss of muscle mass. On the other hand, follistatin-overexpressing mice showed a 327% increase in muscle mass compared to the control group. The remarkable increase in muscle mass observed in these mice was as a result of an increment in both of muscle hypertrophy (27%) and hyperplasia (66%) [82]. Follistatin plays important role in activation of Akt–mTOR signaling through decreases activity of Smad3 since constitutively active Smad3 was found to suppress follistatin-induced muscle growth and mTOR activation. Other potential reason can due to a direct interaction between Smad3 and Akt, as demonstrated in other cell systems, may be involved in cross-talk between the myostatin/activin A and IGF1 pathways in skeletal muscle [72].

1.2.4.5. NF-κB

NF-κB transcription factor is a major pleiotropic transcription factor that modulates immune, inflammatory, cell survival, and proliferative responses. NF-κB activity seems to directly regulate MyoD, which is a myogenic transcription factor, and likely other molecules, such as MuRF1, during atrophy. ROS and TNF-α both activate NF-κB [39]. NF-κB is composed of

a number of proteins (RelA/p65, cRel, RelB, p50 and p52), known as NF-κB family members that cooperate to form a complex. In most tissues, p65/p50 heterodimers are the most frequent and are characterized to be active form of and are involved in most NF-κB signaling in skeletal muscle. NF-κB can be kept in an inactive form in the cytosol through the binding of an IκB. There are seven isoforms of IκB (IκBα, IκBß, IκBγ, IκBε, Bcl-3, p100, p105) in mammals with each form possessing the ability to inhibit NF-κB. Upon certain stimuli, IκBα is phosphorylated by IκB kinase (IKK) in a step that targets IκBα for ubiquitination and subsequent proteolysis, thereby leaving NF-κB unbound. This process allows the unbound NF-κB to translocate to the nucleus where it can affect gene expression by binding NF-κB-target sequences located in the promoter region of specific genes [88]. Although there is little information about the role NF-κB in the onset and progression sarcopenia, however, several studies have found a significant causal role for NF-κB activation in muscle atrophy [89]. One study has reported that the levels of NF-κB was four times higher in the muscles of elderly people compared with their young counterparts.; this increased concentration is associated with anabolic signaling deficiency observed in age related muscle wasting [39]. Mice transgenic for an active form of IKKß exhibit a muscle wasting phenotype. Indeed, inhibition of NF-κB activity in macrophages was associated with reduced muscle degeneration, while systemic treatment with an IKK inhibitor reduced pathologies associated with muscular dystrophy [89]. Aging also affected TNF-α signaling to NF-κB. Intermediary proteins (IKKβ, IκBα, and p65), which are responsible for the transmission of the TNF-α activation of NF-κB, increased with age in the soleus muscle [39].

1.2.4.6. Apoptosis

Recent studies also suggest that apoptosis might be another mechanism involved in sarcopenia [43]. Apoptosis is an important process for cellular function which occurs in multicellular organisms and plays Considerable role in normal development and for maintaining tissue homeostasis [90]. Apoptosis is a programmed cell death process that occurs through a series of coordinated events, resulting in cellular self-destruction without inflammation or damage to other cell components [91]. It is hypothesized that the acceleration of apoptosis in the aging muscle may represent a converging mechanism through which muscle atrophy and physical function decline ensue. Indeed, it has been indicated that

there is a positive correlation between the loss of muscle mass and strength and the rate of increase in apoptosis associated with aging. Apoptosis is initiated by the induction of signals of cell death, which are generated due to unbalance in the regulation of free calcium and alteration in the composition of some protein families. After that, activation of cell surface receptors or mitochondrial pathways, resulted in triggering cytoplasmic and nuclear events that lead to cell death [90]. Broadly, the apoptotic machinery comprises regulatory proteins, endonucleases, protease inhibitors and proteolytic enzymes, known as initiator cysteine-aspartic proteases (caspases). Upon cell death stimulus, caspases initiators (i.e. caspase-8, -9 and -12) are engaged, leading to the activation of effector caspases (i.e. caspase-3, -6 and -7), which are responsible for cellular degradation and DNA fragmentation [91]. Caspases are the major enzymes involved in the beginning and the development of apoptosis. They are considered for proteolytic cleavage of a wide range of cell targets, although they do not alone initiate that process [90]. Two main pathways involved in caspase activation are distinct based on the extrinsic or intrinsic origin of the death-inducing stimulus. In The extrinsic pathway, caspase is activated through the interaction of cell surface death receptors (e.g. tumor necrosis factor receptor (TNF-R)) with their ligands (e.g. TNF-α) [91]. While, intrinsic pathways of caspase activation include those triggered by the ER and the mitochondrion. Under stress conditions, such as calcium dyshomeostasis, the ER-specific procaspase-12 can be activated by m-calpain, leading to caspase-3 activation [31]. Although apoptosis may occur via several mechanisms but it has been shown recently that mitochondria plays considerable contribution in the regulation of apoptosis. Furthermore it is thought that internal cellular stimuli, such as high levels of calcium or reactive oxygen intermediates, may trigger apoptosis by the Cytochrome C (Cyto C)-dependent pathway [92]. Upon stimulation, mitochondria release Cyto C into the cytosol which then complexes with apoptotic protease activating factor-1 (Apaf-1), ATP and caspase-9 forming the apoptosome. The apoptosome then activates caspase-9, which in turn cleaves and activates caspase-3, the final executor of the apoptotic process [30]. The mitochondria can initiates apoptosis independent of caspase activation via the release of Apoptosis-inducing factor (AIF) and endonuclease G (EndoG), both of which can directly execute DNA fragmentation [91]. Since the AIF is located in the mitochondrial intermembrane space, it plays an important role in mitochondrial function, especially for the proper functioning of complex I. AIF acts as a NADH-oxidase activity. After being released from the mitochondria into the cytoplasm, it is then transported into the nucleus where it binds to DNA to induce chromatin condensation. AIF can also together with cyclophilin A constitute an active DNase responsible for fairly large DNA fragmentation. In

response to apoptotic stimulations, AIF along with Cyto C release from mitochondria and activate the caspase cascade. However, AIF is thought to be able for inducing apoptosis independently of caspases. In addition to AIF, under apoptotic conditions EndoG which is a mitochondrion-specific nuclease and necessary for normal cell proliferation, releases from the mitochondria and enters the nucleus, where it participates in oligonucleosomal DNA fragmentation [93]. Since the AIF is located in the mitochondrial intermembrane space, it plays an important role in mitochondrial function, especially for the proper functioning of complex I. AIF acts as a NADH-oxidase activity. After being released from the mitochondria into the cytoplasm, it is then transported into the nucleus where it binds to DNA to induce chromatin condensation. AIF can also together with cyclophilin A constitute an active DNase responsible for fairly large DNA fragmentation. In response to apoptotic stimulations, AIF along with Cyto C release from mitochondria and activate the caspase cascade. However, AIF is thought to be able for inducing apoptosis independently of caspases. In addition to AIF, under apoptotic conditions EndoG which is a mitochondrion-specific nuclease and necessary for normal cell proliferation, releases from the mitochondria and enters the nucleus, where it participates in oligonucleosomal DNA fragmentation [31]. Translocation of Bcl-2-associated X protein (Bax) to the mitochondria in response to apoptotic stimulations, Leads to formation of a pore on the outer mitochondria membrane (OMM), subsequently triggering apoptotic factors stored in the intermembrane compartment into the cytoplasm. In this process the pro-apoptotic factors Bid and/or Bim are involved in activation of Bax and/or Bak, possibly through neutralizing B-cell lymphoma-2 (Bcl-2) and Bcl-XL activity. In addition, the mitochondrial permeability transition pore (mPTP) opening can also cause mitochondrial outer membrane permeabilization (MOMP). The mPTP is a protein structure that is composed of three main considered units: a voltage-dependent anion channel (VDAC) in the OMM, the adenine nucleotide translocase (ANT) located in the inner mitochondrial membrane (IMM), and cyclophilin D (CyPD) in the matrix. Formation of the mPTP requires interaction between ANT and VDAC with CyPD associated with the IMM. Opening of the mPTP permits free diffusion of low-molecular weight solutes across the IMM. This results in a mitochondrial permeability transition (MPT), conditions that lead to uncoupling of oxidative phosphorylation and reduced ATP production [93]. Several stimuli, including calcium, oxidative stress, and TNF-α can trigger apoptotic signaling in aged skeletal muscle. Recently age-related sarcopenia and muscle fatigability have been suggested to be associated with increased ROS production, enhanced mitochondrial apoptotic susceptibility, and reduced transcriptional drive for mitochondrial biogenesis [39]. In addition, an age related,

increased cytosolic Ca2+ levels may have contributed to the activation of the endoplasmic reticulum-mediated apoptotic pathway [39]. An increased expression of pro-apoptotic proteins and caspases and DNA fragmentation have been found, with a concomitant decrease in expression of anti-apoptotic proteins in aged skeletal muscle [45]. Results from human and animal studies have found that caspase-independent pathway is upregulated with age [52]. In this regard, higher levels of the pro-apoptotic protein AIF, with an associated decrease in the apoptotic repressor with a caspase recruitment domain (ARC) has been reported in aged (26 mo) rat gastrocnemius relative to adult (12 mo) [45]. Age related increase in the proportion of apoptotic cells was found generally more in type II fibers in humans [52]. A different age-related pattern of Bcl-2 and Bax expression has also been indicated in rats based on the muscle type. In this regard, Bax content was elevated at old age in the extensor digitorum longus (EDL), whereas no changes were found in the soleus. In contrast, an increased expression of Bcl-2 was observed in both muscles at advanced age [93]. In connection with this, a higher muscle cell apoptotic index was found in the plantaris muscles (9.9-fold) as opposed to the soleus muscles (3.2-fold) in rats following hind limb suspension in aged rats compared with ambulatory control rats. This finding proposes the concept that incidence of muscle cell apoptosis could vary depending on muscle types or even between species [94].

1.3. Therapeutic strategies

Pathology of sarcopenia is multifactorial and there are no effective cure until now [42, 71]. However, due to this multifactorial cause, previous studies have examined alone or in a combination of the different therapeutic strategies, including nutritional and pharmacological intervention [94-97], caloric restriction (CR) [52, 54, 98], exercise training [76, 99-103] and hormone therapy [59, 61, 74, 104]. Here we discuss each in more detail.

1.3.1. Nutritional and pharmacological intervention

The current RDA for protein is 0.8 g/kg/day, but almost 40% of people >70 years do not consume sufficient amounts of dietary protein which leads to a reduction in lean body mass and increased functional impairment [22]. Thus, nutritional interventions may be useful and potential strategy for the prevention and treatment of sarcopenia due to the easy applicability

and safety [70]. It has been indicated that anabolic nutrients increase the phosphorylation of mTOR-associated signaling proteins in human muscle in association with an increase in protein synthesis through both enhanced translation initiation and translation elongation signaling [39].

Numerous studies have examined the effect of protein and amino acid intervention on MPS in the elderly [105-108]. In this regards, Volpi et al. [107] reported that essential amino acid (EAA) are mainly responsible for the stimulation of muscle protein anabolism in the elderly. They compared the muscle protein metabolism response of healthy elderly to oral supplementation of either 18 g EAA or 40 g balanced amino acids in small boluses every 10 min for 3 h. Their result showed that phenylalanine net balance, a reflection of muscle protein balance, increased from the basal state, with no differences between groups, due to an increase in MPS and no change in breakdown [107]. In another study, Katsanos et al. [108] studied the effects of enriching an EAA mixture with leucine on muscle protein metabolism in elderly and young individuals. EAAs were including of whey protein [26% leucine (26% Leu)] or were enriched with leucine [41% leucine (41% Leu)]. No significant increase was observed in fractional synthetic rate in the elderly following ingestion of 26% Leu EAA, but increased following administration of 41% Leu EAA. However, the mean response of muscle phenylalanine net balance was promoted in all groups, with the exception of the 26% Leu elderly group. They then concluded that increasing the proportion of leucine in a mixture of EAA can reverse an attenuated response of MPS in elderly [108]. One possibly underlying mechanisms of leucine's effects, similar to those of IGF-1 treatment, can be due to its effect on phosphorylation of mTOR, p70S6K and 4EBP-1 [109].

It has been suggested that a regime of combination of antioxidants supplementation alone or associated with a diet may possibly increase antioxidant defenses, lower muscle oxidative damage, and improve muscle protein balance during senescence [2]. Sinha-Hikim et al. [94] investigated the effect of administration of a cystine-based antioxidant (F1) on age-specific changes in skeletal muscles. Their result showed that 6 months supplementation increased markers of oxidative stress, inflammation, and muscle cell apoptosis and decreased muscle weight in old mice compared with young mice (5 months old). They then found F1 administration significantly prevented these age-related changes including inactivation of AMPK, increased lipogenesis, activation of c-Jun NH2-terminal kinase, and decreased expression of Delta 1, pAkt, and proliferating cell nuclear antigen in aged skeletal muscle. These data indicate the beneficial effects of F1 to reduced age associated loss of muscle mass

[94]. Positive effects of vitamin D supplementation were also shown, increasing muscle strength and performance and reducing the risk of falling in elderly with low vitamin D levels [40]. Bischoff et al. [110] have reported that supplemental vitamin D in a dose of 700-1000 IU a day resulted in a 19% reduced risk of falling among older individuals [110]. Furthermore, 2–12 months administration of 800 IU of vitamin D to individuals aged 65 years and older, markedly enhanced lower extremity strength or function by 4–11 % after of treatment [111].

Aside from the effect of natural nutrients supplementation, some studies investigated effects of pharmacological strategies for age related muscle wasting. In this regards, pharmacological inhibition of myostatin may have potential therapeutic benefits in the treatment of sarcopenia [22]. LeBrasseur et al. [112] investigated effects of myostatin blocking by anti-human myostatin antibody (PF-354) relative to a vehicle control, on performance and metabolic measures in 24-month-old mice. At the end of study, PF-354–treated mice showed significantly greater muscle weights and more than 30% declines in muscle fatigue. PF-354 was associated with decreased Smad3 phosphorylation and increased PGC-1a expression and decreased MuRF-1 [112]. In another study, Murphy et al. [113] found that PF-354 reduced the age related decline in muscle mass and function of mice by reducing apoptosis. In fact, there result demonstrated that PF-354 prevented the age-related decline in body mass and increased muscle mass. PF-354 also increased fiber CSA by 12% and enhanced maximum in situ force of tibialis anterior muscles by 35%. Myostatin inhibition by PF-354 increased the proportion of type IIa fibers by 114% and enhanced activity of oxidative enzymes (SDH) by 39%. PF-354 significantly reduced markers of apoptosis in TA muscle cross-sections and reduced caspase3 mRNA [113]. These data suggest a therapeutic potential of the pharmacological myostatin inhibition for sarcopenia.

Other pharmacological components such as Angiotensin-converting enzyme inhibitors and losartan (an angiotensin II receptor antagonist) has been candied and studied against sarcopenia [22, 26, 114]. However, further studies are needed to determine their contribution to the prevention and treatment of sarcopenia.

1.3.2. Caloric restriction (CR)

One of the most powerful anti-aging intervention is CR without malnutrition [27], which exerts this effect in multiple ways [54]. CR is generally regarded as consuming 20–40% fewer calories than normal [52]. CR intervention positively modulates both primary aging (natural age-related deterioration) and secondary aging (accelerated aging due to disease and negative lifestyle behaviors) [52]. A number of studies have investigated the effects of CR on sarcopenia [115-118]. Bua et al. [115] studied the role of CR (40% restriction without nutrition deficiencies) in electron transport system (ETS) enzymatic abnormalities in two quadriceps muscles (vastus lateralis and rectus femoris) from ad libitum fed (5, 18, and 36 months) and calorie-restricted rats (36 months). CR reduced the abundance of ETS abnormal fibers in vastus lateralis muscles of the 36-month-old calorie-restricted rats. However, CR did not prevent fiber atrophy in ETS abnormal regions. Their result suggest that CR leads in the producing of less ETS abnormalities, thus affecting/inhibiting a process that ultimately results in fiber loss [115]. CR has been indicated to decreases markers of apoptosis in aging rat skeletal muscle [98]. In this regard, Dirks et al. [116] investigated main proteins involved in apoptotic regulatation in the gastrocnemius muscle of 12 and 26 month old ad libitum fed and 26 month old calorie-restricted male Fischer-344 rats. They found that CR significantly reduced age-elevated levels of pro- and cleaved caspase-3, apoptosis-inducing factor and expression of procaspase-12 compared with their age-matched cohorts. Also increased mitochondrial levels of the ARC, which inhibits Cyto C release, were lower in calorie-restricted rats, can indicate a translocation of this protein to attenuate oxidative stress. They then concluded that CR is able to reduce the potential for sarcopenia by altering several key apoptotic proteins toward cellular survival in aged skeletal muscle [116]. Furthermore, 32 months of CR retarded muscle mass loss in 36 months old rats compared with age match controls. However, CR did not prevent age related muscle mass lose while 36 months old compared with 21 months old rat in CR groups [117]. CR has been shown to decreases oxidant production during aging probably due to an anti-inflammatory effect. This later can happen by decreased MAPK activity and enhanced deacetylasion of SIRT1. It has been hypothesized that SIRT1 deacetylates, and therefore decreases activity of MKP-1, leading to increased PGC-1a function, thus, preventing myofiber dysfunction [54]. Nevertheless, regardless of the benefits of CR on sarcopenia one important problem, which should be taken into consideration, is the exact time frame for starting CR. When started too early in life, it may cause developmental problems and if started too late benefits may not be achieved [71].

1.3.3. Exercise training

It is well accepted that less physically activity in older adult is associated with reduced skeletal muscle mass and increased prevalence of disability [4]. A sedentary lifestyle results in reduced activity levels and loss of muscle mass and strength [95]. One of the effective stimuli for the regulation of multiple metabolic and transcriptional processes in skeletal muscle is physical exercise [81]. Exercise is generally categorized to: endurance training, which is characterized by low resistance work of longer duration, and resistance training, which is characterized by more powerful movements of shorter duration [119]. Both types, such as resistance and endurance exercise interventions have been found to be effective in preventing and postponing age-associated issues that cause sarcopenia [14]. However, the mechanism(s) by which exercise protects skeletal muscle against sarcopenia remain poorly understood; it can be, at least in part, due to decreasing intramuscular adipose tissue, pro-inflammatory cytokine levels, oxidative stress and DNA fragmentation as well as the delay/prevention of telomere shortening and increased sex hormone levels, protein synthesis and growth factors [45].

1.3.3.1. Resistance training

Resistance exercise training (RT) is an effective intervention for preventing and treating sarcopenia due to its ability to stimulate and promote net muscle protein anabolism, resulting in specific metabolic and morphological adaptations in skeletal muscle tissue and also in positive effects on metabolic, cardiovascular, and reproductive systems [14, 80, 120].

Considerable numbers of investigations have examined skeletal muscle responses to both acute and chronic RT in the elderly [51, 76, 78, 99, 100, 119]. However, the results of previous studies are conflicting regarding the acute effect of RT. In this regard, Fry et al. [78] measured intracellular signaling and MPS following an acute bout of RT in young and older subjects. At baseline and at 3, 6 and 24 hours after RT, muscle biopsy was taken from the vastus lateralis. No changes have been seen in phosphorylation for several key signaling proteins, mTOR, S6K1, 4E-BP1 and ERK1/2 after exercise in older group. An increased MPS after exercise from baseline has been found only in the younger group [78]. On the other hand, Ruae et al. [76] Investigated mRNA expression of several key skeletal muscle

myogenic controllers at rest and 4 hours after a single bout of RT in young and old women. Subjects performed 3 sets of 10 repetitions of bilateral knee extensions at 70% of one repetition maximum. RT led to upregulation of MyoD (2.0-fold) and MRF4 (1.4-fold) and downregulation of myostatin (2.2-fold) [76]. Following a series of investigations, Ruae et al. [100] Studied acute bout of RT on mRNA expression of ubiquitin proteasome-related genes involved in muscle atrophy in very old women. The RT protocol consisted of three sets of 10 knee extensions at 70% of one-repetition maximum. Muscle biopsies were taken from the vastus lateralis before and 4 hours after RT. The result demonstrated an induction of atrogin-1 and MuRF-1 gene expression in response to RT. These data suggest that in response to RT the regulation of ubiquitin proteasome-related genes involved in muscle atrophy are altered in very old women (> 80 years) [100] .

In contrast to acute RT, Melov et al. [51] Compared gene expression profiling and a subset of these genes were related to muscle strength in healthy older and younger adult men and women before and after a six-month RT program. In response to RT, strength improved significantly in older adults. Following RT, the transcriptional signature of aging was significantly reversed back towards younger levels for most genes. The authors then concluded that mitochondrial impairment and muscle weakness are favorably regulate altered at the phenotypic and transcriptome level, following six months of RT [51]. In support of the effects of RT on age related changes in mitochondrial function, Luo et al. [99] investigated the signaling pathways that regulate autophagy and apoptosis in the gastrocnemius muscles of 18–20 month old rats in response to 9 weeks of RT. Their finding demonstrated that RT prevented the loss of muscle mass by reduced Microtubule-associated protein 1A/1B-light chain 3 (LC3)-II/LC3-I ratio, reduced p62 protein levels, and increased levels of autophagy regulatory proteins, including Beclin 1, Autophagy-related protein 5/12 (Atg5/12), Atg7, and the lysosomal enzyme cathepsin L. These improvements in autophagy signaling were associated with an upregulation of total AMPK, phosphorylated AMPK, and FOXO3a expressions. Their results also showed that RT inhibited apoptosis by reduced Cyto C level in the cytosol, and inhibited cleaved caspase 3 production. They also found that RT upregulated the expression of IGF-1 and its receptors, but downregulated the phosphorylation of Akt and mTOR. These results suggest an anti-apoptotic role for chronic resistance exercise most likely by the inhibitory effects on mitochondria-mediated apoptosis in aged skeletal muscle [99].

The exact mechanism by which RT stimulates protein synthesis and reduces sarcopenic conditions in aged skeletal muscle is not yet fully understood. However, it is well known that Akt/mTOR signaling and Akt/FOXO3a signaling are both major regulators of skeletal muscle hypotrophy and atrophy. It has been speculated that in response to RT, IGF-1 and its receptors, as well as the Akt/mTOR and Akt/FOXO3a signaling pathways may be modulated [99]. In fact, in response to RT, IGFI/MGF activate PI3K, which leads to membrane translocation and subsequent phosphorylation of Akt by PDKI and PDKII. Once activated, Akt phosphorylates mTOR and GSK3ß, which play a mediator role in protein synthesis, transcriptional and proliferative processes related to hypertrophic response, as well as the control of protein degradation [82]. Other mechanisms that are involved in the synthesis of muscle protein are the MAPK signaling pathways. It has been shown that in response to RT, phosphorylation of ERK1/2 MAPK is increased and mTOR is activated [9]. mTOR activation by the ERK pathway may be through the phosphorylation of TSC2 [82]. It is important to consider that the effects of RT are dependent on the mode of exercise, including intensity, duration and frequency and also on the tissue types [99].

1.3.3.2. Endurance training

In addition to resistance exercise, aerobic endurance training (ET) also have been shown for potential role in the integrity and health of the aged skeletal muscle [121]. One of the serious consequence of aging is a progressive deterioration in aerobic exercise capacity due to reduced quantity or quality of skeletal muscle mitochondria [122], as well as decline in enzyme activities and protein content [53]. It is well known that ET not only improve maximal oxygen consumption (VO2max), mitochondrial density and activity, insulin sensitivity and energy expenditure [70], but can also reduce intramuscular fat and improve muscle functionality in young and older individuals [9]. An increase in the CSA of muscle fibers following ET, supports the notion that ET can contribute to improvement of muscle quality [22]. Numerous studies have investigated the effects of acute [81, 123-125] and chronic [50, 56, 61, 96, 98, 102, 103, 121, 126-132] ET on age related skeletal muscle adaptation in both humans and rodents.

In order to investigation of acute effect on skeletal muscle mitochondria in older subject, Bori et al. [123] Studied a single bout of ET on mRNA levels of genes involved in mitochondrial

biogenesis. They were interested in comparing old sedentary and old physically active individuals in response to acute ET. Compared to old sedentary control, ET resulted in an increased expressions mRNA levels of SIRT1 and AMPK subunit A2, but no change in the levels of PGC-1a, AMPK subunit B2, transcription factor A, mitochondrial (Tfam), Polynucleotide Phosphorylate (PNPase), Mitochondrial uncoupling protein 3 (UCP3), Lon protease, SIRT3. Training also reduced expression of Nrf1, mitochondrial fission protein 1 (Fis1) and mitochondrial fusion 1 (Mfn1) mRNA levels in old sedentary acute ET. an acute exercise bout Led to an significant increase in AMPK subunit A2 and PNPase expressions, maintained levels of SIRT1, PGC-1a, AMPK subunit B2, Tfam, Mfn1, UCP3, Lon protease and SIRT3, but decreased Nrf1 and Fis1 expression mRNA expression levels in old physically active subjects compared to control values [123]. These findings suggest that level of fitness may affect mitochondria adaption following acute ET. In contrast to acute ET, chronic ET appears to have considerably greater effects. Konopka at al. [56] examined the influence of 12 weeks of progressive ET on a cycle ergometer on markers of mitochondrial content in old women. Compared to basal levels, ET significantly increased PGC-1a protein content and levels of Citrate synthases (CS), ß-hydroxylacyl Co A dehydrogenase (ßHAD), succinate dehydrogenase (SDH) and cytochrome c oxidase (Cox) 4. In addition mitofusion or mitofission proteins Mfn1, Mfn2 and FIS1 protein contents were greater after ET [56]. In accordance with the previous results, Bo et al. [121] Found 12 weeks of ET stimulates mitochondrial biogenesis and network and also improves the efficiency of mitochondrial energy transfer in old rats. ET also increased Cox 4 content in trained compared with control old rats. Furthermore, Dynamin-related protein 1 (Drp1) protein, but not Mfn1, significantly increased after ET in the old training group. In addition, in response to training, ATP synthase activity -as an indication of mitochondrial energy production- increased when compared to the control group [121]. Upregulation of PGC-1a signaling is probably one of the main mediators in aged skeletal muscle mitochondrial adaptation to ET [29]. Findings from a study conducted by Kang et al. [131] Demonstrated that 12 weeks of ET increased PGC-1a content by 2.3 fold in trained compared to control old rats. This increased PGC-1a content was correlated with a significant increase in Tfam, Cyto C and mtDNA contents after ET in old rats. In response to ET, there was an increase in upstream signaling, involving PGC-1a activity including AMPK, p38MAPK, SIRT1 and p- cAMP response element-binding protein (CREB) in the old trained vs. old control rats. These data indicate that aging-associated decline in mitochondrial protein synthesis in skeletal muscle can be attenuated following chronic ET [131]. In this regards, another study, conducted by Broskey et al. [50],

investigated the effect of 4-month of ET intervention on proteins involved in mitochondrial biogenesis in sedentary older adults. In response to ET the levels of complexes III, IV, and V were significantly increased. Furthermore, a significant correlation was observed to the increase in Tfam expression levels and increase in PGC1a expression levels after the 4 months of exercise intervention. However, there were no change in Nrf1 and Nrf2 expression levels in responses to ET in older sedentary subjects [50].

One another important mechanism by which ET supports aged skeletal muscle may be due to its role in inhibition apoptosis process [39, 45, 133]. In this regards, Song et al. [98] reported that anti-apoptotic Bcl-2 increased, while significant reduction in DNA fragmentation, cleaved caspase-3, Bax, and Bax/Bcl-2 ratio were observed in the white gastrocnemius and soleus muscles of old rats in response to 12 weeks of ET. Furthermore, age-related decrease in upstream anti-apoptotic NF-B activity was reversed following ET [98]. Recently Marzetti et al [61] confirmed the hypothesis that age-associated apoptosis occurs less in type I muscle fibers, such as the soleus muscle, than type II fibers and therefore less likely to be affected by short-term ET. Their result showed, that in contrast to EDL, there was no significant changes in TNF-R1 expression, cleaved caspase-8 and -3 content, and apoptotic DNA fragmentation in soleus muscle of young and old groups and also in response to ET intervention [61].

A potential role for ET to increase the circulating levels of IGF-1 has also been suggested [134]. In this regard, Poehlman et al. [129] reported that 8 weeks of ET significantly increased fasting levels of IGF-1; more markedly in older men than women. There was also a significant correlation between changes in VO2max and IGF-1 in men, but not in women [129]. In addition, a study conducted by Manetta et al. [125] showed that basal levels of GH, IGF-1, and IGFBP-1 were higher in trained middle-aged men when compared with sedentary control. Furthermore, their data indicated that acute ET in middle-aged men increased the activity of the GH/IGF-1 system [125]. In support of this notion that ET can active anabolic factors, Hansen et al. [124] found that plasma follistatin increased by 7-fold following 3 h of bicycling exercise, but only increased by 2-fold after one-legged knee extensor exercise. These data suggest that increase in plasma follistatin after ET seems to be dependent on several factors, including the intensity and duration of exercise and also the muscle mass recruited during the exercise bout [124]. In accordance, Sakamoto et al. [81] found that Akt activity significantly increased following both acute submaximal and maximal intensity ET. Increases in Akt activity were accompanied by increases in Akt Thr308 and Ser473 phosphorylation [81]. Beneficial effects of ET on anabolic pathway may depend on the

frequency of training. In support of this notion, Pasini et al. [103] Investigated the effects of 8 weeks ET and training frequency (3 (EX3) or 5 days/week (EX5)) on anabolic pathways in the skeletal muscle of old rats. Aging was associated with reduced protein levels of IRS-1 and p-mTOR in aged control rats relative to the young control group. In response to ET, EX3 resulted in reduced IR expression and increased IRS-1 levels compared with old control rats. However, EX5 up-regulated not only IRS-1 and COX activity but also p-mTOR expression [103]. Despite the fact that the precise mechanisms of the age-associated loss of muscle mass is not yet clear, it seems that PGC-1a plays a central role in this process [103, 122]. It has been shown that ET stimulates upstream signaling pathways involved in PGC-1a activity, including AMPK, p38MAPK, SIRT1 and p-CREB [131]. Mitochondrial biogenesis induction by PGC-1a is mediated by the coactivation a large spectrum of transcription factors, including Nrf1, Nrf2 through Tfam, which regulates mitochondrial DNA replication (Figure 6) [50]. Furthermore an increase in mitochondrial function and biogenesis following ET has inhibitory effect on apoptosis initiation, therefore may help to preserve muscle quality and aerobic capacity during aging [98, 122]. However, it has also been suggested that ET may be counted as an effective therapy for sarcopenia not just by its effects on mitochondrial regulation and adaptation, but also by reduced catabolic pathways such as FOXO3A, myostatin and increased anabolic pathways such as IGF-1 and follistatin [56, 103, 124].

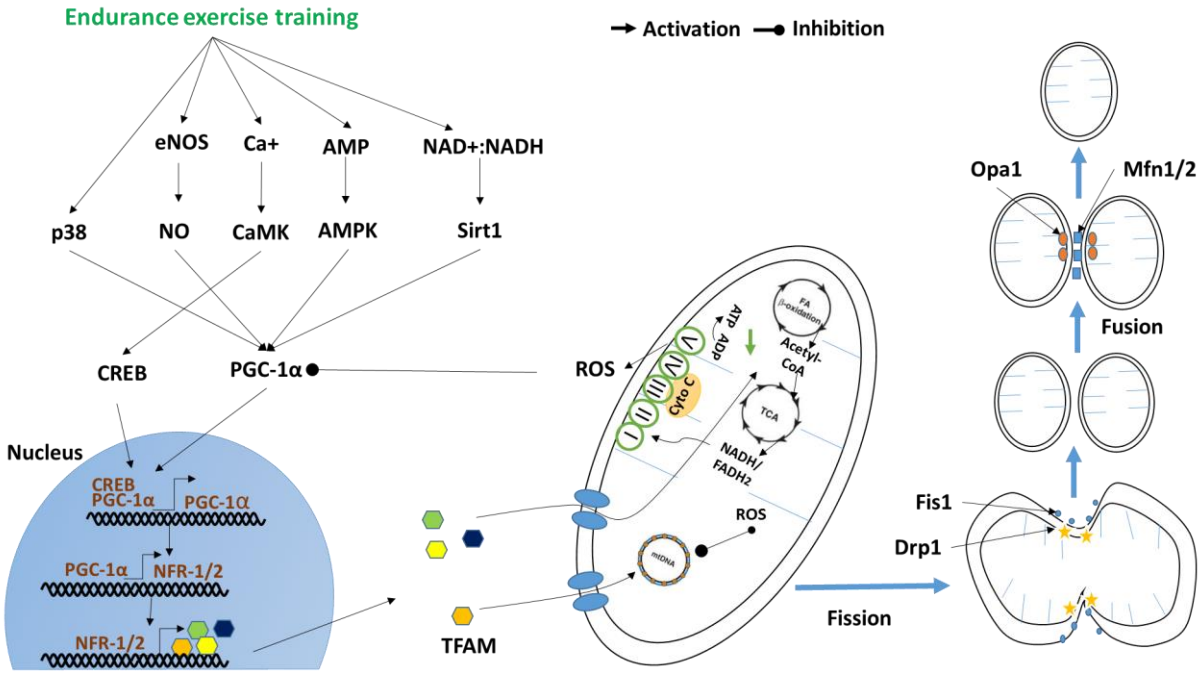

Figure 6. Diagrammatic summary of endurance exercise training signaling pathways involved in mitochondria function in healthy mammalian skeletal muscle cell

Mitochondria biogenesis. PGC-1α is known as a master regulator of mitochondria biogenesis witch its gene expression is mediated by other factors such as AMPK, Sirt1, CaMk, NO and p38. PGC-1α gene expression along with the expression of Nrf1 and Nrf2 induce the expression of Tfam, which is imported into mitochondria. Tfam regulates the expression of the mtDNA gene products, including proteins such as cytochrome c oxidase subunit I (COX I) and also are involved in ATP synthesis.

1.3.4. Hormone therapy

Several hormonal treatments have been proposed for the treatment of sarcopenia including GH, IGF-1, testosterone and estrogens [59, 61, 65, 70, 104, 135]. However, controversial findings have been reported in the literature related to the effectiveness of hormone therapy on sarcopenia. More recently Brioche et al. [59] Investigated 8 weeks of GH administration (2 mg/kg/day) on some cellular markers of sarcopenia in old rats. The result was interesting as GH treatment led to a significant, 100% increase of IGF-1 in old animals. GH supplementation also prevented increased protein and DNA oxidation in old rats. Levels of PGC-1a, Nrf1, and Cyto C as well as citrate synthase activity were significantly lower in old animals than in young ones. These decrements were completely prevented by replacement

therapy with GH. In addition, in response to treatment with GH, the significant decrease in Akt phosphorylation and phosphorylation of p70S6K were completely recovered in the old muscles. GH treatment prevented the elevation of p38 phosphorylation in the muscle of old animals. Myostatin and MuRF-1 are well-known agents involved in proteolysis. GH treatment prevented age associated increases in Myostatin and MuRF-1 [59]. Moreover, GH supplementation has been shown to inhibit apoptosis in aged skeletal muscle [61]. Marzetti et al. [61] found that aging was associated with an elevation of the expression of TNF-R1 and cleaved caspase-8 in the EDL muscle in response to GH administration, both of which were reduced in aged rats. However, they did not find any reduction in the content of cleaved caspase-3 and apoptotic DNA fragmentation by the hormonal intervention. They then concluded that the protective effect of GH supplementation was an early step of the extrinsic pathway of apoptosis in the EDL muscle and did not translate into an effective mitigation of the actual apoptotic events. However, independently of caspase activation, GH administration was associated with increased apoptotic DNA fragmentation in the soleus muscle [61]. However, there are some studies which did not find beneficial effects of GH therapy on muscle strength or muscle mass [34, 40, 136]. Based on the literature, it seems that GH supplementation is more effective in patients with GH deficiency or reduced GH secretion than in those with normal hormonal state [34]. Failure of the regulation of natural GH secretion or the induction of GH-related insulin resistance could be possible reasons for the ineffectiveness of GH treatment in improving muscle mass and strength in the elderly [40]. Regardless of the proposed benefits of GH therapy, numerous side effects have been reported, including soft tissue edema, gynecomastia, orthostatic hypotension, and carpel tunnel syndrome, which pose serious concerns especially in older adults [22, 40, 137] .

Another potential hormone treatment against sarcopenia in women is estrogen supplementation or hormone replacement therapy (HRT). Despite the availability of reports on the effectiveness of hormone therapy [138, 139], however, some studies did not report any significant impact [65, 104]. For instance, it has been demonstrated that there was no difference in the prevalence of sarcopenia in healthy independent older women who were long-term estrogen users compared with older women who did not use estrogen [104]. It has been suggested that HRT may protect against the loss of muscle mass, which occurs in the premenopausal period [22]. Differences in estrogen dose used, the duration of the study, levels of physical activity, diet and medications can be an explanation for the contradictory results between studies [65].

Testosterone is another hormone that has been widely studied for its effects on strength and muscle mass in young and old people. Nevertheless, previous studies have reported conflicting results [22, 24, 40, 45, 66, 71, 91, 135]. Circulatory levels of testosterone are correlated with sarcopenia, muscle mass and function as well protein synthesis. It has been demonstrated that the bioavailable testosterone and the testosterone precursor, DHEA both drop with aging [45]. The exact mechanisms by which testosterone protects against sarcopenia of aging is still unclear. However, it can be due to, at least in part, the suppression of myostatin and the non-canonical TGF-ß pathway through stimulation of Notch signaling, together with the inhibition of JNK mediated apoptosis. Indeed, it has been suggested that the activation of Akt together with the inhibition of JNK may be critical for testosterone-mediated protection against sarcopenia during aging [71, 135]. In this regard, it has recently been reported that suppression of myostatin signaling by testosterone supplementation reduces the extent of myonuclear apoptosis in the gastrocnemius muscle of old mice, while improving muscle mass and fiber cross-sectional area [91]. In support of the anabolic contribution of testosterone, a number of studies have reported an increased IGF-1 protein levels following testosterone administration [24, 45]. However, it has been reported that numerous side effects are associated with testosterone treatment, including increased risk of cardiovascular problems and pedal edema [71, 114].

1.3.4.1. IGF-1

It is crucial that an appropriate treatment strategy should be able to maintain muscle mass, reduce muscle loss and stimulate muscle regeneration that can counteract muscle wasting [140]. At least three major molecular processes are involved in the regulation of skeletal muscle hypertrophy: (1) satellite cell activity; (2) gene transcription; (3) protein translation [141]. Among the different growth factors, IGF-1 has been shown to be involved in many anabolic pathways in skeletal muscle as well as during muscle regeneration [43].

IGF-1, also known as somatomedin C, is a 70–amino acid [142] that is similar to insulin in structure, sharing 50% amino acid identity. However, unlike the insulin gene, the single-copy IGF-1 gene locus encodes multiple proteins with variable amino- and carboxyl-terminal amino acid sequences [140]. IGF-1 exists in at least two isoforms as a result of alternative splicing of the IGF1 gene. IGF1Ea or systemic IGF-1, which is produced in both liver and

muscle tissues and IGF1Eb (rodent form) and IGF1Ec (human form) also known as mechano growth factors (MGFs), which are produced generally by skeletal muscle. Unlike MGFs, IGF1Ea is glycosylated, and this modification protects it from proteolysis and confers a relatively long half-life [60]. Most IGF-1 circulates in blood bound to one of the six high-affinity IGF binding proteins (IGFBPs; IGFBP1 to IGFBP6), which have been shown to modulate IGF-1 availability for action on tissues [125]. It has been shown that overexpression of any of these IGFBP isoforms is associated with decreased in IGF-1 action by inhibiting it's binding to IGF-1R [62]. In tissues, IGFBPs can both decrease or increase IGF1 actions either by detaching IGF1 from the IGF1R or by releasing free IGF1 available for receptor binding [60].

A wide variety of tasks and functions have been related to IGF-1, such as regulation of both proliferative and differentiation responses in muscle cells, promotion and regulation of muscle growth, improvement of sprouting and axonal growth and cell survival on motor neurons, along with the prevention of motor neuron death [43, 142]. The positive regulatory effects of IGF-1 on muscle growth act on several levels, including satellite cell activation, gene transcription, and protein translation [143]. IGF-1 affect both hyperplastic and hypertrophic processes in skeletal muscle. The hyperplastic effect results in the proliferation of muscle satellite cells, while the hypertrophic effect results in increased synthesis of contractile proteins by existing myonuclei [34].

It has been shown that serum and skeletal muscle concentrations of IGF-1 are lower in older adults [144] and this low circulating IGF1 bioactivity and abnormalities of IGF1 may be involved in age-related sarcopenia [60]. Several studies have demonstrated that IGF-1 administration reduce the age-related loss of skeletal muscle mass and strength likely through positive effects on neuronal function and by the prevention of apoptotic death, stimulating axonal sprouting and repair of damaged axons, increasing muscle oxidative enzymes and fatigue resistance [34, 45, 60, 71, 80, 134, 145, 146].

The exact molecular mechanism by which IGF-1 administration improves muscle mass and attenuates age-related muscle atrophy is not completely understood yet. However, it has been demonstrated that IGF-1 /PI3K/Akt and IGF-1 /ERK1/2 MAPK are the two main signaling pathways that are involved in IGF-1-induced cell protection [146].

After binding IGF to its receptor, a conformational change occurs, leading to activation of IRS-1 [62]. Phosphorylated IRS-1 can activate PI3-K, leading to Akt phosphorylation, which

in turn enhances protein synthesis through mTOR and p70S6 kinase activation and also mediating the antiapoptotic effects of the IGF1R through phosphorylation and inactivation of BAD. Indeed, activation of Ras by phosphorylated IRS-1 or SHC leads to the stimulation of the RAF-1/MEK/ERK pathway and downstream nuclear factors, leading to the induction of cell proliferation [60].

IGF-1 treatment can also increase protein synthesis and reduce protein degradation via downregulation of ubiquitin ligases. Activation of PI3K/Akt in turn leads to the phosphorylation and inactivation of FOXO transcription factors resulting in the reduction of MuRF1 and atrogin-1 expression thereby, a reduced protein degradation by the 26S proteasome in skeletal muscle [97] [147].

Taken together, it seems that the effectiveness of hormone therapy such as GH, estrogen, testosterone and IGF-1 on age-related loss of skeletal muscle mass is explained by the decreased the rate of protein degradation than increasing protein synthesis due to the modulation of IGF-1/FOXO, IGF-1/NFkB and IGF-1/ERK1/2 signaling pathways.

1.3.5. Combination of exercise and nutritional and pharmacological supplementation

It seems that exercise in combination with nutritional and pharmacological intervention is more effective against sarcopenia [32]. A wealth of data exists which demonstrate beneficial effects of the combination of exercise and nutritional interventions on skeletal muscle adaptation in older person [39, 46, 61, 66, 134]. For instance, it has been found that combined wheel running and mild CR significantly preserved a higher muscle mass/body mass ratio and fiber CSA [39]. Guo et al. [66] demonstrated that 2 months of testosterone administration together with low-intensity physical training (T/PT) improved grip strength, spontaneous movements, and respiratory activity in old mice. T/PT was associated with increased mitochondrial DNA copy number and expression of markers for mitochondrial biogenesis. Furthermore, 2 months of T/PT led to an increased expression of markers for mitochondrial fission-and-fusion and mitophagy and reduced tissue oxidative damage, while also improved muscle quality [66]. In contrast, Li et al. [134] have reported that there were no significant increases in Akt-1 and p70 S6K phosphorylation following an acute bout of ET and IGF-1 injection in old mice [134]. However, the combination of exercise and IGF-1 has shown a modest effect on reducing aged-related wasting of skeletal muscle [148]. More recently,

McMahon et al. [148] investigated the effect of long term wheel-running on the prevention of sarcopenia in IGF-1Ea overexpressing transgenic mice compared with wild-type. Their results demonstrated that the combination of IGF-1 and exercise prevented the reduced mass of the quadriceps muscles in 28 months-old mice compared with wild type. However, there was no improvement in muscle function as assessed by grip strength [148].

1.4. Summary of introduction

Along with increased longevity, the prevalence and cost of sarcopenia are likely to rise [9]. It is associated with elevated risk of cardiac, pulmonary, and metabolic disease processes, which further contributes to the socioeconomic burden [126]. This global aging phenomenon has led to an increased morbidity and greater need for hospitalization and/or institutionalization. Healthy style of life is essential for older people to remain independent and to continue to actively take part in family and community life [14]. Developing new therapies and strategies to prevent and treat sarcopenia not only will help to improve the quality of life for patients, but also will help to reduce the economic and productivity burdens for society in general [9, 80].

In this regard, in order to provide further insight into the development of more effective therapy, it is important to explore the mechanism and etiology of sarcopenia [42]. Among factors, free testosterone, physical activity, cardiovascular disease, IGF-1 in men and total fat mass and physical activity in women are significantly associated with muscle mass [149]. In spite of the documented benefits of aerobic exercise on cardiovascular and metabolic health for older adults, the influence of this type of exercise on skeletal muscle mass and function is less understood [128]. Furthermore, comparison of master athletes with sedentary controls demonstrated that training may not be sufficient to prevent skeletal muscle loss in older adults [46].

Therefore, it seems that factors, such as nutrition in conjunction with appropriate physical activity can help attenuate the age related physical and muscle mass decline and maintain quality of life [44].

2. Objectives

As mentioned in the previous chapter, reduced systemic anabolic hormone levels, especially IGF-1 and reduced mitochondrial efficiency with aging are two potential factors involved in sarcopenia. Despite strong evidence for the effectiveness of RT, the effect of ET on the prevention and reduction of age-related muscle loss is still not well understood. Furthermore, available data from previous studies on hormone therapy is ambiguous. Although, it has been shown that the combination of exercise and hormone supplementation is more effective than either alone in attenuating muscle atrophy.

In our study, we aimed to elucidate the effects of endurance training with or without IGF-1 administration on four of the main mechanisms, involved in the onset and progression of sarcopenia including, decreased rate of protein synthesis, increased protein degradation, alteration in mitochondrial biogenesis and increased apoptotic signaling.

It was hypothesized that endurance exercise training alone and in combination with IGF-1 treatment has beneficial effects on age-related muscle atrophy, and in general can attenuate the process of sarcopenia.

The following assumptions were made:

H1. Aging negatively influences the cellular markers involved in sarcopenia.

H2. Endurance exercise training positively influences the cellular markers involved in sarcopenia.

H3. IGF-1 administration enhances positive effects of endurance exercise on the cellular markers involved in sarcopenia.

3. Methods

3.1 Subjects

Fifteen young (3 months old) and 15 old (26 months old) male Wistar rats were used in the study. We chose 26-month-old rats because previous studies have reported that sarcopenia is evident at 22 months age in this species [59, 150]. Subjects then were assigned to one of the following groups: young control (YC), young exercised (YE), young exercised and IGF-1-treated (YEI), old control (OC), old exercised (OE), and old exercised and IGF-1-treated (OEI).

The investigation was carried out according to the requirements of The Guiding Principles for Care and Use of Animals of the European Union and approved by the local ethics committee.

3.2 Exercise protocol

Exercised rats were introduced to treadmill running for 3 days; then for the next 2 weeks the running speed was set at 10 m/min, with a 5% incline for 30 min/day, 5 days per weeks. The running speed and duration of the exercise were gradually increased to 60% of VO2 max of the animals. Therefore, on the last week of the 6-week training program, young animals ran at 22 m/min, on a 10% incline, for 60 min, whereas old animals ran at 13 m/min, on a 10% incline for 60 min (Figure 7). At the end of the study the animals were anesthetized with intraperitoneal injections of ketamine (50 mg/kg) and were sacrificed. This occurred two days after the last exercise session, to avoid any metabolic effects of the final run (Figure 8). Quadriceps muscle was carefully excised and stored at -80 ◦C.

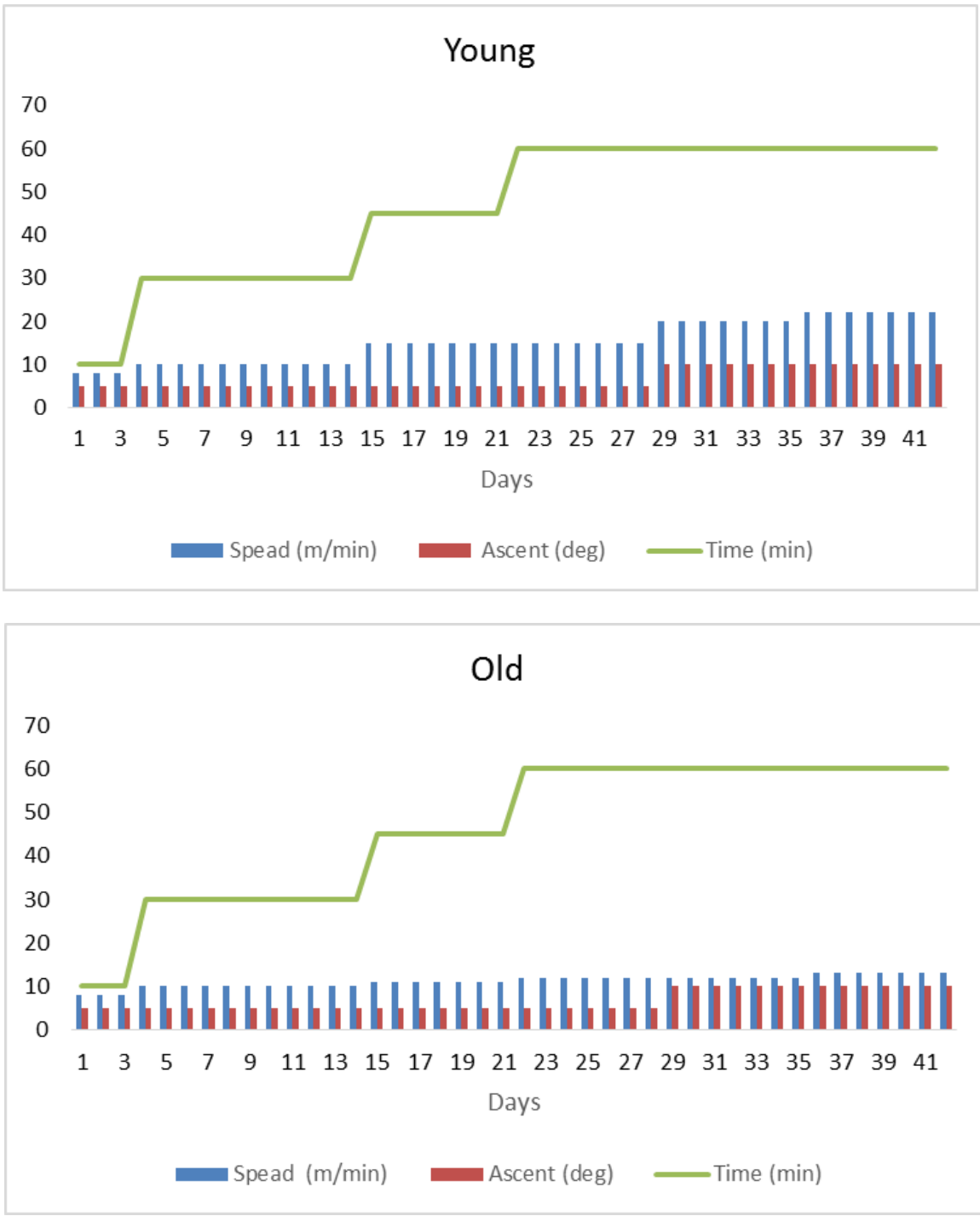

Figure 7. The figures represent Speed, time and incline (ascent) of 6 weeks of endurance training on treadmill in young and old rats

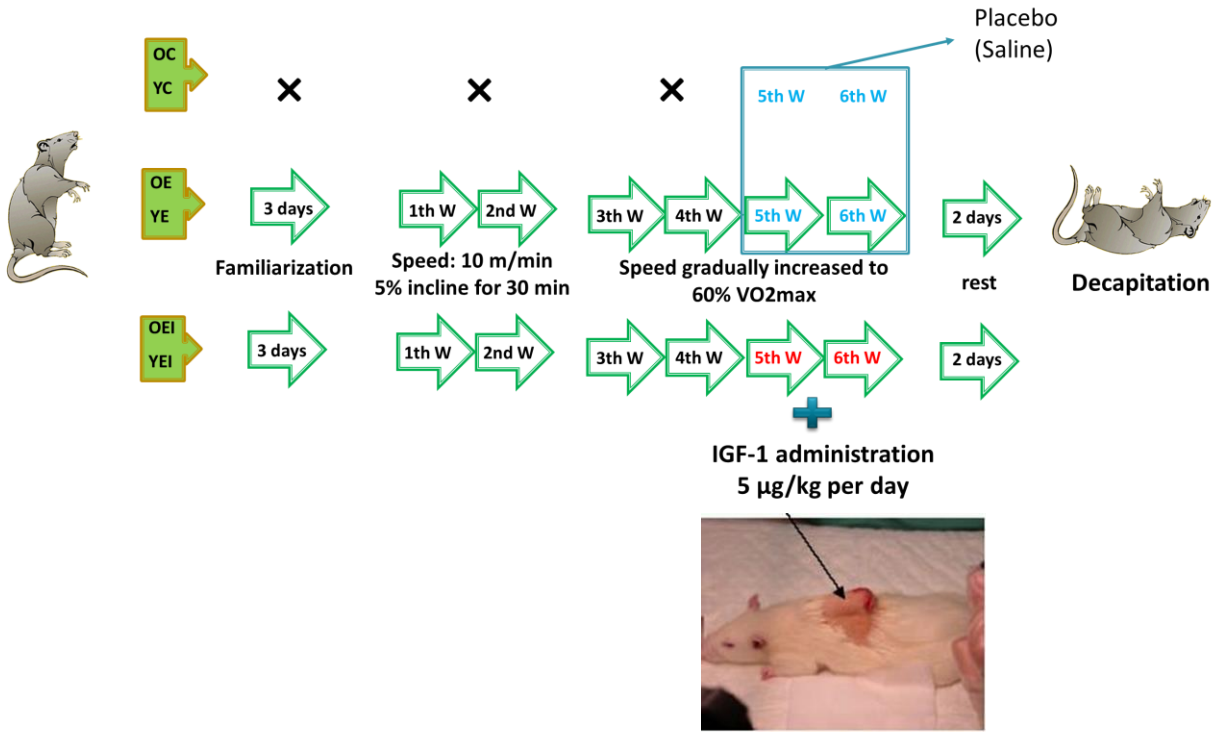

Figure 8. Schematic design of the study protocol

OC (old control), YC (young control), OE (old exercise), YE (young exercise), OEI (old exercise and IGF-1 treatment), YEI (young exercise and IGF-1 treatment) and W (week).

3.3 IGF-1 administration

3.3.1 Alzet pump

An Alzet pump (Alzet mini-osmotic pump model 2002. Durect Corporation #0000296) was inserted subcutaneously in all animals. Alzet pump operate via an osmotic pressure difference between a compartment within the pump, called the salt sleeve, and the tissue environment in which the pump is implanted. The high osmolality of the salt sleeve causes water to flux into the pump through a semipermeable membrane, which forms the outer surface of the pump. As the water enters the salt sleeve, it compresses the flexible reservoir, displacing the test solution from the pump at a controlled, predetermined rate. Because the compressed reservoir cannot be refilled, the pumps are designed for single-use only. The rate of delivery by an Alzet pump is controlled by the water permeability of the pump's outer membrane. Thus, the delivery profile of the pump is independent of the drug formulation dispensed. Drugs of

various molecular configurations, including ionized drugs and macromolecules, can be dispensed continuously in a variety of compatible vehicles at controlled rates. The molecular weight of a compound, or its physical and chemical properties, has no bearing on its rate of delivery by Alzet pumps. The volume delivery rate of Alzet pumps is fixed at manufacture. Alzet osmotic pumps are available with a variety of delivery rates between 0.11 and 10 µL/hr and delivery durations between 1 day and 6 weeks. While the volume delivery rate of the pump is fixed, different dosing rates can be achieved by varying the concentration of agent in the solution or suspension used to fill the pump reservoir.

A more complete and technical explanation of the operation of Alzet osmotic pumps can be found in the http://www.alzet.com/.

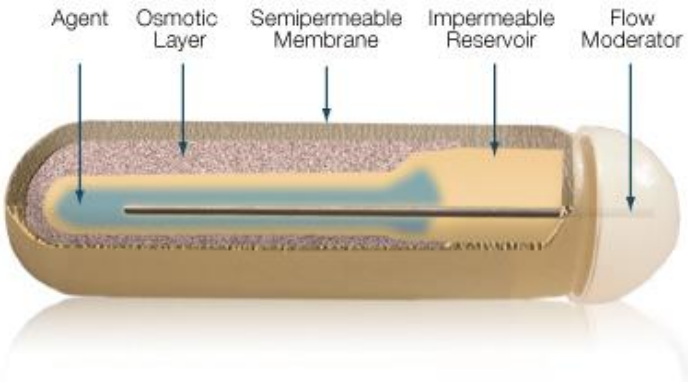

Figure 9. Schematic representation of an Alzet osmotic pump

3.3.2. IGF-1 supplementation

In the last 2 weeks of the study, treated animals received 5 µg/kg per day, 0.5 µL/hr IGF-1 (Sigma #13769), whereas non-treated animals received saline via the pumps. With the help of the Alzet pumps, the 2-week supplementation of IGF-1 or saline could be maintained at constant flow, thus avoiding daily injections and their possible disturbance of behavioral and cognitive functions of the animals.

3.4. Tissue preparation

Frozen vastus lateralis samples were weighed (~100 mg) and homogenized (1:10 w/v) in ice-cold buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 2% NP-40, 10% glycerol) supplemented with phosphatase and protease inhibitors. The homogenates were incubated at 4 °C for 30

min, and then centrifuged at 12,000 g for 20 min at 4 °C. Then, the supernatant was collected and Bradford assays were used to determine supernatant protein concentrations. Proteins were diluted in 2× SDS sample buffer (1:1) and then heated to 95°C for 5 minutes.

3.5. Western blot analysis

Ten to 20 μg of protein were electrophoresed on 6–15% vol/vol polyacrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with 5% milk–Tris-buffered saline with 0.1% tween-20 solution and then incubated with a primary antibody overnight at 4°C. Primary antibodies are described in Table 1 and were diluted 1:500 to 1:1000 with 5% milk–Tris-buffered saline with 0.1% tween-20. Blots were incubated with horseradish peroxidase–conjugated secondary antibody diluted 1:3000 with 5% milk–Tris-buffered saline with 0.1% tween-20. After incubation with the secondary antibody, membranes were washed repeatedly and then were incubated with chemiluminescent substrate (Thermo Scientific, SuperSignal West Pico Chemiluminescent Substrate), and protein bands were visualized on X-ray films. The bands were quantified by ImageJ software and normalized to B-actin, which served as an internal control.

3.6. Measurement of IGF-1 level

After sacrificing the animals, blood was collected, supercharged ethylenediaminetetraacetic acid (EDTA) was added, and the samples were centrifuged at 3000 × g, for 10 min at 4°C. Plasma was separated and kept at −80°C. A Quantikine Mouse/Rat IGF-1 Assay Kit (R&D Systems, cat. no. MG100) was used to detect IGF-1 levels according to the description of the supplier. Briefly this assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat IGF-1 has been pre-coated onto a microplate. Standards, control, and samples were pipetted into the wells and any rat IGF-1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat IGF-1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate

solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the Stop Solution was added. The intensity of the color measured was in proportion to the amount of rat IGF-1 bound in the initial step. The sample values were then read off the standard curve. The optical density of each well were determined within 30 minutes. microplate reader and wavelength was set to 450 nm and 570 nm respectively.

Table 1. Description of primary antibodies used in the study

Antibody/antigen	Dilution ratio	company	Catalog number
Follistatin	1: 500	Santa Cruz	SC-30194
Akt	1: 1000	Cell signaling	#9272S
p-Akt (Ser473)	1: 1000	Cell signaling	#9271S
mTOR	1: 500	Santa Cruz	SC-8319
pmTOR (Ser2448)	1: 500	Cell signaling	#5536
ERK1/2	1: 1000	Cell signaling	#9102
pERK1/2 (Thr202/Tyr204)	1: 500	Cell signaling	#9106
Myostatin (GDF-8)	1: 500	Santa Cruz	SC-6884
Ubiquitin	1: 1000	Cell signaling	#3936
MuRF1	1: 500	Santa Cruz	SC-32920
MuRF2	1: 500	Santa Cruz	SC-49457
PSMA6	1: 1000	Cell signaling	#2459
PGC-1α	1: 1000	Millipore	ST1202
SIRT1	1: 500	Santa Cruz	SC-15404
SIRT3	1: 500	Sigma	S4072
Nrf2	1: 500	Santa Cruz	SC-722
Cyto C	1: 1000	Santa Cruz	SC-13560
Cox 4	1: 500	Santa Cruz	SC-69359
TNF-α	1: 500	Santa Cruz	SC-1350
p53	1: 500	Santa Cruz	SC-99
Bcl-2	1: 500	Santa Cruz	SC-492
Bax	1: 500	Santa Cruz	SC-493

3.7. Measurement of ROS level

Intracellular oxidant and redox-active iron levels were estimated using modifications of the dichlorodihydrofluorescein diacetate (H2DCFDA) staining method. The oxidative conversion of stable, nonfluorometric DCFDA to highly fluorescent 2′7′-dichlorofluoorescein (DCF) was measured in the presence of esterases, as previously reported [151]. This assay approximates levels of reactive species, such as superoxide radical, hydroxyl radical, and hydrogen peroxide. The method has been widely used in the literature but does have the problem of not being particularly specific, and results can be strongly affected by release of labile iron or copper [152]. Briefly, the H2DCFDA (Invitrogen-Molecular Probes #D399) was dissolved to a concentration of 12.5 mM in ethanol and kept at −80 °C in the dark. The solution was freshly diluted with potassium phosphate buffer to 125 μM before use. For fluorescence reactions, 96-well, black microplates were loaded with 150 ul of 50 mM potassium phosphate buffer (13.969 g K2HPO4 + 2.71 g KH2PO4 in distilled water up to 200 ml, pH 7.4) to a final concentration of 152 μM/well. Then eight μl diluted tissue homogenates and 50 μl 125 μM dye (20 ul 12.5 mM H2DCFDA in 1980 ul 50 mM potassium phosphate buffer) were added to achieve a final dye concentration of 25 μM. The change in fluorescence intensity was monitored every five minutes for 30 minutes with excitation and emission wavelengths set at 485 nm and 538 nm (Fluoroskan Ascent FL) respectively. Data obtained after 15 min were used. The fluorescence intensity unit was normalized to the protein content and expressed in relative unit production per minute.

3.8. Statistical analyses

Statistical significance was assessed by the IBM SPSS program version 21. Data were tested with Shapiro-Wilk's W normality test. Parametric data were analyzed by one-way ANOVA, followed by Tukey's post hoc test. Kruskal-Wallis ANOVA followed by Mann-Whitney U test was applied to evaluate the differences in non-parametric results in case of those variables where post-hoc analysis was required. The significance level was set at $p < 0.05$.

4. Result

4.1 Cellular markers involved in protein synthesis

The IGF-1/Akt and mTOR pathways are two evolutionarily conserved pathways that play critical roles in regulation of cell proliferation, survival, and energy metabolism. Moreover, it is possible that the increased IGF expression might contribute to skeletal muscle hypertrophy induced by follistatin [153]. Indeed, the IGF-ERK1/2 pathway can stimulate several substrates, such as p90RSK (p90 ribsosomal S6 kinase), resulting in the activation of transcription factors and the ribosomal subunit S6. ERK1/2 may also increase activity of kinases associated with protein translation, such as MAPK-interacting kinase 1 (Mnk 1) and its downstream substrate, eIF4E [39]. Therefore, we measured the IGF-1 level and phosphorylated and total protein content of Akt, mTOR, ERK1/2 and follisatin.

The levels of circulating IGF-1 decreased with aging. There was significant increase in OEI compared with OC group (Figure 10). The expression levels of follisatin was also lower in OC vs. YC ($p<0.05$) while both exercise and IGF-1 treatment significantly increased ($p<0.05$) follistatin levels in OE and OEI (Figure 11). Total protein content of Akt and pAkt was higher ($p<0.05$) in OC vs. YC but there was no effect of exercise and IGF-1 interactions among groups (Figure 12B and Figure 12A respectively). The results showed that protein levels of mTOR and pmTOR didn't change with age, however, exercise resulted in an increase ($p<0.05$) in pmTOR among YE vs. YC, while significantly increases were observed in both mTOR and pmTOR levels ($p<0.05$ and $p<0.01$ respectively) following IGF-1 supplementation in OEI vs. OC (Figure 13B and Figure 13A respectively). Nevertheless, aging and exercise training didn't have significant effect on the ratio of pAkt: Akt (Figure 12C) and pmTOR: mTOR (Figure 13C), however, IGF-1 treatment led to a significant increase ($p<0.05$) of pmTOR: mTOR in OEI compared to OE (Figure 13C), whereas YEI showed a significant reduction ($p<0.01$) compared to YE flowing IGF-1 administration (Figure 13C). Old subject showed lower level ($p<0.05$) of pERK1/2 (Figure 14B). Exercise training decreased the protein content of ERK1/2 ($p<0.01$) in YE vs. YC, while IGF-1 treatment led to markedly reduction in OE and OEI ($p<0.05$ and $p<0.01$ respectively) (Figure 14A). Aged subjects had significant increase ($p<0.05$) in pERK1/2: ERK1/2, while no effect of exercise and IGF-1 treatment was observed despite of a tendency for a decrease in OE and OEI, however, this ratio was lover ($p<0.01$) in YEI vs. YC (Figure 14C).

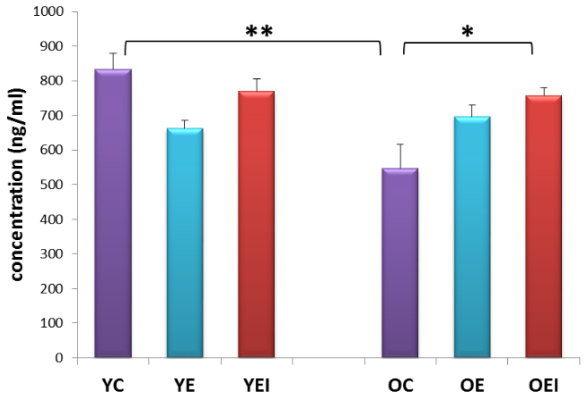

Figure 10. Effect of age, exercise and combination of exercise and IGF-1 administration on plasma level of IGF-1

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

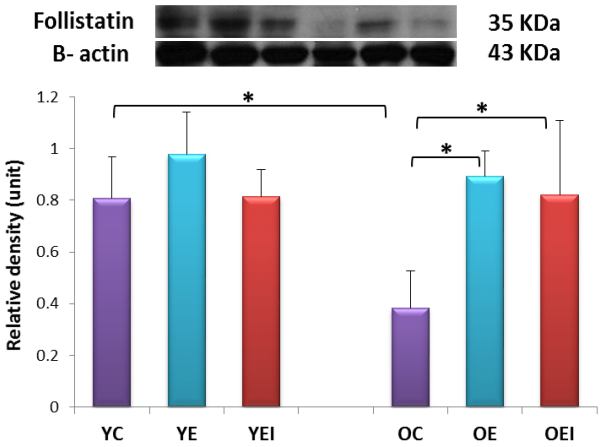

Figure 11. Effect of age, exercise and combination of exercise and IGF-1 administration on follistatin protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

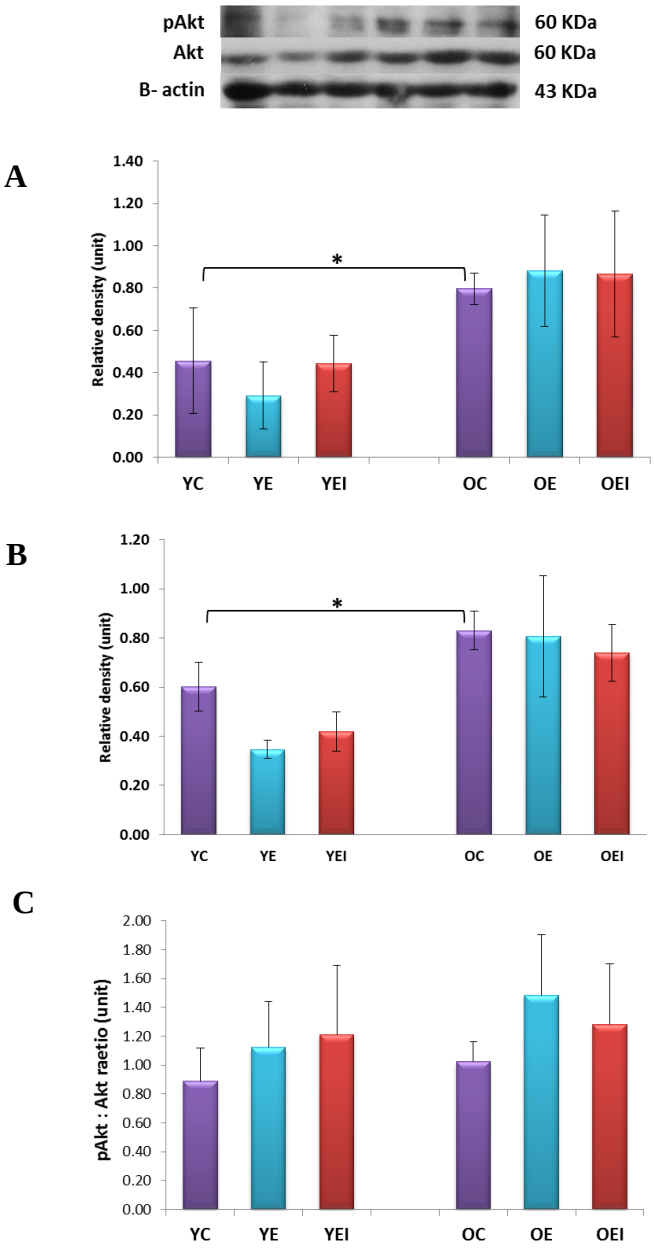

Figure 12. Effect of age, exercise and combination of exercise and IGF-1 administration on levels of pAkt (A), total Akt (B) and pAkt: Akt ratio (C)

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

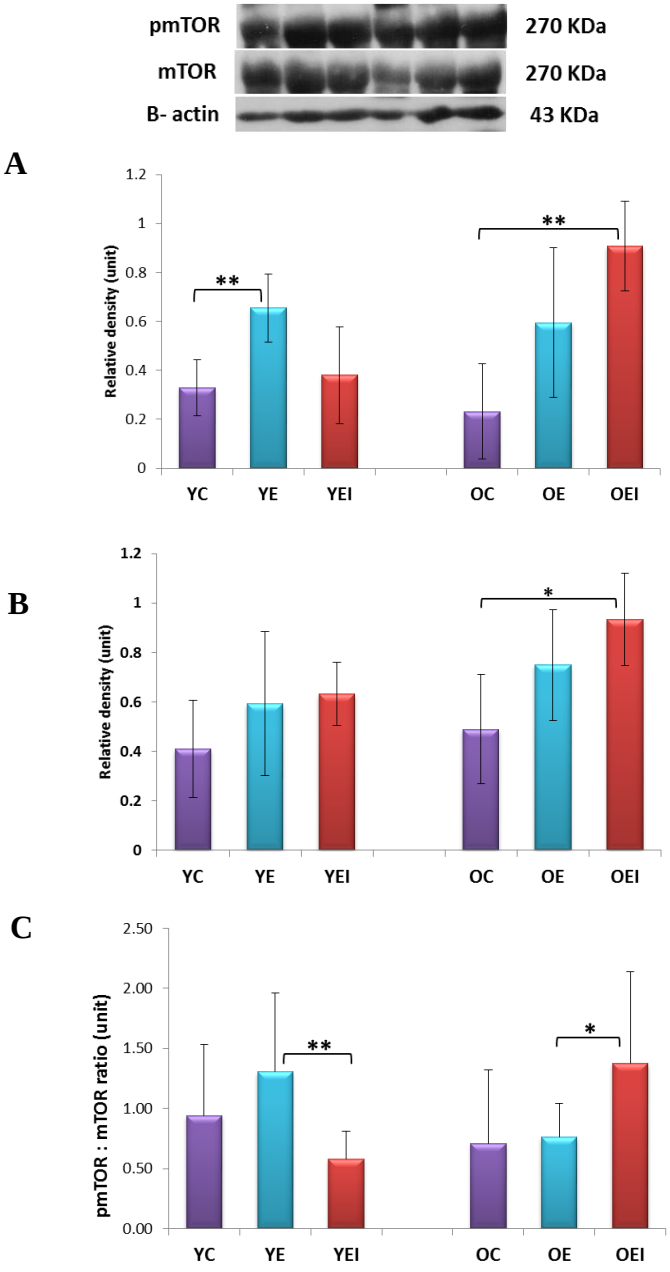

Figure 13. Effect of age, exercise and combination of exercise and IGF-1 administration on levels of pmTOR (A), total mTOR (B) and pmTOR: mTOR ratio (C)

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

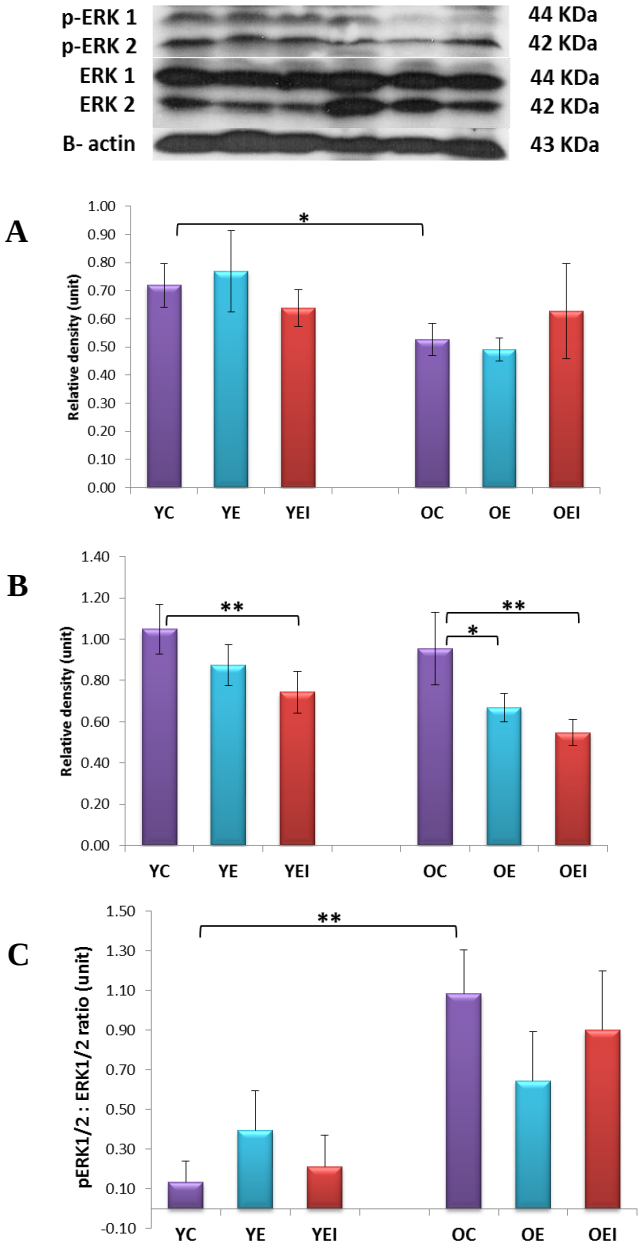

Figure 14. Effect of age, exercise and combination of exercise and IGF-1 administration on levels of pERK1/2 (A), total ERK1/2 (B) and pERK1/2:ERK1/2 ratio (C)

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

4.2 Cellular markers involved in protein degradation

In recent years there has been a significant interest in growth and differentiation factor-8 (GDF-8), or myostatin, which functions as a powerful negative regulator of muscle growth [154]. On the other hand, the ubiquitin proteasome system is one of the major pathways that regulates muscle protein degradation, playing a central role in controlling muscle size. MuRF1 and MuRF2 and proteasome subunits have been proposed to regulate protein degradation and gene expression in muscle tissues [80, 155, 156].

Therefore, we measured the expression levels of Myostatin, Ubiquitination, MuRF1, MuRF2 and PSMA6.

Myostatin expression levels was significantly higher in OC vs. YC ($p<0.05$) and it was lower ($p<0.01$) in YEI then YC (Figure 15). Ubiquitinated level was higher in OC vs. YC, whereas exercise and IGF-1 administration led to a significant increase in YE and YEI ($p<0.05$ and $p<0.01$ respectively) (Figure 16). Expression levels of MuRF1 (Figure 17) and MuRF2 (Figure 18) was significantly higher in OC then YC ($p<0.05$). Exercise training decreased MuRF1 and Murf2 levels in OE ($p<0.05$). Exercise also decreased MuRF2 protein content in YE ($p<0.05$) (Figure 18). Administration of IGF-1 also decreased MuRF2 levels in OEI ($p<0.01$) compared to OC (Figure 18) and increased ($p<0.05$) MuRF1 levels in YEI compared to YE (Figure 17). PSMA6 showed an increased with aging ($p<0.05$) and exercise training significantly increased its protein content both in YE and OE ($p<0.05$). IGF-1 supplementation led to an evident increase ($p<0.01$) in YEI (Figure 19).

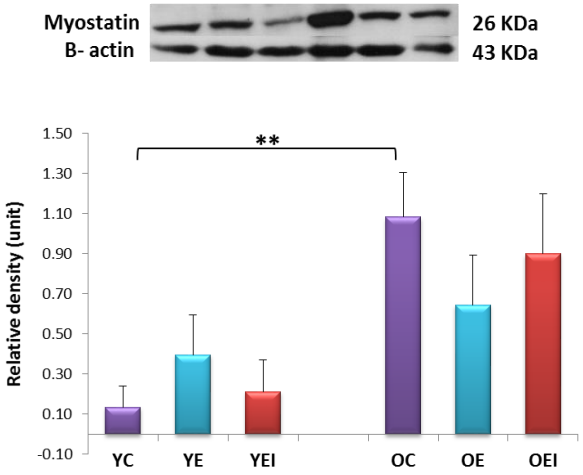

Figure 15. Effect of age, exercise and combination of exercise and IGF-1 administration on Myostatin protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

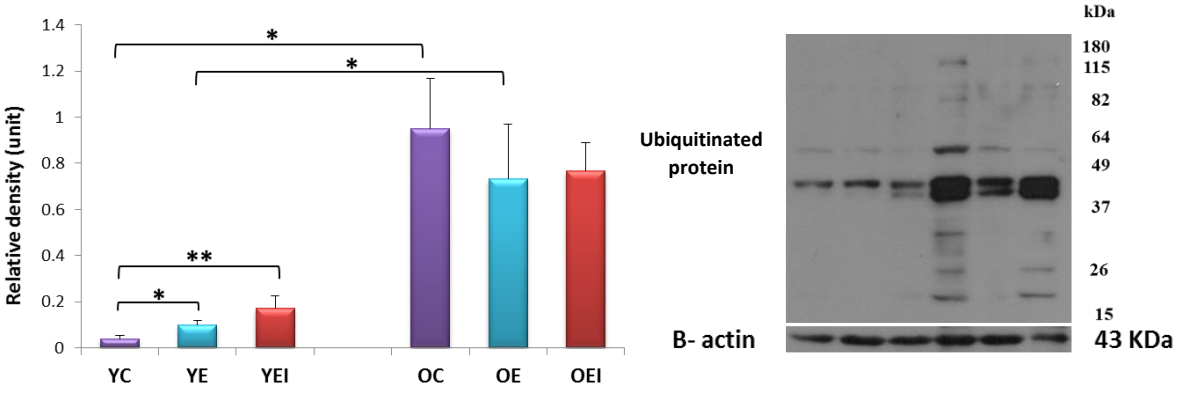

Figure 16. Effect of age, exercise and combination of exercise and IGF-1 administration on ubiquitinated protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

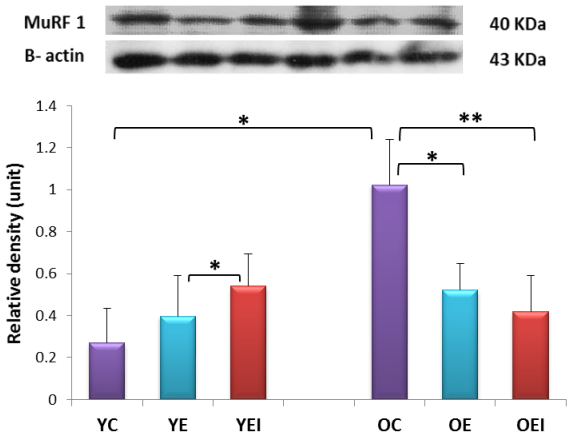

Figure 17. Effect of age, exercise and combination of exercise and IGF-1 administration on MuRF1 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

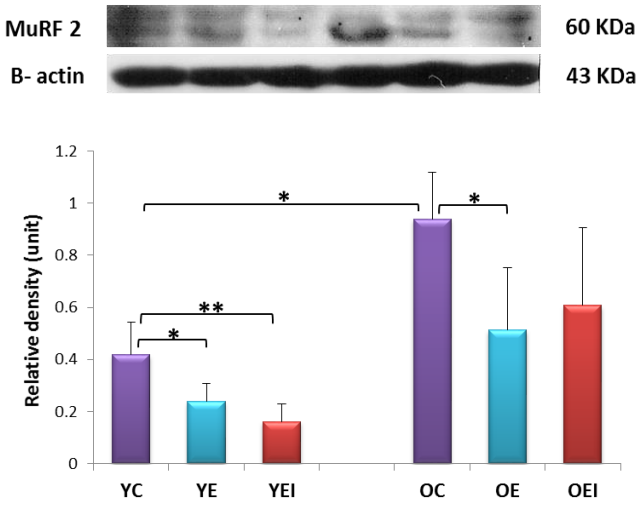

Figure 18. Effect of age, exercise and combination of exercise and IGF-1 administration on MuRF2 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

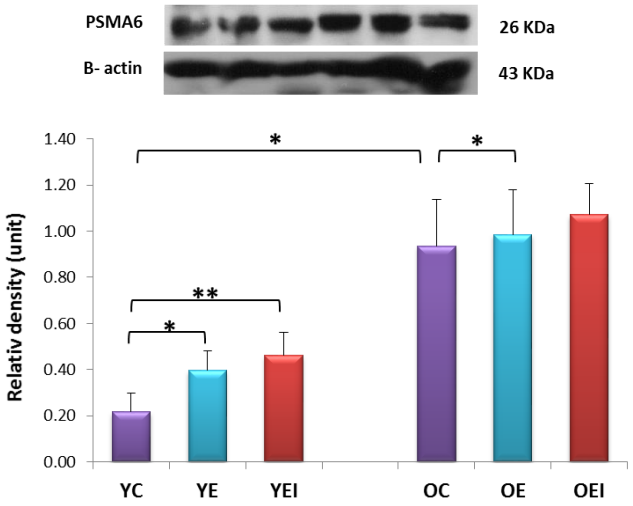

Figure 19. Effect of age, exercise and combination of exercise and IGF-1 administration on PSMA6 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

4.3 Cellular markers involved in mitochondria biogenesis

Loss of mitochondrial functional integrity is centrally involved in muscle degeneration during aging and other atrophying conditions [55]. In particular, oxidative stress-induced damage to mtDNA impairs mitochondrial function, which can lead to further increases in ROS production and exacerbate the intracellular ROS-induced damage [157]. Therefore, we measured the ROS levels and expression levels of SIRT1, PGC-1a, SIRT3, Cyto C, Cox 4 and Nrf2.

OC showed elevated levels ($p<0.05$) of ROS compared to YC. There was no effect of exercise and IGF-1 treatment on ROS level (Figure 20). There was no difference between groups in PGC-1α and SIRT1 protein contents (Figure 21 and Figure 22 respectively). SIRT3 level was significantly lower ($p<0.01$) in OC, while markedly increased SIRT3 protein content was observed in OE and OEI ($p<0.05$ and $p<0.01$ respectively) following exercise and IGF-1 administration (Figure 23). Cyto C and Cox 4 protein contents decreased with age ($p<0.05$) whereas endurance training led to a significant increase ($p<0.05$) of both Cyto C and

Cox 4 in OE (Figure 24 and Figure 25 respectively). Nrf2 was also lower ($p<0.01$) in OC compared to YC, while exercise and IGF-1 treatment resulted an increase ($p<0.05$ and $p<0.01$ respectively) in OE and OEI (Figure 26).

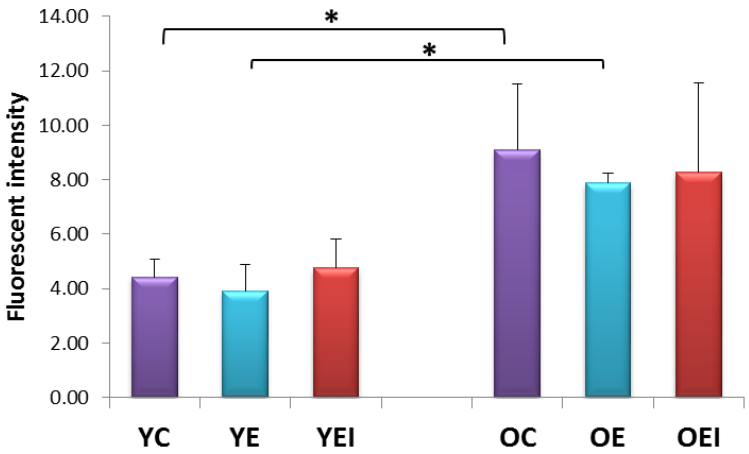

Figure 20. Effect of age, exercise and combination of exercise and IGF-1 administration on ROS level

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

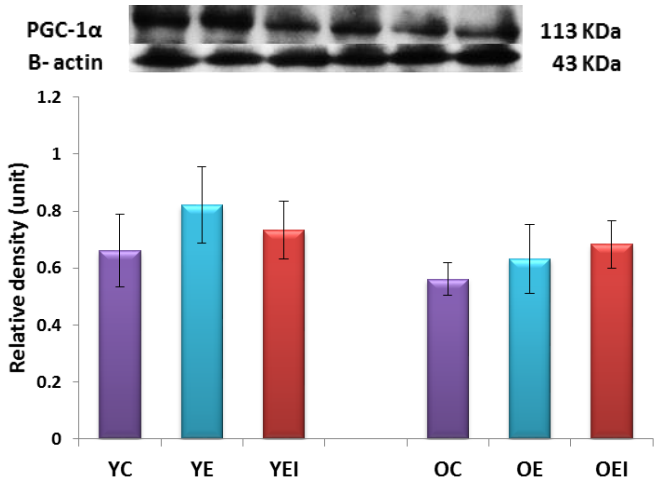

Figure 21. Effect of age, exercise and combination of exercise and IGF-1 administration on PGC-1α protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group.

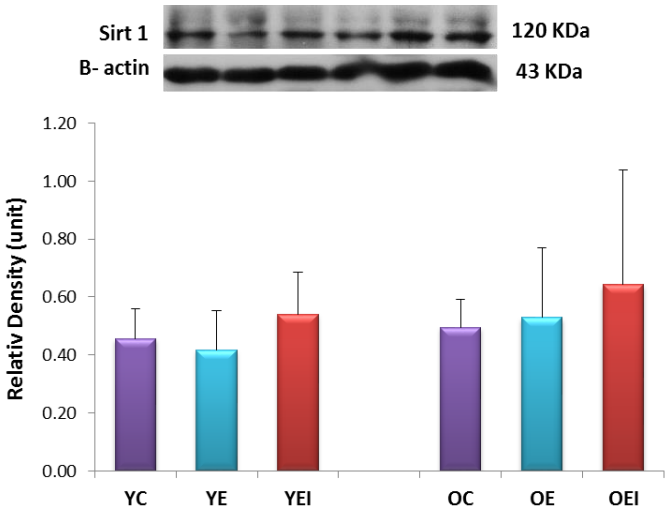

Figure 22. Effect of age, exercise and combination of exercise and IGF-1 administration on SIRT1 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group.

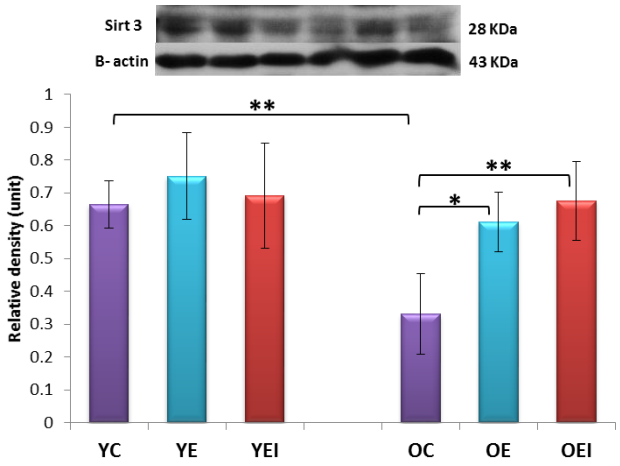

Figure 23. Effect of age, exercise and combination of exercise and IGF-1 administration on SIRT3 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

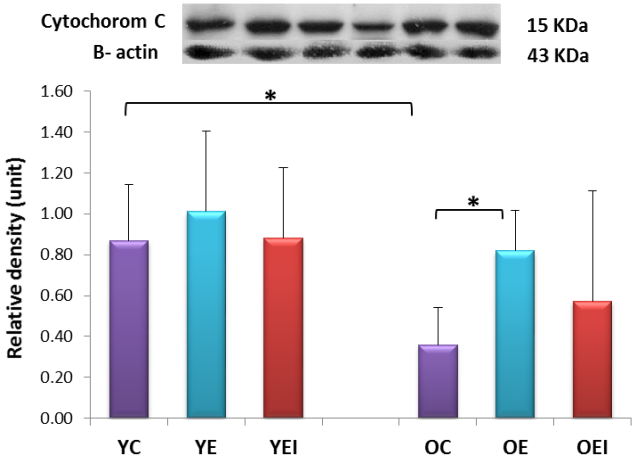

Figure 24. Effect of age, exercise and combination of exercise and IGF-1 administration on Cyto C protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

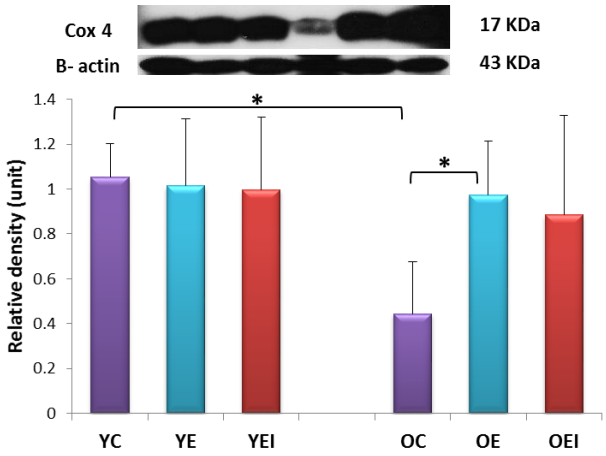

Figure 25. Effect of age, exercise and combination of exercise and IGF-1 administration on Cox 4 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

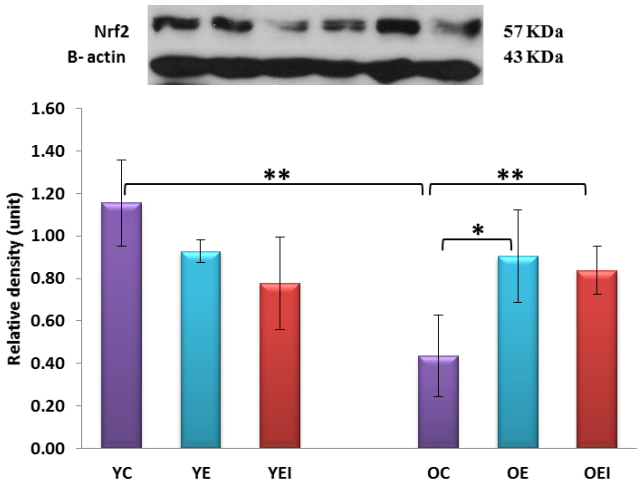

Figure 26. Effect of age, exercise and combination of exercise and IGF-1 administration on Nrf 2 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

4.4 Cellular markers involved in apoptosis

Available evidence supports the hypothesis that excessive myonuclear apoptotic elimination may drive the onset and progression of sarcopenia. The extrinsic pathway is initiated by the interaction of cell surface death receptors (e.g. tumor necrosis factor receptor, TNF-R) with their ligands (e.g. TNF-α) [91]. Recent studies suggest a fundamental role for p53 in organismal senescence. Several mouse models that display chronic p53-activation or chronic cell stress pathway activation display premature aging associated with pronounced tissue atrophy [158]. A critical event in mitochondrial-driven apoptosis is the formation of permeable membrane pores, regulated by the balance between competing anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and pro-apoptotic proteins, including Bax [159, 160]. Therefore, we measured the expression levels of TNF-α, p53, Bcl-2 and Bax. Also the ratio of Bax to Bcl-2 as an apoptotic index was calculated.

There was no effect of age on TNF-α protein content, but administration of IGF-1 led to a significant reduction ($p<0.05$) in OEI vs. OC (Figure 27). p53 was higher ($p<0.05$) in OC than YC. Although there was a tendency of reduced p53 levels following exercise and IGF-1 treatment, however, significant decrease ($p<0.05$) was only observed in YEI (Figure 28). Bcl-2 protein content significantly decreased with aging ($p<0.05$), but IGF-1 supplementation markedly enhanced ($p<0.05$) its levels in OEI (Figure 29). Unlike in old subjects, YE and YEI showed a significant lowering ($p<0.05$ and $p<0.01$ respectively) of Bcl-2 levels in response to exercise and IGF-1 treatment (Figure 29). Bax protein content was obviously higher ($p<0.05$) in OC vs. YC. There was no significant effect of exercise and IGF-1 supplementation, in spite of the tendency for a decrease in OE and OEI (Figure 30). The ratio of Bax to Bcl-2 as an indicator of apoptotic condition inside the cell, however, was significantly higher ($p<0.05$) in OC compared with YC. This ratio markedly decreased ($p<0.05$) following IGF-1 administration in OEI compared with OC (Figure 31).

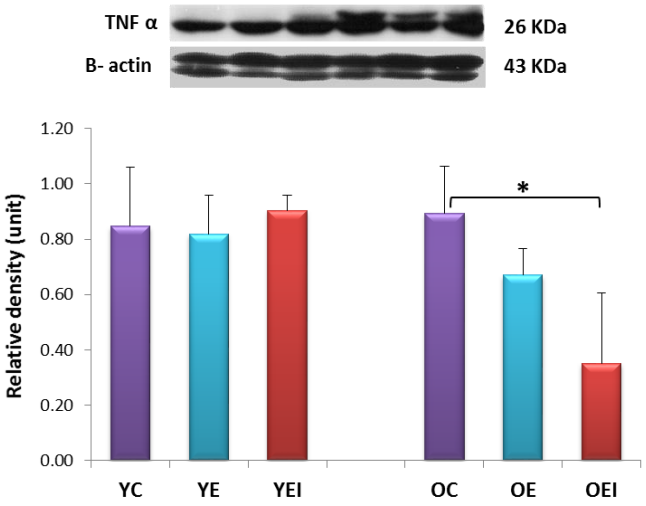

Figure 27. Effect of age, exercise and combination of exercise and IGF-1 administration on TNF-α protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

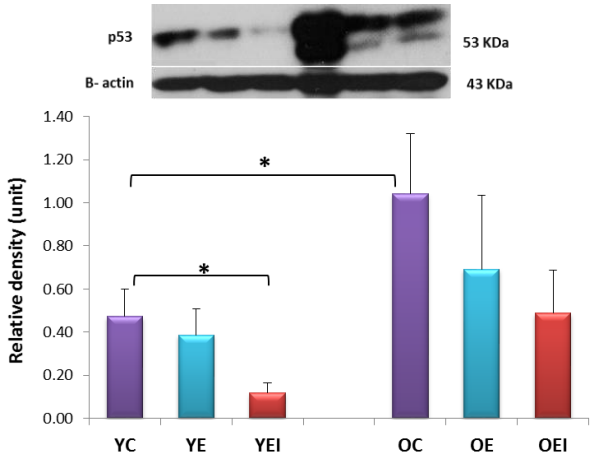

Figure 28. Effect of age, exercise and combination of exercise and IGF-1 administration on p53 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

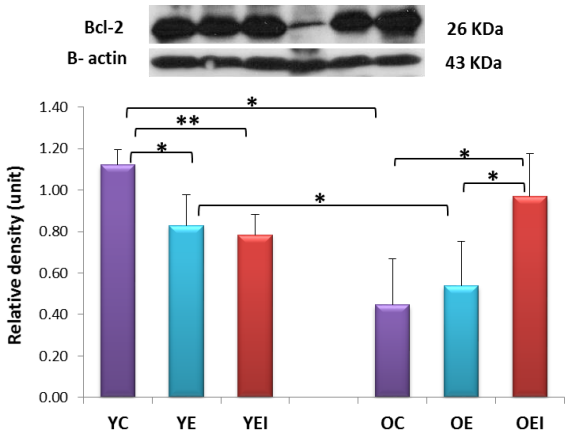

Figure 29. Effect of age, exercise and combination of exercise and IGF-1 administration on Bcl-2 protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

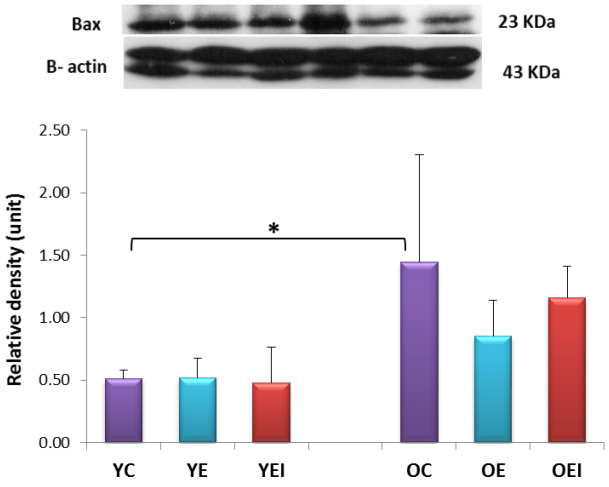

Figure 30. Effect of age, exercise and combination of exercise and IGF-1 administration on Bax protein content

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$.

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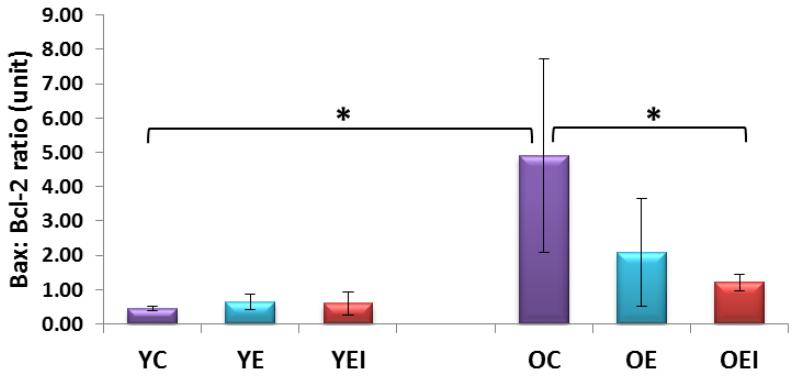

Figure 31. Effect of age, exercise and combination of exercise and IGF-1 administration on apoptotic index (Bax: Bcl-2 ratio)

YC (young control); YE (young exercised); YEI (young exercised IGF-1 treated); OC (old control); OE (old exercised); OEI (old exercised IGF-1 treated). Values are means ± standard error (SE) for 5 animals per group. (*) $p < 0.05$; (**) $p < 0.01$.

5. Discussion

Sarcopenia, often defined as the age-related decrease in lean body mass, has become a topic of significant investigation since it affects so many people, healthy and ill, and utilizes significant medical resources. The direct costs approached $18.5 billion in the United States in 2000; by comparison, the annual cost of osteoporotic fractures in 1995 was $16.3 billion [161]. The precise reasons for sarcopenia are not well understood, however, the general age-related decrease in hormonal and nervous system capacity are proposed as contributors with interactions between many systemic and local factors at the tissue level [162]. The purpose of this study was to examine age-related changes in cellular markers involved in sarcopenia in response to endurance exercise with or without IGF-1 treatment and to highlight some of the potential underlying factors that appear to contribute to the skeletal muscle wasting with aging.

5.1 Aging negatively affected cellular markers involved in sarcopenia

Maintaining muscle mass is achieved by balance between protein synthesis and protein degradation. An increase in muscle mass can come about due to either an increase in protein synthesis or a decrease in degradation, while a decrease in muscle mass can occur as a result of decreasing protein synthesis or increasing protein degradation [80]. Two major signaling pathways control skeletal muscle growth: the IGF1–Akt/PKB–mTOR pathway acts as a positive regulator of muscle growth, and the myostatin–Smad3 pathway acts as a negative regulator [72].

Our result demonstrated that aging was associated with a reduced level of IGF-1 and expression level of follistatin -two important protein synthesis stimulators in mammalian skeletal muscle- in old rat.

IGF-1 acts mainly through three downstream signals that are mediated by PI3K-Akt. Each pathway plays a different role in various aspects of muscle growth. In the first pathway, IGF-1 activates MAPK through PI3K-Akt, which eventually leads to the proliferation of myoblasts and satellite cells. PI3K-Akt-mTOR-P70S6K is the second pathway involving IGF-1 and its signals are transduced mainly through PI3K-Akt and mTOR to P70S6K. In the

third pathway, the activation of Akt inhibits the activities of GSK-3ß, thereby promoting the synthesis of specific proteins [163]. In accordance with our findings, age-related changes in systematic and local IGF and their binding proteins has been reported in human [164-167] and rat [168, 169]. Follistatin is also essential for skeletal muscle development and growth [170] and it is possible that the increased IGF expression might contribute to skeletal muscle hypertrophy induced by follistatin [153]. Hamrick et al. (Hamrick et al., 2012) reported that follistatin levels decreased by ~30% in mouse EDL with age. They then concluded that age-associated loss of muscle mass in the predominantly fast-twitch EDL muscle may be due, in part to declining levels of follistatin [171]. Our result also demonstrated that the amount of Akt and pAkt was higher in old animals, while no effect of aging observed for the amount of mTOR and pmTOR. The phosphorylated and total amount of Akt and mTOR may be varied among muscle types in response to aging. Paturi et al. (Paturi et al., 2010) studied the effects of aging on muscle mass in the F344BN rat model. They compared Akt and mTOR in the slow soleus and fast extensor EDL muscles of 6, 30 and 36-month male rats. Their results were interesting. In soleus muscle the abundance of Akt protein was lower in 36-month-old relative to that observed in 6-month-old animals. However, compared to 6-month-old male animals the amount of p-Akt (Ser 473), p-mTOR (Ser 2448) were 47% and 28% lower and p-Akt (Thr 308) was 38% higher in 36-month-old male animals. In the EDL muscle, relative to 6-month-old male animals, the amount of Akt, mTOR, p-Akt (ser 473) and p-mTOR (Ser 2448) were 38%, 182%, 73% and 91% higher in the 36-month-old male animals [172]. A decrease in IGF-1 mRNA by 45%, along with a 2.5-fold increase in total Akt, but not phosphorylated Akt, has been reported in older males compared to the young subjects [19]. This increase in phosphorylation and expression of Akt protein in muscle seems to be a compensatory response to aging. Akt plays a number of roles that may be important in sarcopenia. These roles can be included at least due to the decrease in apoptosis and protein degradation in skeletal muscle by increasing phosphorylation and inhibition of the pro-apoptotic protein Bad and FOXO transcription factors, respectively [39]. The mTOR signaling pathway is also important for translation initiation and is therefore critical for muscle protein synthesis. One mechanism that activates mTOR signaling is the IGF-1/PI3k/ Akt pathway. Downstream effectors of mTOR signaling include p70s6k, 4E-BP-1, eIF-4E, and S6K [45]. It has been reported that a reduced amount of mTOR, pmTOR, S6 ribosomal protein are present in aged rodents, consequently impairing mTOR signaling and mRNA translation. Therefore, impairment in protein synthesis in aged muscle is evident [173-175]. However, there are some studies, which reported increased amounts of phosphorylated

mTOR and p70S6K in the tibialis anterior and increased level of phosphorylated p70S6K, eukaryotic initiation factor 2 subunit B (eIF2B) activity in gastrocnemius muscle of senescent rats [176, 177]. Our finding showed that there was no difference in phosphorylated and total expression of mTOR protein between old and young subject. Therefore, aging did not commonly modulate the PI3-K/Akt/mTOR-linked molecules in skeletal muscle under sedentary conditions [77], suggesting that other pathways, such as the MAPK signaling pathway should also be considered. MAPK signaling transduction pathway acts in a wide variety of physiological and pathophysiological cellular processes including cell proliferation, differentiation, apoptosis, migration, inflammation, metabolic disorders and diseases. In skeletal muscle, it plays a critical contribution in muscle fiber specialization, muscle mass preservation, damage-induced muscle regeneration and muscle diseases. MAPK pathway consists of at least 4 subfamilies that include ERK 1/2, p38a/ß/?/ d MAPK, JNK 1/2/3, and ERK5 [178]. The ERK1/2 pathway is involved in activation of several substrates, such as p90 ribosomal S6 kinase (p90RSK), leading to the activation of transcription factors. ERK1/2 can also activate kinases associated with protein translation such as Mnk 1 (MAPK-interacting kinase 1) and its downstream substrate, eukaryotic initiation factor 4E (eIF4E) [39]. We found that baseline content of ERk1/2 was lower in aged rats whereas no difference was observed in pERK1/2 between the young and old groups, however, activity of ERK1/2 (pERK1/2: ERK1/2 ratio) was higher in aged rats compared with their young counterparts. Recently, a study investigated activation and total protein content of MAPK signaling cascade proteins (ERK 1/2, p90RSK, Mnk 1, eIF4E, p38 MAPK, JNK/SAPK, and MKP 1) at rest and following exercise in sedentary young and old men. The results demonstrated a higher baseline levels of ERK 1/2, p90RSK and Mnk 1 in the old men compared with young counterpart [179].

Among the several pathways which are participated in the pathogenesis of muscle mass maintains, MAPKs is considered for having an important role. MKP-1 is a phosphatase which by dephosphorylating acts as an inhibitor for MAPKs. It has been found that overexpression of MKP-1 in skeletal muscle fibers induced profound muscle fiber atrophy possibly through the ubiquitin-proteasome pathways. This anti-atrophic effect of MAPKs may be through ERK1/2 signaling pathway. It is believed that ERK1/2 pathway counteracts muscle wasting through enhanced protein synthesis by its control of ribosomal RNA gene expression [178]. These findings may explain the higher baseline levels of ERK 1/2 old rat

from the current study, possibly as a compensatory response of skeletal muscle along with aging.

As mentioned above, age related increased in protein degradation in skeletal muscle plays an important role in sarcopenia [77, 87]. We found much higher level of Myostatin, ubiquitination, MuRF1, MuRF2 and proteasome subunit PSMA6 expression in old subjects compared with young ones. Our finding are in line with previous studies which reported increasing TGF-ß/myostatin pathways [19, 135, 180-182] and ubiquitin proteasome system activity [100, 177, 183, 184] in old humans and animals. Myostatin and TGF-ß are generally located in an inactive form in the muscle extracellular matrix and after activation can bind to their receptors resulting in activation of the Smad2/3 and TAK1/p38 MAPK signal transduction cascades. Smad2 and Smad3 are transcription factors which are able to bind DNA and directly regulate the expression of target genes. Smad2/3 can also bind to the FOXO family to regulate gene expression. In addition, myostatin signaling can suppress the IGF-1/PI3K/Akt axis and reduce p70S6K activation [80].

The UPS is mostly responsible for the degradation of misfolded proteins, as well as long-lived proteins. The substrate specificity of the ubiquitin conjugation cascade is mediated by hundreds of E3 ubiquitin protein ligases. MuRF proteins MuRF1, -2, and -3 comprise a subfamily of the RING-finger E3 ubiquitin ligases that are expressed specifically in cardiac and skeletal muscle. The main target of MuRF1 is titin at the M-band of the sarcomere that has an important role in maintaining the stability sarcomeric M-line region. MuRF2 can also bind to the titin kinase domain and is contributed in the serum response factor signal transduction pathway [185]. Since it has been demonstrated an enhanced proteolysis by the UPS in aged skeletal muscle, which may enhance their capacity to eliminate misfolded proteins, hence they appear to be involved in the sarcopenia [183]. Nevertheless, it should be noted that not all studies have reported significant age-related differences in Myostatin and MuRF1in protein and mRNA expression at baseline [19, 186-188]. One possible reason for these inconsistency can be due to differences in the age of the subjects between the studies that can be related to the degree of muscle mass loss, because individuals >80 years old have a greater prevalence of sarcopenia, and more severe muscle atrophy compared to individuals only a decade younger [39].

There are a number of lines of evidence supporting the hypothesis that mitochondrial dysfunction is a characteristic of human aging in skeletal muscle [51]. The mechanism for

age-associated decline of mitochondrial biogenesis and dysfunction is still unknown and under intense investigation [131]. Currently, it is generally accepted that ROS play a primary role in the aging process, mitochondrial production of ROS has been shown to increase in skeletal muscle along with increasing age [2, 27]. Excessive production of ROS has been shown to be a key signal for the onset of several musculoskeletal diseases [189]. Old animals in our study showed much higher level of ROS compared to young animals. ROS also stimulate negatively the mitochondrial biogenesis [68]. These include decreased mitochondrial biogenesis and turnover, and oxidative damage to mitochondrial enzymes, structural proteins and membrane lipids. It has been suggested PGC-la plays a critical role in age-related reduction of mitochondria biogenesis [93].

However, there was no significant change in PGC-1a protein content between old and young rats in our study. This finding can be supported by the notion that overall levels of PGC-1a protein did not change with age [190]. In other hand, however, the changes in mitochondrial energy metabolism may be due to a decline in PGC-1a activity with age [191]. In contrast to our result, Kang et al, reported PGC-1a mRNA expression and protein content decreased by 35% and 19% ($P < 0.01$ and $P < 0.05$ respectively) in old rats compared to young rat rats [131]. Koltai et al. also found a lower amount of PGC-1a in old rats than young ones [192]. Different muscle types that were used in these studies (soleus) [131, 192] compared to our study (vastus lateralis) can explain, at least in part, this difference observed for PGC-1a protein content in responses to aging. Previously it has been shown that type II fibers are vulnerable to age-associated atrophy in humans and in non-human primates, while type I fibers generally are not. These data suggest that fibers of distinct structural and metabolic profiles are differentially impacted by age [190]. Among the upstream enzymes and transcription factors known to control PGC-1a gene expression and activity, such as AMPK, p38MAPK, SIRT1 and CREB [131], we found that SIRT1 level did not show a significant age-related change, thus, its unchanging expression could be a reason for the lack of difference in PGC-1a protein level between old and young age in current study. In support of our findings, Kang et al. [131] reported that there was no difference in expression levels of SIRT1 in muscle of old vs. young rats. In our study we did not measure SIRT1 and PGC-1a activity, however, pervious study reported that levels of nicotinamide phosphoribosyltransferase (NAMPT) were lower in tissue from old animals. NAMPT is a key enzyme in the NAD salvage pathway that positively regulates SIRT1 activity in skeletal muscle. A decline in NAMPT would be predicted to lower SIRT1 activity, which would

negatively influence PGC-1a localization and activity [190]. SIRT1 deacetylates and activates PGC- 1a [193], while SIRT3, another one of mammalian Sirtuins, which is localized to mitochondria plays a major role in deacetylating and modifying the enzymatic activities of several mitochondrial proteins [194]. In agreement with previous studies [193, 195, 196], we found a significant reduction of the protein content of SIRT3 in aged rats compared to younger ones. SIRT3, a mitochondrial NAD+-dependent deacetylase, has been shown to play a crucial role in controlling cellular ROS homeostasis [197]. SIRT3 deacetylates and activates many mitochondrial enzymes involved in fatty acid ß-oxidation, amino acid metabolism, the ETC, and antioxidant defenses [198]. Interestingly, this decreased levels of ROS and SIRT3 in our study was associated with a remarkable reduction in Cyto C and Cox 4 in old subjects. In consistent with our results, data reported from a study on 146 healthy men and women aged 18–89 years demonstrated that mtDNA and consequently abundance of mRNA for Cox 4, which is encoded by mtDNA and nuclear DNA, declined with advancing age [199]. Kontani et al. [200] Found that Cox 4 protein level was also reduced considerably in the aged gastrocnemius muscles with atrophy [200]. Moreover, COX activity, an established biochemical indicator of mitochondrial volume, was 30% lower ($P < 0.05$) in the muscle from old, compared to young animals [201]. On the other hand, our further measurements showed that Nrf2, a nuclear transcription factor that activates the proximal promoter of the rat Cox 4 gene significantly decreased with aging [202]. Taken together, our findings support age related mitochondrial dysfunction in the old rat.

Apoptosis have also been shown as a potential mechanism involved in sarcopenia [43, 91, 118]. Oxidative stress, chronic inflammation, and impaired insulin sensitivity seems to be potential candidates for the activation of myonuclear apoptosis at old age [43]. Proteolytic enzymes, known as caspases, perform the dismantling of the cell and are normally present as inactive zymogens (procaspases). Upon appropriate stimuli, initiator caspases (i.e., caspase-8, caspase-9, caspase-12) are activated, leading to the activation of effector caspases (i.e., caspase-3, caspase-6, caspase-7) responsible for the cellular degradation and DNA fragmentation via a caspase-activated DNase (CAD) [31]. Two major pathways of caspase activation are distinguished based on the extrinsic or intrinsic origin of the death-inducing stimulus. The extrinsic pathway is initiated by the stimulation of cell surface death receptors (e.g. TNF-R) by their ligands (e.g. TNF-α). The intrinsic pathway is activated through the triggering signaling from mitochondria or the endoplasmic reticulum [91]. The release of apoptotic triggers appears to be modulated through two mechanisms: (1) the balance of

proapoptotic (e.g., Bax) and anti-apoptotic proteins (e.g., Bcl-2), particularly from the Bcl-2 family, which control OMM stability and form the mitochondrial apoptosis-induced channel (MAC), and (2) the mPTP [52]. The balance between these mediators (e.g., Bax-to-Bcl2 ratio) is considered a fundamental control point for the cell fate by regulating OMM stability as Bax promoting mPTP opening, while the antiapoptotic Bcl-2 possess an inhibitory effect [31]. The mPTP opening then results in the release of apoptotic factors that are stored in the intermembrane compartment [93]. In addition, p53 can also promote apoptosis via inducing Bax activity [203-205].

Our result demonstrated that a significant increase in p53 protein content was associated with a remarkably decreased Bcl-2 and a significant increase of Bax protein expression in old vs. young subjects. Consequently, cell antiapoptotic ability (Bax to Bcl-2 ratio) was also lower in the aged rats. However, no age-related difference was found in TNF-α protein level. Increased p53 content observed in the current study would be probably due to much higher levels of ROS in aged rats compared to young ones.

Recent studies have indicated that cellular concentration and distribution of p53 has different cellular function, and ROS can act as an upstream signal that triggers p53 activation [206]. In agreement with our findings, a number of studies reported increased expression of Bax and reduced levels of Bcl-2 in the skeletal muscle of old rodents [55, 98, 207, 208]. In contrast, however, some investigators have found that Bcl-2 family proteins increased in old muscles [209, 210]. These elevations of Bcl-2 detected in aged muscles could be interpreted as a compensatory response to aging. It was demonstrated that increased expression of Bcl-2 in the gastrocnemius muscle of old mice was paralleled by enhanced serine-phosphorylation and subsequent inactivation of Bcl-2, which prevented its anti-apoptotic actions in spite of the elevated expression [55].

5.2 Endurance training positively reversed some cellular markers involved in sarcopenia

Physical inactivity is a significant contributing factor to age-related sarcopenia. It is well established that sedentary elderly have less skeletal muscle mass and high prevalence of disability [4]. Exercise training is considered as a simple, feasible, and inexpensive strategies available to prevent the onset of sarcopenia and reduce the rate of functional decline [46].

Previous studies have clearly shown the effectiveness of resistance-type exercise interventions on skeletal muscle mass and functional capacity in the elderly [211-217]. In addition, endurance type exercise training has been shown to improve muscle mass and strength, and increase performance capacity in both the young and elderly [75, 103, 126, 128, 218-223].

The data from our study showed that 6 weeks endurance exercise training did not lead to significant changes in the level of IGF-1 and protein content and phosphorylated of Akt, pAkt, mTOR, pmTOR, pERK1/2, but significantly increased the amount of follistatin and decreased pERK1/2 in the old exercise group compared to old control.

Number of studies found an increased size and contractile properties of old slow (MHC I) and fast (MHC IIa) myofibers, following endurance training [128, 220, 224]. One study reported that 12 wk of cycle ergometer training increased MHC I fiber size 16 +/- 5% and MHC I peak power 21 +/- 8% while MHC IIa peak power was unaltered [220]. In one other study, 78 healthy, previously untrained men and women aged 19-87 years were evaluated before and after 4 months of bicycle training or control (flexibility) activity. They found that mixed muscle protein synthesis declined with age at the whole body level at the rate of 3.5% per decade. Exercise training improved overall aerobic capacity 9%, while mixed muscle protein synthesis increased by 22%. This study also demonstrates that aerobic exercise can enhance muscle protein synthesis irrespective of age [222]. However, the mechanism(s) by which endurance exercise affects aged skeletal muscle remain poorly understood. A number of studies have examined the effect of endurance training on IGI-1 and IGF-1 binding proteins among older subjects [45, 125, 129, 225]. Poehlman et al. studied 8 weeks cycling exercise, three times per week at 75% of their Vo2max, on older individuals. They found that endurance training significantly increased fasting levels of IGF-1only in men ($r = .79$, $P < .02$), while there was no mean group change in IGFBP-1 or IGFBP-3 [129]. A comparison between sedentary middle-aged (MAsed) and active middle-aged (MAcy)- almost 12 hours of cycling per week for the past 11 ±1.4 (SE) years- showed that basal IGF-1, IGFBP-1, and IGFBP-3 were higher (61%, 127%, and 21%, respectively, $P <0.05$) in MAcy than in MAsed [225]. In addition, an increased activity of the GH/IGF-1 system by endurance training in middle-aged men has also been reported [125].

However, there are some documents which reported that endurance training did not lead to remarkable changes in muscle mass, maximal strength, and power [112, 127, 221, 223].

Possible explanations for this difference could be due to differences in age (middle-age vs. aged) and gender (men vs. women) of the subjects, and especially the various exercise training protocols used (i.e., intensity and duration). For example, 2 weeks of endurance training at moderate intensity did not increase IGFBP-3 in an aged sedentary population, whereas specific, intense training in elite athletes performed over several months increased this binding protein [225]. In accordance with our results, Gielen et al. [127] found that 4 weeks endurance training on treadmill did not change muscle mRNA levels of IGF-1 in elderly men. Also, LeBrasseur et al. [112] reported that 4 weeks treadmill exercise training did not change Akt protein abundance and phosphorylation of Akt relative to total in aged muscle of old mice. Furthermore, Pasini et al. [103] investigated the effects of treadmill exercise and training frequency on anabolic pathways in skeletal muscle of old rats. Aged male Wistar rats were trained on a treadmill for 3 (EX3) or 5 days/week (EX5) during 8 weeks and compared with age-matched sedentary controls (SED). Compared with SED rats, the expression of p-mTOR was unaffected by EX3. However, EX5 up-regulated p-mTOR expression [103]. Taken together, these data suggest that IGF-1/Akt/mTOR pathway is not the main target of endurance exercise training and its effects on skeletal muscle protein synthesis probably occur through other pathways involved in sarcopenia. In this regard, we found that follistatin protein amount significantly increased in old rats after endurance training compared with control.

Follistatin is expressed in different tissues and acts as an antagonist of different family members of TGF-ß [82]. Increased protein synthesis can also be due to decrease in the protein breakdown as a result of inhibitory extracellular binding proteins, such as follistatin, whose effect is even greater than the lack of myostatin [72]. In agreement with our result, Hansen et al. [124] found that endurance exercise induces increased levels of follistatin in the circulation. The kinetics revealed that plasma follistatin increased markedly during the recovery after exercise both in humans and in mice. The increase in plasma follistatin after exercise appears to be dependent on both the intensity and duration of exercise. Thus, 3 h of bicycling exercise induced a 7-fold increase in plasma follistatin, whereas 2 h of one-legged knee extensor exercise only increased plasma follistatin by 2-fold. The exercise-induced increase in follistatin may also be dependent on the muscle mass recruited during the exercise bout [124].

Sarcopenia is the result of imbalance between protein degradation and synthesis, Although the exact contribution of each of these factors is unknown, however it is believed that the

increase in muscle proteolysis associated with aging can make a significant contribution in the development of muscle degradation [90]. Unlike protein synthesis, a larger number of studies have examined the effects of Endurance Training on the mechanisms involved in protein breakdown in aged-skeletal muscle and conflicting results have been reported [112, 126, 127, 226, 227].

The results of our study revealed that endurance training significantly decreased protein expression of MuRF-1 and Murf-2 and increased PSMA6 levels in aged rats, while no change was found in Myostatin and ubiquitination levels. Similar reduction in MuRF-2 content and elevation in PSMA6 level has been seen in young rats following training.

Our results are in line with previous studies, which demonstrated reduced levels of mRNA and protein expression of MuRF-1 [112, 127] and no changes in myostatin [127] following endurance exercise in old humans and animals. The effect of myostatin is mediated by the transcription factors Smad2 and Smad3, which also interact with IGF1-Akt signaling [63]. LeBrasseur et al. also found that Smad3 protein abundance did not change following 4 weeks endurance training in old mice [112]. Nevertheless, in contrast, some studies have reported reduced levels of myostatin mRNA and protein expression in response to endurance exercise [126, 226, 227]. One possible reason for these conflicting results could be due to differences in muscle fiber type used in these studies. For example, Ko et al. [227] showed that treadmill exercise improved muscle mass and strength through suppression of myostatin mRNA and protein expression in the gastrocnemius (versus vastus lateralis muscle in our study). Protein synthesis and muscle adaptation are regulated differently with aging in different muscle types [172] and signal transduction protein concentrations vary between fast and slow muscles [228]. To our knowledge, this is the first investigation to report significant reduction in protein content of MuRF-2 in aged skeletal muscle in response to endurance training. It has been indicated that combined inhibition of MuRF1/MuRF2 can lead to stimulation of striated muscles anabolism and protestation muscles from sarcopenia during aging [155].

Aging is characterized by a progressive deterioration in aerobic exercise capacity and this attenuation in cardiovascular efficiency may be linked to reduced quantity or quality of skeletal muscle mitochondria [122]. Endurance training can correct the age related decline in enzyme activities or protein content in older individuals [53]. Muscle mitochondrial adaptations to aerobic training appear to be the result of exercise-induced increases in the transcription of mitochondrial genes [122].

The findings of our study revealed that 6 weeks endurance training significantly increased protein expression of SIRT3, Cyto C, Cox 4 and Nrf2 while there was no effect of exercise training on ROS levels and the amount of SIRT1 and PGC-1α.

Among the upstream enzymes and transcription factors known to control PGC-1α gene expression and activity, such as AMPK, p38MAPK, SIRT1 and CREB [131], we found that SIRT1 levels did not show a significant change in response to exercise training. Thus, this lack of response could be a possible reason for the unchanged PGC-1a protein expression with endurance training at old age. SIRT1 is known to activate PGC-1a by deacetylation, and SIRT1/PGC-1a work as regulatory axis to control mitochondrial function during aging [229]. Previous studies have reported conflicting results regarding the effect of endurance training on PGC-1a activity and expression in old human and rodents. In agreement with our result, findings of LeBrasseur et al. [112] Showed that 4 weeks endurance training did not change PGC-1a protein expression in 24-month-old male mice. In contrast, 12 weeks endurance training showed a 2.3 and 1.8-fold higher PGC-1a content in old exercised than old and young control rats, respectively ($P < 0.01$) [131]. Moreover, after 12 weeks of aerobic exercise training on a cycle ergometer in older women, PGC-1a protein content was 20 ± 5% lower ($p < 0.05$) [126]. A possible explanation for these conflicting results could be due to, at least in part, differences in muscle types that have been examined in these studies (e.g. soleus muscle [131] vs. vastus lateralis muscle in [126] and our study). Skeletal muscle fibers are classified into three types: type I, type IIa, and type IIb [230] and PGC-1α is expressed preferentially in muscle enriched in type I fibers [231]. Coactivation of PGC-1a induces Nrf1 and 2, which promote the expression of most nuclear-encoding mitochondrial proteins, as well as Tfam that directly stimulates mitochondrial DNA replication and transcription [131]. Nrf2 downstream signaling are believed to be involved in redox homeostasis preservation and protection of the structure and function of skeletal muscle [189]. Nrf2 is regarded as a master regulator of antioxidant transcription and binds to the antioxidant response element (ARE) in the promoter of target antioxidant genes and tightly regulates its transcription [232]. Furthermore, SIRT3 activity can reduce ROS levels by directly modulating key antioxidant enzymes, thereby acting as a shield against oxidative damage. SIRT3 exerts its antioxidant effects in an interaction with manganese superoxide dismutase (MnSOD) and isocitrate dehydrogenase 2. MnSOD is the primary mitochondrial antioxidant enzyme that converts O•-2 to H2O2, which is further converted to water by catalase. The ability of MnSOD for scavenging ROS in mitochondria can be significantly enhance due to directly deacetylation

by SIRT3 [198]. In line with previous studies [233-236], our results demonstrated that protein contents of both Nrf2 and SIRT3 significantly increased, suggesting an adaptive response in the intracellular antioxidant system following endurance training in old rats. Indeed our findings showed improved mitochondrial biogenesis due to remarkably increased Cyto C and Cox 4 in old exercised rats. Kang et al. reported that a treadmill running program for 12-weeks resulted in 1.4-fold increase in Cyto C protein content ($P < 0.05$) in old trained vs. old sedentary rats [131]. Indeed, 12 weeks of aerobic exercise training on a cycle ergometer in nine older women (70 ± 2 years) demonstrated that Cox 4 was elevated 33 ± 7% after training, suggesting that the training program resulted in mitochondrial biogenesis [126]. Moreover, an increased muscle protein content of Cox 4, the marker of mitochondrial biogenesis, has been reported in response to endurance training among old humans and rodents [56, 103, 121]. Taken together, our result indicates that endurance exercise training improved mitochondrial biogenesis in old rats.

In connection with the factors involved in sarcopenia, accumulating evidence suggests that enhanced activation of apoptosis takes place in aged skeletal muscle, likely contributing to the development of sarcopenia [93]. In general, aging is associated with increased mitochondrial dysfunction and pro-apoptotic signaling through the mitochondrial Bcl-2 pathway. The ratio of pro- to anti-apoptotic Bcl-2 family proteins (e.g., Bax/Bcl-2) can be used as an indicator of cell apoptosis which is involved in myonuclei integrity and cell survival by controlling mitochondrial membrane permeability and activation of caspases. Exercise training is well known to convey benefits across a spectrum of biological processes, including adaptations in apoptotic pathways [237]. A number of studies have investigated the effect of endurance training on cellular apoptotic markers in old subjects [61, 98]. One study found that twelve weeks of treadmill exercise training increased anti-apoptotic Bcl-2, while markedly reduced Bax, and Bax/Bcl-2 ratio in the white gastrocnemius and soleus muscles of old rats [98]. However, in contrast, our results indicated that six weeks of treadmill exercise training did not change the protein contents of TNF-α, p53, Bcl-2, Bax and Bax/Bcl-2 ratio in the vastus latralis muscles of old rats. In addition to differences in the duration of the studies (12 weeks [98] instead 6 weeks in our study), another important potential factor for these inconsistent results may be due to the differences in muscle fiber types used. In this regard, Marzetti et al. [61] reported that type I muscle fibers, like soleus, are less susceptible to age-associated apoptosis than type II fibers and therefore less likely to be affected by short-term

exercise training. However apoptotic potential is elevated in type II muscle fibers at old age and may be attenuated by interventions, such as life-long CR and ET [61].

In summary, our results indicated that 6 weeks endurance training positively regulated some cellular sarcopenic markers by reducing proteins involved in muscle proteolysis and increasing some proteins involved in mitochondria biogenesis in the vastus latralis muscle of old rats.

Changes in fiber-type-specific myosin isoform and in mitochondrial energy metabolism point to PGC-1α regulated pathways in the metabolic transition at mid-age. Although overall levels of PGC-1α protein did not change with age, the changes in mitochondrial energy metabolism are consistent with a decline in PGC-1α activity with age. Immunohistological detection of PGC-1α indicates that its localization to the nucleus is impaired with age, suggesting a possible mechanism for diminished PGC-1α activity. The NAD-dependent deacetylase SIRT1 is an activator of PGC-1α [238-240] that can also regulate PGC-1α cellular distribution [241]. The lower NAD/NADH ratio detected in mid-age is predicted to negatively influence SIRT1. Attempts to measure levels of SIRT1 in tissue homogenates were not successful; however, levels of NAMPT were lower in tissue from old animals. NAMPT is a key enzyme in the NAD salvage pathway that positively regulates SIRT1 activity in skeletal muscle in mice [242]. A decline in NAMPT would be predicted to lower SIRT1 activity, which would negatively influence PGC-1a localization and activity. The finding that subsarcolemmal mitochondria were more sensitive to the impact of age is of interest because this population of mitochondria is known to be most responsive to changes in PGC-1α activity [190, 243].

5.3 Endurance training combined with IGF-1 administration positively affected some cellular markers involved in sarcopenia

Our third study question was about the effect of combination of endurance training and IGF-1 treatment on some cellular markers involved in sarcopenia. To the best of our knowledge, our study is the first, which has studied the effects of combined endurance training and IGF-1 treatment on skeletal muscle cellular markers of sarcopenia in old rats. Just recently, one study examined the effect of IGF-1 expression within skeletal muscles with or without exercise on the prevention of sarcopenia [148]. They used four-month-old male transgenic

mice that were assigned to be sedentary, or had access to free-running wheels, until 18 or 28 months of age. They found that in wild-type mice, the mass of the quadriceps muscles was reduced at 28 months, but exercise rescued such loss, without affecting the CSA of the myofibers. In contrast, they reported that elevated IGF-1 level alone was insufficient, while the combination of exercise and IGF-1 was augmentative in maintaining the diameter of myofibers in the quadriceps. Their findings showed that exercise and IGF-1 had a mild effect on reducing aged-related skeletal muscle loss, but there is no improvement in muscle function when assessed by grip strength [148]. However, previous studies investigated the effects of combined endurance training and administration of testosterone [66], growth hormone [244, 245], fresh red orange juice (ROJ) [246], melatonin [247], resveratrol [248] , 17 beta-estradiol and perindopril [249], and caloric restriction [250] on aged skeletal muscle in humans and rodents.

We found that two weeks of IGF-1 administration significantly increased serum IGF-1 levels in OEI compared to OC. Consequently, this lead to a significant increase in protein expression of follistatin, mTOR and pmTOR and reduction in pERK1/2 following combination of endurance training and IGF-1 supplementation in OEI vs. OC. This activation of protein synthesis pathway was along with a remarkable reduction in the protein content of MuRF-1.

In agreement with our results, Guo et al. [66], reported that testosterone injection plus low intensity physical exercise training (T/PT) or vehicle plus physical training (V/PT) for 2 months increased mRNA expression of IGF-1 and FGF21, while reduced mRNA expression of MURF1 and MAFbx in skeletal muscle (triceps) in old mice [66]. It is well known that in addition to stimulating protein synthesis and hypertrophy, IGF-1 also inhibits protein breakdown [62]. The exact mechanism by which increased IGF-1 levels lead to a reduction in the level of MuRF1 is currently unclear. However, it was shown that binding of IGF-1 to its receptor induces a conformational change in the IGF-1 receptor tyrosine kinase, resulting in a multiple auto-phosphorylation cascade. As a consequence, PI3K is activated resulting in Akt phosphorylation. Activation of PI3K/Akt, in turn inhibits FOXOs activity. Consequently, Inhibition of translocation of FOXOs into the cell nucleus then suppress transcription of the atrogin-1 and MuRF1 [97]. Interestingly, we found that protein expression of follistatin and total mTOR corresponded with a significant reduction in MuRF1 protein amount in OEI vs. OC. In this regard, it is suggested that in addition to the AKT/FOXO-1 pathway, mTOR also blocks MuRF-1 and MAF box transcription [62]. This inhibitory effect of mTOR can be

mediated by follistatin while these effects were attenuated by the inhibition of mTOR or the deletion of S6K1/2. Furthermore, Smad3 is an important intracellular regulator that is able to mediate the effects of follistatin on mTOR signaling. In fact, Smad3 can prevent skeletal muscle growth through suppression follistatin downstream signaling. Interestingly, follistatin can regulate Smad3- and mTOR activity independent of myostatin [170]. Other mechanisms that mediate the process of apoptosis is by IKK, which phosphorylates IRS-1 on serine 307 to reduce IGF-1 stimulated signaling [62]. NF-κB transcription factors are expressed in skeletal muscle and are activated by inflammatory cytokines, particularly TNF-α. The increase in the TNF-α level induces activation of an IKKß complex that phosphorylates IκB, resulting in its ubiquitination and proteasomal degradation [72]. It has been demonstrated that IKK deletion is associated with increased activity of AKT and P70S6K, along with increased protection against atrophy. Cytokines activate NF-κB signaling which can directly attenuate IGF-1 stimulated protein synthesis; this NF-κB activation enhances muscle atrophy and upregulates MuRF-1 [62]. In this regard, we observed that two weeks of IGF-1 administration combined with 6 weeks endurance training markedly decreased TNF-α and MuRF-1 protein contents and elevated IGF-1 levels in OEI compared with OC.

The most remarkable result to emerge from our data is that protein expression of Bcl-2 was significantly higher in OEI than in OC and OE. Furthermore, Bax to Bcl-2 ratio as an apoptotic index was significantly lower in OEI vs. OC.

Alterations of the mitochondrial Bcl-2 family pathway may be a potential mechanism leading to apoptosis in aging skeletal muscle [98]. The Bcl-2 gene family regulates the apoptotic process through the balance of pro-apoptotic (Bax, Bcl-XS) and anti-apoptotic products (Bcl-2, Bcl-XL) [251]. The ratio of pro- to anti-apoptotic proteins (e.g., Bax/Bcl-2) regulates myonuclei and cell survival by controlling mitochondrial membrane stability. Decreased mitochondrial membrane stability and increased pore formation initiate the release of Cyto C, formation of the apoptosome, catalyzed by Apaf-1 (apoptotic protease activating factor-1), and followed by the cleavage and activation of caspase-9 and caspase-3 [98]. The mechanism by which IGF-1 protects cells from apoptosis is not yet completely understood. However, it has been argued that IGF-1 increased the phosphorylation of the pro-apoptotic factor Bad and the levels of the anti-apoptotic protein Bcl-2 [252]. In fact, interaction of the IGF-1R with IRS-1 activates PI3-ki, which in turn activates Akt/ PKB. The concluding step is the phosphorylation, by Akt/PKB, of BAD, one of the members of the Bcl-2 family of proteins [253].

Our result also demonstrated that protein contents of SIRT3 and Nrf2, but not SIRT1, PGC-1a, Cyto C and Cox 4, were significantly increased in OEI compared with OC.

Indeed critical roles of SIRT3 and Nrf2 in mitochondrial biogenesis have been described [254] and both of them play an important role in antioxidant response element signaling pathway [255, 256]. As mentioned before, elevated levels of SIRT3 and Nrf2 proteins could be the result, at least in part, of adaptive responses to endurance training. However, IGF pathways can also contribute to mitochondria biogenesis and function. In this respect, Guo et al. [66] reported that increased mRNA expression of IGF1 and FGF21 following 2 months of testosterone injection combined with low intensity physical exercise training led to increased mitochondrial DNA copy number and expression of markers for mitochondrial biogenesis [66]. In support of this notion, it has been shown just recently that suppression of IGF-1 and mTOR contributes to impairments in mitochondrial biogenesis and function in aging G3 mTerc-/- mice (telomerase RNA component knockout) [257].

In summary, our findings demonstrated that 6 weeks endurance training combined with 2 weeks IGF-1 administration positively affected some cellular markers involved in sarcopenia via increased IGF-1 levels and activation of its downstream pathways.

6. Conclusions

In conclusion, our findings demonstrate that aging is associated with activation of signaling pathways involved in sarcopenia. Based on our study, it seems that among cellular factors that are responsible for protein synthesis and degradation, increased activity of proteolysis signaling, such as FOXO and apoptosis are contributed more to the onset and progression of sarcopenia than decreased anabolic pathways.

In regard to our finding, short term ET may attenuate sarcopenic conditions in old rats. This protective effect of ET seems to be due to, at least in part, improvement of mitochondria biogenesis and function as well as reduced levels of factors involved in protein degradation, such as MuRF1, in aged skeletal muscle.

According to the results of this study, combination of ET and IGF-1 administration can enhance the effects of ET alone on sarcopenic rats. This reinforcing effect of IGF-1 is likely to be through the modulation of activator and inhibitor factors of apoptosis such as TNF-α and Bcl-2, respectively.

However, in order to achieve the best combination therapy it is essential for future researches to examine the impact of the intensity and duration of ET, as well as that of different doses of the hormone at various level of sarcopenia in both old female and male subjects.

7. Summary of thesis

7.1 Summary in English

Introduction: The aging process is associated with reduced physiological and functional of several body systems. Loss of muscle mass with aging, also known as sarcopenia, is associated with elevated risk of cardiac, pulmonary, and metabolic disease processes, which further contributes to the socioeconomic burden. It has been well documented that IGF-1 and mitochondria signaling pathways play important roles in protein synthesis and degradation in skeletal muscle of mammalian. In this regard, we hypothesized that endurance training and IGF-1 treatment can positively affect cellular markers involved in sarcopeni. **Methodology:** Fifteen young (3 months old) and 15 old (26 months old) male Wistar rats were were assigned to one of the following groups: young control (YC), young exercised (YE), young exercised and IGF-1-treated (YEI), old control (OC), old exercised (OE), and old exercised and IGF-1-treated (OEI). Exercised rats were introduced to treadmill running for 3 days; then for the next 2 weeks the running speed was set at 10 m/min, with a 5% incline for 30 min/day, 5 days per weeks. The running speed and duration of the exercise were gradually increased to 60% of VO2 max of the animals. In order to protein measurement, frozen vastus lateralis samples were weighed and homogenized and western blot were used. Data were analyzed by SPSS program version 21 and significance level was set at $p < 0.05$. **Result:** Our result demonstrated that aging was associated with a reduced level of anabolic factors including IGF-1 plasma levels and expression level of follistatin and an increased in catabolic agents including Myostatin in old rat. The data from our study showed that 6 weeks endurance exercise training significantly increased the amount of follistatin and mitochondria function such as Cyto C, Cox 4 and Nrf2 and also decreased levels of catabolic factors including MuRF 1 and 2. Our findings demonstrated that 6 weeks endurance training combined with 2 weeks IGF-1 administration positively affected some cellular markers involved in sarcopenia via increased IGF-1 levels and activation of its downstream pathways. **Discussion and conclusion:** According to the results of this study, combination of ET and IGF-1 administration can enhance the effects of ET alone on sarcopenic rats. This reinforcing effect of IGF-1 is likely to be through the modulation of activator and inhibitor factors of apoptosis such as TNF-α and Bcl-2, respectively.

7.2 Summary in Hungarian (Összefoglalás)

Bevezetés: az öregedés folyamata során számos fiziológiai és funkcionális változás következik be az élő szervezetekben. Az idősödő szervezet vázizom vesztése, más néven szarkopénia, pulmonáris- kardiológia és nem utolsó sorban metabolikus elváltozásokkal hozható összefüggésbe. Ezek betegségek igen jelentős szocioökonómiai terhet jelentenek társadalmunk számára. Egy ideje jól dokumentált tény, hogy emlős szervezetek vázizomzatában az IGF-1 és a mitokondriális jelátviteli útvonalak egyaránt fontos szerepet játszanak a fehérjék szintézisben és lebontásban. Ennek fényében feltételeztük, hogy az állóképességi edzés és az IGF-1 kezelés pozitív hatást fog kifejteni a szarkopéniával összefüggésbe hozható sejtmarkerek szintjére.

Anyag és módszer: vizsgálatunk során fiatal (3 hónapos, n=15) és idős (26 hónapos, n=15) wistar patkányokkal dolgozunk. Mind az idős, mind a fiatal populációból 3 csoportot képeztünk: Fiatal kontrol (YC), fiatal edző (YE), fiatal edző és IGF-1 kezelt (YEI), továbbá idős kontrol (OC), idős edző (OE), idős edző IGF-1 kezelt (OEI). Az állatok edzését futópadon végeztük: a háromnapos szoktatási időszak után az álatok 2 hétig 10m/min sebességgel 5% emelkedő mellet edzettek 30pecgig heti öt napon. Ezt követően a terhelés fokozatosan a maximális relatív oxigén felvételük 60%-hoz lett igazítva. Az IGF-1 kezelést a vizsgálat utolsó két hetében alkalmaztuk. A kombinált csoport egyedei naponta ~5μg/kg mennyiségű IGF-1 et kaptak 0.5 μL/hr emissziós rátával, a kontrol csoportnak placebóként fiziológiás sóoldatot adagoltunk. A fehérje összetétel maghatározást Western Blott analízissel végeztük. A vizsgálati állatok vastus laterális izmait folyékony nitrogénnel fagyasztottuk, majd tömegmérés után homogenizáltuk. A csoportok közti különbség meghatározásánál SPSS (vol.21) programot használtunk, a szignifikancia szintet $p < 0,05$ értékben határoztuk meg.

Eredmények: méréseink során demonstráltuk, hogy az öregedés során csökkenek az anabolikus folyamatok indikátorai, ezek közül kiemelten a plazma IGF-1 mennyiség és a follisztatin expresszió. A katabolikus markerek tekintetében a myosztatin mennyiség növekedett az idős patkányokban. Eredményeink szerint 6 hetes állóképességi edzés szignifikánsan képes növelni a follisztatin mennyiséget és fokozza a mitokondriális funkciókat, melyeket Cyto C, Cox4 és Nrf-2 fehérjék emelkedése szemléltet. Mind e mellet MuRF-1 és MuRF-2 katabolikus faktor csökkenés is tapasztalható a kezelés hatására. A

kombinált kezelés számos esetben pozitívan befolyásolta a szarkopénia markerek szintjét, feltehetően az IGF-1 jelátviteli útvonal aktiválódásán keresztül.

Diszkusszió, összefoglalás: Eredményeink alapján, az állóképességi edzés és az IGF-1 szuplementáció együttalkalmazása tovább tudja fokozni az testmozgás jótékony hatását szarkopéniás patkányok esetében. A tapasztalt szinergikus hatás úgy tűnik az apoptózist gátló és aktiváló folyamtok modulációjában rejlik, kiemelve a TNF-alfa és a Bcl-2 fehérjék által vezérelt útvonalakat.

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9. Bibliography of the author's publications

9.1. Publications related to this study

Mosaferi Ziaaldini M, Koltai E, Csende Z, Goto S, Boldogh I, Taylor AW, Radak Z. (2015) Exercise training increases anabolic and attenuate catabolic and apoptotic processes in aged skeletal muscle of male rats. Experimental Gerontology. In press. (Impact Factor: 3.529)

Torma F, Koltai E, Nagy E, **Ziaaldini MM**, Posa A, Koch LG, Britton SL, Boldogh I, Radak Z. (2014) Exercise Increases Markers of Spermatogenesis in Rats Selectively Bred for Low Running Capacity. PLoS One. 9(12): e114075. (Impact Factor: 3.53)

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