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Junjie Xiao *Editor*

Muscle Atrophy

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Part I
Overview

Chapter 1
An Overview of Muscle Atrophy

Shengguang Ding, Qiying Dai, Haitao Huang, Yiming Xu, and Chongjun Zhong

Abstract Muscle is the most abundant tissue in human body, and it can be atrophy when synthesis is inferior to degradation. Muscle atrophy is prevalent as it is a complication of many diseases. Besides its devastating effects on health, it also decreases life quality and increases mortality as well. This review provides an overview of muscle atrophy, including its prevalence, economic and health burden, and clinical therapy. Its clinical therapy includes exercise training, nutritional therapy, electrical stimulation, and drugs such as testosterone and ghrelin/IGF-1 analogues. More large-scale, long-term clinical trials are needed for therapies for muscle atrophy. In addition, more therapeutic targets are highly needed.

Keywords Muscle atrophy · Overview

1.1 Introduction

As the most abundant tissue in the human body, muscle occupies around 40% of the body weight. It stores the most amount of amino acids which can be utilized by other organs under certain situations [1, 2]. In response to physical or pathological stimuli, muscle tissue changes fiber content, capillary distribution, and the components of intracellular connective tissue. All these changes may finally lead to pathologic consequences like atrophy or hypertrophy [3]. Muscle metabolism is important for the dynamic balance of protein degradation and synthesis [3, 4]. Two different

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AKT signaling pathways are responsible for the balance. Muscle protein synthesis is controlled by the AKT/mTOR (mammalian target of rapamycin) pathway, while the AKT/FOXO (forkhead box O) pathway regulates the degradation process [5, 6]. Myostatin, a member of the transforming growth factor-ß (TGF-ß) superfamily, is the key factor involved in the cross-talk between these two AKT pathways. Overexpression of myostatin induces muscle atrophy by downregulating phosphorylation of AKT and FOXO transcription factors. Muscle atrophy occurs when synthesis is inferior to degradation, followed by reduced muscle strength and function [7]. Causes of muscle atrophy can be divided into three types: diffuse deconditioning like denervation, microgravity, or immobilization, nature aging, and chronic diseases [8–10]. Muscle atrophy is very prevalent as it is a complication of numerous diseases. Besides its devastating effects on health, it also reduces life quality and increases mortality [10].

1.2 Prevalence

In the USA, muscle atrophy occurs in about 20,000,000 patients with chronic kidney disease, which leads to spiraling healthcare costs [11]. Heart failure (HF) is another common cause of muscle atrophy. With advanced healthcare, people with HF tend to live longer. It is reported that people over 65 years old account for 80% of HF patients. The combination of cardiac dysfunction and aging significantly impairs normal muscle metabolism. Around half of HF patients suffer from muscle atrophy. As much as 68 in 100 patients have the symptoms of muscle atrophy. Many factors contribute to the HF-related muscle atrophy. Fibrosis between muscle fibers is also observed in HF samples. Tissue from HF rat models showed a lower capillary-to-fiber ratio and capillary density [12]. Alterations in muscle structure like switching muscle fiber types and decreasing the numbers of mitochondria occur during HF. With all these modifications, muscle metabolism change to a state where there is less oxidative metabolism but more proteolysis [13–16]. In the end, cardiac cachexia developed, with a remarkable feature of body wasting, especially the loss of muscle tissue [15, 17, 18].

The key factor that gives rise to muscle atrophy is sarcopenia. Sarcopenia was first proposed in 1989 by Irwin Rosenberg through Greek to describe the decrease of skeleton muscle mass and strength which is related to the growing age [19–22]. Later on, a great number of researches have revealed that sarcopenia has a wide clinical prevalence. It is conservatively estimated that nowadays over 50 million people have been affected by sarcopenia and 150 million more will be affected in the following four decades [23].In western countries, sarcopenia prevalence is around 5–40% in the common population. Sarcopenia is positively related to age. When people are in their 70s, prevalence of sarcopenia is about 5–13%. When the age increases to over 80, prevalence shoots up to 11–50% [23, 24]. Females age over 80 have a prevalence range of 16%, which is almost doubled compared to that of under 70 [25–27]. On the other hand, socioeconomic status affects sarcopenia

distribution. Generally, higher socioeconomic status is associated with better outcome [25, 27]. The difference may be due to some other biological changes, such as obesity and fat infiltration [23, 28, 29]. Sarcopenia is coupled with other muscle atrophy syndromes as well, such as cachexia, frailty, and obesity. Cachexia is a complicated metabolic syndrome which presents with insulin resistance, protein degradation, and inflammation [30–33]. Sarcopenia acts as one of the factors to cause cachexia [23, 30]. Frailty happens frequently in old people and is associated with a lot of disabilities and frequent falls. Sarcopenia and frailty can occur at the same time. People with sarcopenia are frail, and frail people can also have certain degree of sarcopenia [34]. Sarcopenic obesity is a state with the coexistence of both sarcopenia and obesity. When there is a high fat mass component, the condition is known as sarcopenic obesity [35].

In order to set out a diagnostic criteria and operational definitions for clinical practice, an organization, named the European Working Group on Sarcopenia in Older People (EWGSOP), was established by the European Union Geriatric Medicine Society (EUGMS) [23, 36]. The organization established the famous EWGSOP principles to identify sarcopenia with a study involving 103 community-dwelling older people in the UK. The study found that the rate of sarcopenia of 6.8% is the lowest third marker of dual-energy X-ray absorptiometry and lean mass, while the rate of sarcopenia of 7.8% is the lowest third marker of skinfold-based fat-free mass [36]. EWGSOP definition studies have been carried out to detect the prevalence of sarcopenia. It was found that the prevalence of sarcopenia in community-dwelling older adults varied from 3.9% to 7.3% in Taiwan [37]. In Italy, about 20% of community-dwelling people had reduced muscle mass. In Barcelona, every ten men and every three older women suffer from muscle wasting [38]. In Germany, the prevalence rate of sarcopenia is 4.5% in community-dwelling females over 70 years old. In the same study, 252 participants with osteoarthritis at the hip and lower limbs showed 3 times higher rates of sarcopenia [39]. In China, the prevalence rate of sarcopenia is 9.8%. Sarcopenic women account for about 12%, which is almost doubled compared to men. Also, the rate is two times higher in people who live in rural areas than those who live in urban areas [40]. According to Baumgartner criteria, the prevalence of sarcopenia in Korea was 1.3% in men and 0.8% in women over 60s. Every one fifth women aged over 65 years showed a decrease in muscle mass, and 7.6% of them showed a decrease in both muscle mass and strength [41–43]. A report including 31 studies and 9416 participants showed 17.0% of elderly people in Brazil have sarcopenia. Among these people, women account for 20.0% and men account for 12.0% [44]. In another report involving 59,404 people, the overall prevalence of sarcopenia was 10% in men and 10% in women, and the rate is lower in Asians compared to non-Asian people [45]. Sarcopenia prevalence increases with age. It was found that in patients aging from 73 to 89 years, the rate of sarcopenia could be as high as 31% [46]. Residence also influences the distribution of sarcopenia. In patients who live in convalescent rehabilitation ward, 343 of 637 were identified to have sarcopenia [47]. Chronic disease is another factor that contributes to sarcopenia. For example, intestinal failure is strongly associated with malabsorption, which directly impacts muscle metabolism balance. Patients with

this disease are found to have significant higher risk of developing sarcopenia. 72.7% of intestinal failure patients were found to have sarcopenia [48]. Alcohol abuse is another common condition that is related to malnutrition. Prevalence of sarcopenia in female alcoholics who drank weekly or daily was 2.8 times higher than social drinkers. Even after adjusting covariates (age, body mass index, energy intake, and physical activity), alcoholics are still 3.9 times more likely to suffer from sarcopenia [49]. Organic disease can cause sarcopenia by inducing chronic inflammation. Sarcopenia was more commonly observed in patients with advanced kidney disease and is associated with worse outcomes [50].

1.3 Economic and Health Burden

High prevalence of sarcopenia brings tremendous economic burden on healthcare [51, 52]. On one hand, sarcopenic patients are more likely to be dependent on medical care, which has made great impact on public finance expenditures. On the other hand, muscle weakness creates more accidental falls [53]. In the USA, direct healthcare costs for sarcopenia was $18.5 billion, with $10.8 billion for men and $7.7 billion for women. It nearly occupied 1.5% of total healthcare expenditures in 2000 [54]. It was evaluated that every year 1.1 billion dollars would be saved if the prevalence of sarcopenia can be reduced by 10% [54]. In addition, other healthcare costs, such as productivity, psychological problems, and life quality will be saved along with sarcopenia reduction [55–57].

1.4 Clinical Therapy

Considering the great economic and societal burden that sarcopenia could bring, effective treatment and prevention system are necessary. Physical exercise training has been proven to be the most doable and effective therapy. However, it is not applicable for all patients, because one needs to have certain muscle strength to participate physical therapy. Patients who are bedbound or extremely fragile are not suitable for the physical therapy [58, 59]. In order to create new and doable therapy for this disease, researchers have been doing their best to elucidate mechanism of sarcopenia in molecular level [5, 15, 60–62].

1.5 Exercise Training

Exercise training has been studied for years. It is easy to perform and has been used prevalently in all medical facilities. It remains the most commonly used therapy for sarcopenia.

A clinical study involving 60 patients with HF found that oxygen uptake peak was increased in HF patients after 1 month of exercise training. Further biological study detected the expression of MuRF-1 (a component of the ubiquitin-proteasome system participated in muscle proteolysis) in HF patients and healthy controls. MuRF-1 expression was significantly decreased after exercise training, which meant that exercise suppressed the activity of ubiquitin-proteasome system [63].

Muscle growth could be affected by exercise, depending on its intensity. Sixty-four people over 65 years old are randomly assigned to different exercise regimens: high-resistance concentric-eccentric training (H) 3 days per week (HHH); H training 2 days per week (HH); 3 days per week of mixed model consisting of H training 2 days per week separated by 1 bout of low-resistance, high-velocity, concentric-only (L) training (HLH); and 2 days per week mixed model consisting of H training 1 day per week and L training 1 day per week. After 4 weeks, HLH group presented with significant benefits over others. Also, HLH showed greatest improvement in body lean mass, thigh muscle mass, and knee extension maximum isometric strength, while HHH induced the expression of pro-inflammatory cytokine receptors in muscle [58, 64].

It is common to see high prevalence of muscle atrophy in hemodialysis patients. Chronic systemic inflammation impairs mitochondria function and endothelial hemodynamics and then leads to muscle atrophy. Exercise therapy could improve these problems and also increase the muscle fiber number [65].

In old people, declined muscle mass and strength are always accompanied with mitochondrial volume decrease [66]. Exercise could induce up to 40% increase of the mitochondrial volume. This volume increase consists of increase in cross-sectional area and longitudinal growth [66, 67]. On the other hand, moderate exercise training improves mitochondrial biogenesis through mitochondrial transcription factor A (TFAM)-dependent pathway [68].

In molecular levels, exercise training protects individuals from muscle atrophy by suppressing oxidation-related injuries. Reactive oxygen species (ROS), which could be induced in any stimulation, damages muscle fibers. One theory proposes that ROS accelerates muscle fiber degradation by inducing ubiquitin-proteasome pathway [68–72]. Exercise training reverses this process by activating antioxidant enzymes [73–76]. Besides, many other nonenzymatic antioxidants could be induced by exercise training to act as ROS antagonists, like glutathione (GSH) [77]. Endurance exercise training can increase the expression of GSH [77–79]. Other nonenzymatic antioxidants, such as α-lipoic acid and bilirubin, are regulated by exercise training as well [76, 79–81].

Aggravated chronic inflammation is a key factor in age-induced muscle atrophy. Elderly people with a smaller muscle area, less appendicular muscle mass, and a lower knee extensor strength seem to have a higher plasma concentration of inflammatory cytokines including IL-6 (interleukin-6) and TNF-α (tumor necrosis factor-α). Both of them have inhibitory effects on muscle protein synthesis, which also promotes insulin resistance. In addition, IL-6 can prohibit the expression of insulin-like growth factor-1 (IGF-1) [82]. A significant decrease of IL-1 and TNF-α was observed after exercising training for about 12 weeks in the elderly [83]. Other

anti-inflammatory cytokine or cytokine inhibitors, such as IL-10, IL-1ra (IL-1 receptor antagonist), sTNF-r1, and sTNF-r2 (TNF receptors), could be suppressed by exercise too. By decreasing these inflammatory signals, exercise training alleviated inflammation-mediated muscle damage [76, 84–87].

1.6 Nutritional Therapy

Increasing studies have found that nutrients, mainly protein, play an important role in muscle damage treatment, especially in chronic disease caused by muscle atrophy [88–91].

Forty-one sarcopenic patients were randomized into amino acid treatment group and placebo group. The treatment of amino acids was implemented twice per day in the morning and afternoon with a content of 8 g of essential AA snacks. After 6 months and 18 months, muscle tissue mass was measured by dual-energy X-ray absorptiometry as well as fasting blood glucose and insulin resistance. Patients who received amino acid treatment have higher muscle tissue compared to placebo counterparts. Moreover, serum TNF-α and IGF-1 concentrations were decreased significantly without any side effects in the treatment group [92]. Whey protein intake combined with additional supplements is also demonstrated to benefit muscle mass [93, 94].

Not only the amino acid supplementation helps improve sarcopenia; daily consumption of dairy products also has similar effects. It was found that additional daily ricotta cheese could improve sarcopenia symptoms [95].

Another study was conducted using fish oil-derived n-3 (omega-3) PUFA to treat 60 men and women aged 60–85 years old. After n-3 PUFA (n = 40) or corn oil (n = 20) treatment for 6 months, isokinetic leg exercises were used to access muscle status and exercise ability. People from n-3 PUFA group have an improvement in average isokinetic power, thigh muscle volume, handgrip strength, and one-repetition maximum muscle strength. PUFA treatment is considered as a novel therapy for muscle atrophy in older individuals [96].

1.7 Electrical Stimulation

Exercise therapy is not applicable in patients who are bedbound or sedated. Neuromuscular electrical stimulation (NMES) is a kind of electrical stimulation that uses a device to send electrical stimulations to nerves. This stimulation will cause muscle contraction. Unlike exercise therapy, NMES does not require any muscle strength to participate in treatment. Passive muscle contraction initiated by the electrical stimulation is found to be effective in treating muscle atrophy [97]. A study was conducted in six patients. For experimental group, one patient leg was subjected to neuromuscular electrical stimulation twice a day, while the others

served as control. Later, muscle fiber-type-specific cross-sectional area was assessed from the quadriceps muscle biopsies of both groups. Moreover, muscle protein synthesis was compared. Muscle cross-sectional area was reduced by 20% in the control legs, while no muscle atrophy was detected in electrically stimulated legs. Phosphorylation level of mTOR (mammalian target of rapamycin) was increased by 19% in the treated legs, but no change was found in the control ones [98].

1.8 Drugs

Several medications have been studied to be potentially effective in treating muscle atrophy.

1.8.1 Testosterone

It is reported that serum testosterone is closely relevant to muscle myopathy and mortality [99–102]. Testosterone increases muscle volume by inducing muscle fiber hypertrophy, in a dose-dependent manner [103, 104]. In order to explore its medical benefit, a study detected maximal exercise capacity, ventilatory efficiency, baroreflex sensitivity, insulin resistance, and muscle strength in 35 heart failure patients after 12 weeks of testosterone administration. Compared to control group, peak VO(2), peak torque, insulin sensitivity, and quadriceps maximal voluntary contraction were all significantly increased in testosterone group [105]. Similar results had been observed in another study involving female patients [106]. Further study demonstrated that the effect of continuous testosterone treatment was more effective than monthly testosterone administration [107]. Although testosterone is proved to be effective in treating muscle atrophy, its side effects including increasing risk of cancer and multiple behavior abnormalities prevent it from becoming a standard treatment [101, 108–113].

Encouraged by the positive findings on testosterone, nonsteroidal selective androgen receptor modulators (SARMs) were subsequently studied in the field of muscle atrophy [114–117]. SARMs are frequently used to treat testosterone-related disease, like benign prostate hyperplasia. The advantage of SARMs is that they stay at target organs without affecting luteinizing hormone or cross-activating with other steroid receptors. Many clinical trials had suggested the benefit of SARMs in treating cancer-related cachexia and prostate surgery-related sarcopenia [118–120]. Enobosarm is one of SARMs being studied in the current clinical trial. A 12-week double-blind phase II clinical trial revealed a dose-dependent improvement in lean body mass and insulin resistance [120, 121]. Another phase II clinical trial supported the protective effects of enobosarm as well as its safeties in cancer patients [122].

1.8.2 Ghrelin/IGF-1 Analogues

Ghrelin, a peptide with 28 amino acids, is mainly produced by gastrointestinal tissues, especially the stomach [16, 123, 124]. It maintains body weight and muscle volume by assisting food absorption and controlling the expression of IGF-1 and growth hormone in certain levels [125, 126]. In addition, ghrelin plays an important role in depressing chronic cancer or cachexia-induced chronic inflammation [127–129]. In general, it increases the level of anti-inflammatory cytokine interleukin-10 and decreases the pro-inflammatory cytokines interleukin-1β, IL-6, and TNF-α [130–132]. However, its short half-life limits its clinical use [133, 134]. For this reason, anamorelin, a non-peptidic ghrelin mimetic, was developed, which could be taken orally and has a longer half-life [135, 136]. Healthy participants received various doses of anamorelin or placebo for 5–6 days, and an increased level of IGF-1 and growth hormone was detected in anamorelin group. A positive relation between anamorelin and body weight was found as well [137]. The following studies had been done to further validate its clinical applications [138–141]. However, any agents which increase the level of IGF-1 or growth hormone may lead to diabetes or insulin resistance diseases [125, 142–145]. Clinical trials with long-term follow-up should be conducted to evaluate these side effects.

1.9 Conclusion

With various pathogenic factors and wide prevalence, muscle atrophy remains a great challenge in clinical practice [146]. Several treatments mentioned above, exercise therapy, NMES, and drugs, have been proven to be effective. Medication therapy for muscle atrophy has received great achievements in the recent studies. However, their long-term effects remain unknown, and most of the studies only follow up patients for several months. More large-scale, long-term clinical trials are needed [5, 60, 147–150].

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Part II
Basic Aspects of Muscle Atrophy

Chapter 2
Myofibers

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Abstract Muscle tissue is a highly specialized type of tissue, made up of cells that have as their fundamental properties excitability and contractility. The cellular elements that make up this type of tissue are called muscle fibers, or myofibers, because of the elongated shape they have. Contractility is due to the presence of myofibrils in the muscle fiber cytoplasm, as large cellular assemblies. Also, myofibers are responsible for the force that the muscle generates which represents a countless aspect of human life. Movements due to muscles are based on the ability of muscle fibers to use the chemical energy procured in metabolic processes, to shorten and then to return to the original dimensions. We describe in detail the levels of organization for the myofiber, and we correlate the structural aspects with the functional ones, beginning with neuromuscular transmission down to the biochemical reactions achieved in the sarcoplasmic reticulum by the release of Ca^{2+} and the cycling of crossbridges. Furthermore, we are reviewing the types of muscle contractions and the fiber-type classification.

Keywords Skeletal muscle · Myofiber · Myofibril · Sarcomere · Slow-contracting muscle fiber · Fast-contracting muscle fiber

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2.1 General Description of Skeletal Muscle Structure

Movement is one essential characteristic of living creatures, its forms becoming varied and highly complex in the humans for which it is specific. Due to active movements, humans gain greater independence toward changes in their environment. Motor actions, results of contractions and relaxations of the muscles, represent the expression of the volitional aspect of the act of communication, while mimic muscles, voice, and writing express aspects of the human personality. In this sense, the nervous and muscular systems form a functional unit.

In the human body, the skeletal muscles represent about 40% of the total weight, being the most abundant tissue. Skeletal muscles are specially designed to perform contractions based on their characteristic properties such strength, flexibility, and plasticity [1]. They allow various actions to be taken from writing to weight lifting or jumping. Muscle contraction is involved in a series of important physiological processes such as breathing or heat generation, in maintaining normal body temperature. Human skeletal muscles are made up of muscle fibers (myofibers) and other different types of cells (adipocytes, fibroblasts, satellite cells, smooth and endothelial cells which are part from the vessel walls, neurons, and Schwann nerve cells) [2]. The main source of energy that provides ATP for contraction is glycogen. After contraction, there are three major systems for the replenishment of ATP: the phosphagen system (ATP–creatine phosphate system), the glycolytic system, and the mitochondrial oxidative phosphorylation system [3].

2.1.1 Embryology and Postnatal Development of the Myofibers

Skeletal muscles are derived from the paraxial mesoderm, along the embryonic development being divided into somites [4]. Each group is divided into three divisions: sclerotome (vertebrates), dermatome (which forms the skin), and myotome (which forms muscles) [5]. During development, myoblasts (muscle progenitor cells) that originated from mesenchymal stem cells may remain in somites to compose muscles of the spine; otherwise they participate in the formation of other muscles [6]. In the development of striated muscle fibers of the postnatal period, the satellite cells are also involved, and they are also responsible for the regeneration of the muscles in the adult [7, 8]. Skeletal muscle fibers develop through the fusion of myogenic progenitors (myoblasts) forming muscles in a process known as myogenesis [9]. Myogenesis is regulated by a series of transcription factors, including Pax 3, Pax 7, and Gli, and four myogenic regulatory factors: MyoD, Myf-5, myogenin, and MRF-4 [10, 11].

2.1.2 Organizational Hierarchy of Skeletal Muscle

Skeletal muscles are hierarchically comprised of muscle fascicles and muscle fibers, which are made of myofibrils (arranged in parallel), are further divided into myofilaments and sarcomeres (arranged in series), and are ultimately broken down into structural proteins. In skeletal muscles, there is a close relationship between the muscle fibers and the connective tissue responsible for providing the nourishment of the muscle and the transmission of the force. Thus, each striated muscle is surrounded on the outside by a fibrous structure called fascia (dense lamellar connective tissue), which is anchored by epimysium (dense semi-coordinated connective tissue) [12]. The epimysium, consisting of collagen, reticular, and elastic fibers, provides the shape of the muscle and contains blood vessels and nerves. From the epimysium start connective septa – perimysium – which delimits and wraps muscle bundles. The internal perimysium envelops the primary muscles, and the external perimysium covers the secondary and tertiary muscle bundles [13]. Several muscle fibers form a primary fascicle, some primary fascicles form a secondary fascicle, and some secondary fascicles form a tertiary fascicle. In the connective tissue of perimysium, there are vessels, nerves, and proprioceptors (neuromuscular spindles, Vater-Pacini corpuscles, Ruffini corpuscles). Each muscle fiber is wrapped in endomysium, composed mainly of reticulin fibers (type III collagen) and rare type I collagen fibers. Endomysium contains numerous blood capillaries and nerve fibers, but there are no lymph capillaries (Fig. 2.1). All these connective structures represent 10–15% of the volume of the muscle and form a sort of "skeleton" of the muscle that modulates and controls its activity [14]. The number of fibers ranges from several hundred in small muscles to >1 million in large muscles. Muscle fibers are innervated by somatic efferent (motor) neurons which participate in the formation of a motor unit consisting of axonal terminals and skeletal muscle fibers that it innervates [15]. Each muscle is formed by tens or hundreds of motor units, each with own specificity that allows the same muscle from the same species and in different species to be used for various tasks [16]. These vary from continuous low-intensity activities, like posture keeping in humans and supporting their body weight, to performing movements in a large variety of situation (e.g., locomotion) that involve repeated submaximal contractions and fast and strong maximal contractions (jumping, kicking) [16]. To deal with these divergent activities, muscle cells have been provided with large differences in their contractile properties and metabolic profile, the nerve activity being a major determinant of the fiber-type profile [16].

2.1.3 Skeletal Muscle Cells: General Characteristics and Morphological Aspects

The skeletal muscle fiber is a cylindrical cell, with a length that can range from 2–3 cm up to 50 cm (with an average of 10 cm in men) and a thickness between 10 and 100 μm. From the ultrastructural point of view, skeletal striated muscle fibers

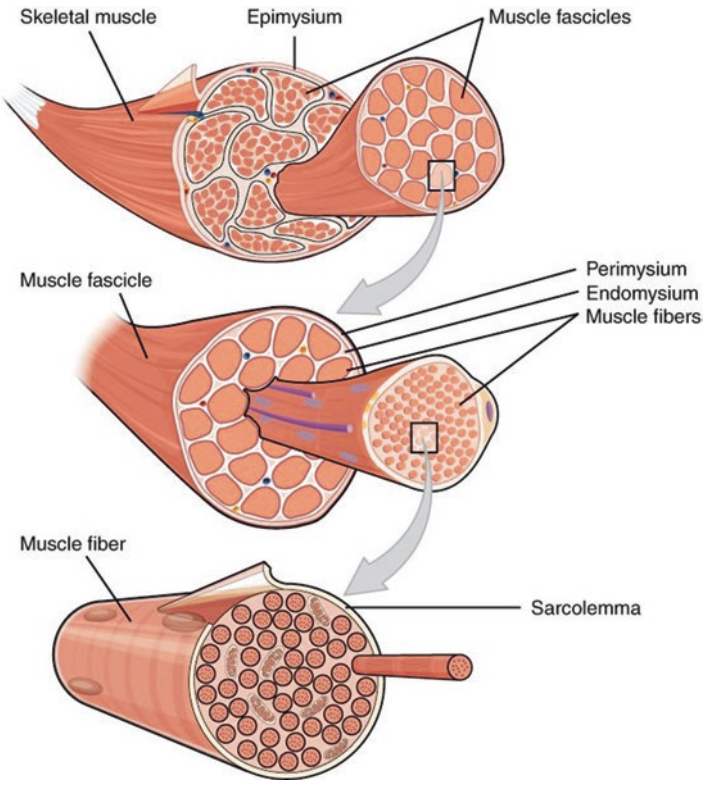

Fig. 2.1 The three connective tissue layers of a skeletal muscle. The muscle is surrounded by a connective tissue sheath called epimysium. Bundles of muscle fibers, called fascicles, are covered by the perimysium. Each skeletal muscle fiber is covered by the endomysium. (Image credit: download for free at http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.119)

describe all three classical components of a cell: membrane (sarcolemma), cytoplasm (sarcoplasm), and numerous peripheral nuclei. The myofiber contains up to 100–200 nuclei representing the largest cell in the body. Each myofiber contains long, thin, cylindrical rods, called myofibrils, usually 1–2 μm in diameter, which run parallel to the long axis of the muscle fiber occupying most of the intracellular space [17]. As a consequence, cell organelles, like mitochondria and nuclei, are pushed to the periphery of the sarcoplasm. Myofibrils are about 2500 per fiber, and each one contains approximately 8000 repetitive units called sarcomeres (2.7 μm in length for the human muscle), which are joined end to end [18]. Each sarcomere is delineated between two Z lines and is made up of myofilaments comprised of thick and thin filaments (Fig. 2.2), the thick one consisting in myosin and the thin composed of actin, troponin, and tropomyosin [19]. In fact, sarcomere periodicity is responsible for the distinctive banding pattern of striated muscle, which can be observed in light and electron microscopy. Myofibrils are specific contractile

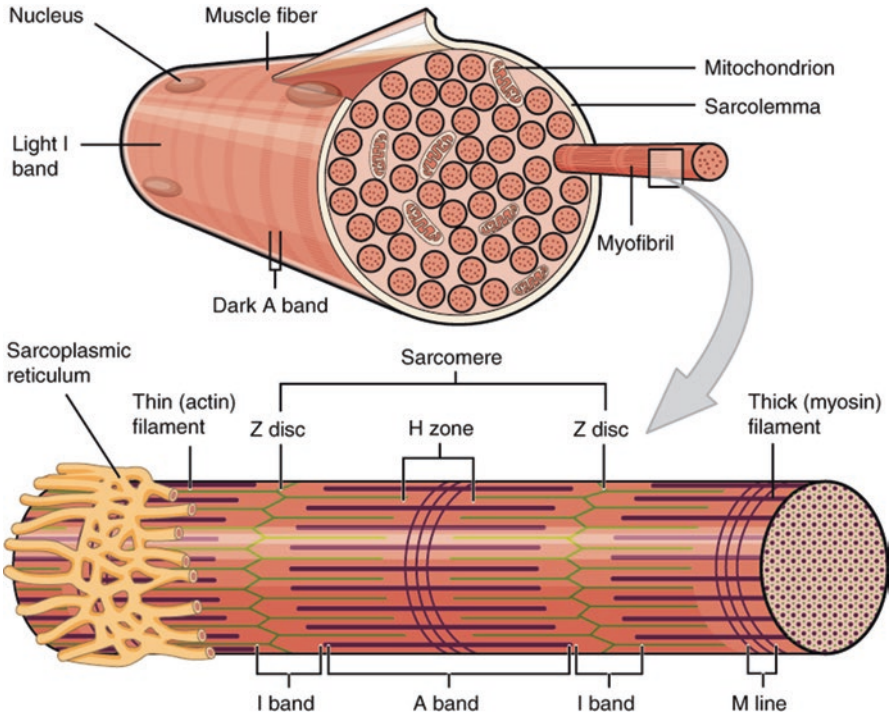

Fig. 2.2 Muscle fiber. A skeletal muscle fiber is surrounded by a plasma membrane called the sarcolemma, which contains sarcoplasm, the cytoplasm of muscle cells. A muscle fiber is composed of many myofibrils, which give the cell its striated appearance. Each myofibril is a succession of sarcomeres. Each sarcomere is delineated between two Z lines. (Image credit: download for free at http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.119)

organelles, arranged parallel to each other and to the longitudinal axis of the muscle fiber. They can take up between 80 and 86% of the cell volume. Myofibrils are composed of thin and thick myofilaments, parallel to each other. Myofilaments are accompanied by regulatory proteins (tropomyosin and troponin) and stabilizing proteins [17].

In a longitudinal section, skeletal muscle fibers appear as parallel, organized, multinucleated structures (plasmodial aspect), with hundreds of fallen, pliable nuclei distributed across the length of the fiber and placed subsarcolemmally. Sometimes the round-oval nuclei of the satellite cells can be seen outside the myofiber [20]. Sarcoplasm is almost entirely occupied by striated myofibrils. These are parallel to the long axis of the skeletal muscle fiber and placed so that all the clear and dark disks overlap perfectly, giving the fiber the striated appearance (Fig. 2.3a). These transverse strains are less obvious in the usual staining techniques but readily detectable with Heidenhain's hematoxylin. By this method, it is possible to emphasize, especially in the immersion objective, the alternation of clear I band bisected

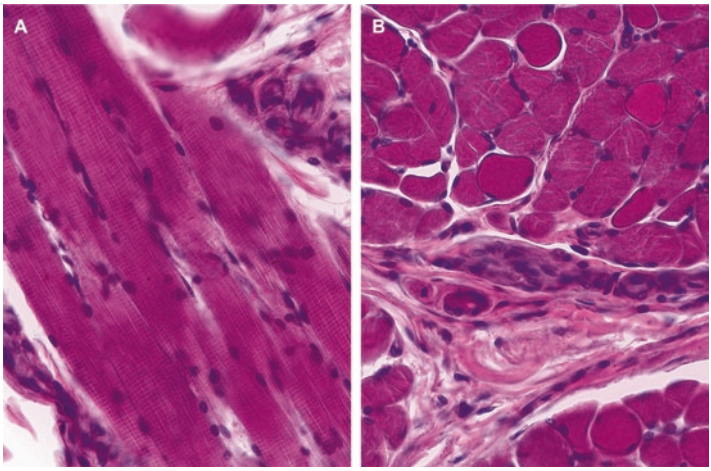

Fig. 2.3 Light microscope slide of skeletal muscle stained by H&E. (**A**) Longitudinal section depicting the A bands which are stained dark and the I bands which are lighter forming the so-called striations. (**B**) A cross section of skeletal muscle – one cannot see the striations, but in the bundles of circles that contain mosaic-like figure formed by a group of myofibrils separated by a clear interstitial substance called "Cohnheim fields," you can identify the peripherally located nuclei (dense purple spots around the large pink fibers). Courtesy of Dr. Adrian Dumitru

by Z line (for Zwischen-Scheibe meaning interim disk) and dark A band containing the clear H band (for HelleScheibe), halved by M line (for mittel – middle). The myofibrils are grouped in bundles called Leydig colonnettes (Koelliker) separated from each other by acidophilic sarcoplasm [21].

In the cross section, the muscle fibers have a polygonal contour (due to tight wrapping of the cells) or round-oval, with 1–3 nuclei surprised in the section field, and there is a punctual aspect given by the organized myofibrils in the Cohnheim areas or fields (clusters of points delimited by clear spaces) in the cytoplasm (Fig. 2.3b). The cross-sectional area of an individual muscle fiber ranges from approximately 2000 to 7500 μm^2.

As observed in the transmission electron microscope, sarcolemma has the classical structure of a plasmalemma and is surrounded by a glycoprotein/glycosaminoglycan layer similar to a basal lamina of epithelia. Reticular fibers are also present in its structure, mingled with those from the endomysium. At each end of the muscle fiber, this surface layer is lost between the tendinous fibers with which it merges. Satellite cells are located between the basal lamina of the muscle fiber and sarcolemma, closely intimate with the muscle fiber whose sarcoplasm is deformed to the inside by the satellite cells, the outer surface of the fiber being not deformed [22, 23].

Sarcolemma has inward extensions (invaginations) into the sarcoplasm and forms the T (transverse) tubule system – T system:

– It builds a very branched network filled with extracellular fluid that prolongs the extracellular space in the depth of the cell up to the vicinity of the contractile structures; this system together with a pair of terminal cisterns of the sarcoplasmic reticulum forms triads [24]; T tubules penetrate to all levels of the muscle fiber.
– It is perpendicular to the plane of the membrane at the junction where the A and I bands of the myofibrils overlap and where a mesh surrounding each myofibril is formed. In this way, ions and signal molecules can reach up to the contractile structures [25].
– Sarcolemma of the T tubules is intertwined with a large number of L-type calcium channels, designed to propagate the potential of action initiated at the neuromuscular junction within the muscle fiber.

Sarcolemma itself contains the integral proteins and ion pumps (ATPase, adenylate cyclase, 5′-nucleotidase) to control plasma ATP concentration. Also, at the level of the sarcolemma are described the costameres – structural-functional components. Costameres are subsarcolemmal assemblies of proteins aligned across the circumference of the skeletal fiber at the Z lines and have the role of physically coupling the force generated by sarcomeres with sarcolemma, tethering the sarcomere to the cell membrane [26–28]. The DAG (dystrophin-associated glycoprotein) complex contains various integral and peripheral proteins, such as dystroglycan and sarcoglycan, which are thought to be responsible for the connection between the internal cytoskeletal system of myofibers (actin) and the structural proteins within the extracellular matrix (such as collagen and laminin) [29]. Through this complex, sarcolemma ensures the binding of the sarcomere to the extracellular connective tissue. If the complex comes to be associated with desmin, the respective regions turn out to be involved in signaling. Proteins associated with dystrophin-glycoprotein complex might be dysfunctional, leading to myopathies, which manifest by progressive muscle damage and impairments in regeneration [29]. Caveolae are sarcolemmal invaginations existing in the regions of the membrane microdomains rich in caveolin-3 and organized into multilobed structures which provide a large reservoir of surface-connected membrane underlying the sarcolemma. Besides acting as cellular devices involved in the concentration and functional regulation of various signal molecules [30], caveolae can protect the muscle sarcolemma against damage in response to excessive membrane activity [31].

The skeletal muscle fiber contains numerous nuclei (30–40 nuclei/cm long), oval-elongated (8–10 μm) and rich in heterochromatin. The nuclei are disposed in the peripheral sarcoplasm immediately beneath the sarcolemma, with their long axis parallel to the fiber and in alternate positions. Their number is higher at the level of the motor end plates and the myotendinous junctions, where they form agglomerations [12].

Sarcoplasm is a component found among myofibrils and can vary in quantity depending on the type of skeletal fiber in which it is found (red muscles, rich in cytoplasm; white muscles, little sarcoplasm) [32]. It also contains common and specific organelles and various inclusions (glycogen, lipid, pigments).

Common Organelles Mitochondria are located in the sarcoplasm in the vicinity of the nucleus or among the bundles of myofibrils – intermyofibrillar [33]. The number of mitochondria is higher at the Z line and in the I band where they have a long axis parallel to the long axis of the muscle fiber and are very numerous in high-speed skeletal fibers.

Specific Organelles Sarcoplasmic reticulum (SR) can be considered as a muscle-specific organelle, although it is, actually, the smooth endoplasmic reticulum specialized in calcium release/storage [34]. The sarcoplasmic reticulum describes a dilated portion (junctional SR) in contact with the T tubules and a binding portion (free SR). In the SR lumen, calcium is linked to calsequestrin and has a concentration of 10^4–10^5 times higher than cytoplasmic calcium. The action potential of the sarcolemma is led up to the neighborhood of the SR through the T-tubes and determines the release of calcium from SR cisterns through membrane ion channels. The calcium concentration in the sarcoplasm increases from 10^{-7} to 10^{-6} and triggers the contraction. Calcium reuptake is performed by an enzyme, the Ca^{2+} pump, with ATP consumption, against the concentration gradient, the consequence being the decrease of calcium in the sarcoplasm followed by relaxation [35].

Muscle contraction is triggered by electrical activity induced at the level of the transverse tubules and the membrane cell surface. The scientific research is currently focusing on the correlation between two major components, respectively, SR and T tubules. This interaction is mediated by the dihydropyridine receptors (DHPRs) and by ryanodine receptors (RyRs). These channels are implicated in calcium release mechanism. Optimal functioning of the skeletal muscles requires three essential processes, respectively, storage, discharge, and recovery of calcium. In these mechanisms are implicated three classes of SR calcium-regulatory proteins: luminal calcium-binding proteins, SR calcium release channels, and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps. The first category includes calsequestrin, histidine-rich calcium-binding protein, junctate, and sarcalumenin and is involved in calcium storage, while the second category (type I ryanodine receptor or RyR1 and IP3 receptors) is implicated in calcium release. Calcium recovery is provided by SERCA pumps [36]. Triads are specialized complexes consisting of a centrally located T tubule and flanked by two junctional sarcoplasmic reticulum cisterns [37, 38]. They are located adjacent to the boundary between A and I bands and are designed to ensure a smoothing of muscle fiber contraction.

Myofibrils are the specific contractile organs parallel to each other and the longitudinal axis of the muscle fiber, occupying between 80 and 86% of the cell volume. Myofibrils are composed of thin and thick myofilaments, parallel to each other, and are responsible for the striated nature of the muscle fiber. The skeletal fiber-specific band (cross striations) can be seen in optical microscopy as an alternation between dark A bands (anisotropic under polarized light, dark in phase contrast) and bright I bands (isotropic under polarized light, bright in phase contrast). In the middle of the bright bands, the narrow, dense lines, the Z lines or Z disks, can be seen (Fig. 2.4). The orderly arrangement of myofibrils is conferred by solidarization, by means of

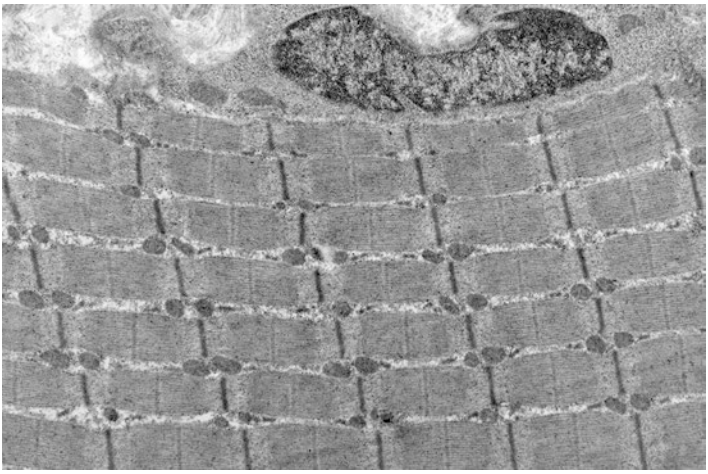

Fig. 2.4 Transmission electron micrograph (TEM) of a longitudinal section through the skeletal muscle. The striations are due to the presence of sarcomeres consisting of the darker bands – A bands (includes a lighter central zone, called the H band) – and the lighter bands, I bands. Each I band is bisected by a dark transverse line called the Z line flanked by mitochondria. Paired mitochondria are on either side of the electron opaque Z line. The Z Line marks the longitudinal extent of a sarcomere unit

intermediate filaments of desmin. The Z disks are solidarized between the adjacent myofibrils via plectin. The segment comprised of two Z-membranes (disks) is a sarcomere (the Krause muscular box) – the morpho-functional unit of the ribbed myofibril. The sarcomere is the functional unit of the myofibril and consists of an A band and two clear halves of I band and has a length of 2–3 μm. In electron microscopy, it is observed that the A band (1.5 μm long) is electron-dense and is crossed through by a clear area – H band (Hensen) through which a fine membrane passes – the M line (Mittel – middle line), hard to observe in optical microscopy. The I band (0.8 μm long) is transparent to the electron beam. The middle of clear bands is crossed by a thin membrane – Z (Stria Amici or Krause's membrane) membrane. Myofilaments include:

– Thick filaments, ~ 1.500 per sarcomere (15 nm in diameter and 1.5 μm long), disposed in the middle of the sarcomere and forming the A band.
– Thin filaments, ~3000/sarcomere (7 nm in diameter and 1.0 μm long), form the I band but also participate in A band formation.

While A band contains thick and thin filaments (a thick filament is surrounded by six thin filaments), I band is formed only from thin myofilaments. The H band is composed only of thick myofilaments solidified at the M band by cytoskeletal filamentous proteins. The Z band consists of actin-like filament anchor proteins: α-actinin, CapZ, and nebulin.

2.1.4 Molecular Organization of Myofilaments in Striated Muscle Fiber

The myofibrils are composed of proteinaceous structures, called myofilaments, which are different in size. Myofilaments are the actual contractile-specific organelles of striated muscles, made of individual filamentous polymers of myosin II (thick filaments) and actin and specifically associated proteins.

Thin Filaments Thin myofilaments contain actin, tropomyosin, troponin, and other associates. The thin filaments are mostly made up of a globular monomeric protein called G-actin (**g**lobular) – about 300 individual molecules. They measure 8 nm in diameter and extend from the Z line for a length of ~ 1.0 μm [19]. The G-actin monomers combine to form a long polymer chain F-actin (filamentous). Each G-actin molecule of the thin filaments has a myosin-binding site, which in resting stage is protected by tropomyosin molecule. Because all the actin monomers are oriented in the same direction, actin filaments have a distinct polarity and their ends (called the plus and minus ends). Two such actin polymers intertwine in a helical fashion to form a thin filament strand. Thin filaments are oriented in opposite directions at each Z line of a sarcomere, which is essential for the production of contractile forces [39]. Tropomodulin is intended to cover the end of the actin by preventing the addition of new actin G monomers. The F-actin filament has a specific polarity with a tropomodulin-coated end that penetrates the thick filaments which is called minus (−) end and a plus (+) end that anchors to the Z membrane by the CapZ protein when the filament reaches the right length. Then, the plus end of each filament is bound to the Z line by α-actinin (bundles thin filaments into parallel arrays and anchors them at the Z line) with nebulin assistance [40]. The minus end extends toward the M line and is protected by tropomodulin, an actin capping protein. Nebulin anchors through the terminal carboxyl-terminus at the Z lines and with the amino-terminal ends at the A band [41]. Nebulin is an inelastic filamentous protein that twists around the actin filament by packing with actin, troponin, and tropomyosin molecules [41]. The nebulin is linked with thin filaments through tropomodulin and Z line proteins, being involved in establishing their length [26].

Tropomyosin is a fibrous protein consisting of rods (40 nm each) linked head-tail and is located in the grooves of the double helix of actin F. Tropomyosin has two α-helical polypeptides that bind laterally to seven contiguous actin subunits as well as head to tail to neighboring tropomyosins, forming a continuous strand along the whole thin filament. Troponin is a complex oligomeric protein and has three components: troponin C (Ca^{2+}-binding), troponin I (inhibitory), and troponin T (tropomyosin binding) [42].

In striated muscles, the concentration of Ca^{2+} influences the complex formed from tropomyosin molecules and troponins; thus at low calcium concentration,

muscles do not contract. If the level of Ca^{2+} is higher, muscle contraction is initiated [26, 43].

Thick Filaments These filaments are 12–16 nm in diameter and ~ 1.6 μm long and are packed in a hexagonal array on 40–50 nm centers throughout the A bands [19]. Each thick myofilament contains approximately 250 myosin II molecules arranged antiparallel and associated with myomesin, titin, and protein C. The myosin II class includes various muscle myosins and cytoplasmic myosins that also have two heads and long coiled tails. The assembly of tails into bipolar filaments allows myosin II to pull together oppositely polarized actin filaments during muscle contraction. Myosin II, a 510 kDa, long, rod-shaped, actin-associated motor protein, is an asymmetric dimer composed of two heavy polypeptide chains (222 kDa each) and four light chains (two regulatory chains and two essential chains). Heavy chains form a structure called a tail or stick, twisted in the form of a helix, but it also enters the constitution of a large part of the globular ends. The ends of the myosin molecule contain, besides heavy chains, the associated light chains, one of 20 kDa (LC20) and one of 17 kDa (LC17). LC20 comprises the phosphorylation site by MLCK (myosin light chain kinase).

Myosin molecules in striated muscle aggregate tail to tail to form bipolar thick myosin filaments; the tails overlap so that the globular heads protrude from the thick filament at regular intervals to form transverse bridges. In the middle of the filament, there are not any globular projections.

The regions of the myosin heads contain distinct actin-binding sites, ATP hydrolysis, and association of light chain subunits. By limited proteolysis, myosin can be divided into two functional domains due to the presence of protease-sensitive sites in the hinge region and the head-tail junction. Under the controlled action of trypsin, light meromyosin (LMM) is formed – the region in which myosin molecules interact to form filaments – and heavy meromyosin (HMM) is the transverse bridge (the tail and the two globular ends). HMM can be cleaved under the action of papain in two subfragments: S2 representing the rest of the tail and S1 (representing the two globular ends) containing the ATP and actin-binding sites.

Several accessory proteins stabilize thick filaments. The M line in the center of the sarcomere is a three-dimensional array of protein cross-links that maintains the precise registration of thick filaments. M line proteins include myomesin, M protein, obscurin, and muscle creatine phosphatase. The interaction between the heavy and light chains determines the speed and strength of muscle contraction. The myosin head has two specific binding sites, one for ATP with ATPase activity and one for actin [26].

Myomesin is a protein that solidarizes the filaments at the level of line M. The protein C binds to the myosin in the vicinity of the M line at the end of the thin filament at the intersection of A and I bands.

Titin is a large (2500 kDa) protein, which spans half of the sarcomere, and is responsible for the axial periodicity of myofilaments because it maintains

three-dimensional relationships by keeping the thick and thin filaments in proper alignment. Titin is named after the mythological giants, due to its remarkable size: more than 30,000 amino acids folded into a linear array of 300 immunoglobulins and fibronectin II measuring more than 1.2 μm long. The amino terminus end of the titin molecule completely crosses the Z lines and is anchored to α-actinin. At the Z band, the titin molecules in the adjacent sarcomeres overlap. The carboxy terminus end traverses the entire M line and overlaps the titin molecules in the other half of the sarcomere and binds to the myomesin. At I band, titin interacts with actin molecules and at A band interacts with protein C. If titin molecules are broken experimentally, thick filaments slide out of register toward one Z disk during contraction.

Desmin helps to align the sarcomere laterally by linking each Z disk to its neighbors and to specialized attachment sites on the plasma membrane (intermediate filaments that interconnect adjacent myofibrils).

The interaction of these myofibrillar proteins allows muscles to contract.

2.2 Skeletal Muscle Contraction Mechanism

2.2.1 Neuromuscular Transmission

Skeletal muscle works under voluntary control. Muscles will contract or relax when they receive signal from the nervous system. The control of skeletal muscle fibers is performed by alpha motor neurons located in the anterior horns of the spinal cord and in motor nuclei of the origin of the cranial nerves. A neuron, along with the specific muscle fibers that it innervates, is called a motor unit. The axons of the neurons branch as they are adjoining the muscle, giving rise to terminal branches that end on individual muscle fibers. The neuromuscular junction is the site of the signal exchange where synaptic bulb of an axon and a muscle fiber connect. The axon ending is a typical presynaptic structure which contains numerous mitochondria and synaptic vesicles that contain the neurotransmitter acetylcholine (ACh). The neuron that carries the action potential is known as the presynaptic cell and the cell receiving it (muscle cell) as the postsynaptic cell. The neurotransmitter is released in the synaptic cleft, the space between the axon terminal and the muscle cell (the space contains amorphous basal lamina matrix). Motor end plate is a region of the sarcolemma that participates in the synapse having ACh receptors. The nicotinic ACh receptor in striated muscles is a transmitter-gated Na^+ channel. Binding of ACh opens Na^+ channels, causing an influx of Na^+ into striated muscle cell. These channels are not voltage-gated, and they will open only when the ACh attaches to them. Once open, they will allow the passage of sodium ions into the muscle cell, down their electrochemical gradient.

2.2.2 Excitation-Contraction Coupling (Exposure of Active Sites)

When sarcolemma is depolarized, an action potential (AP) is generated and triggers muscle cell contraction. The AP initiated on the membrane surface spreads radially in all directions, spanning the entire surface and then penetrating deep into the cell via T tubule (invaginations of the sarcolemma). Due to these tubules, the action potential can spread along the muscle cell evenly and quickly [44]. As the AP reaches the membrane of the sarcoplasmic reticulum, it makes it permeable to calcium ions. Once the calcium is inside the cytosol, it can interact to thin filaments to initiate contraction. T tubules show numerous L-type voltage-dependent Ca^{2+} channels. The change in potential difference opens the Ca^{2+}channels and allows the calcium to penetrate into the cell according to the concentration gradient. This type of calcium channels is also called dihydropyridine (DHP)-dependent channels because they can be blocked by dihydropyridine. The amount of Ca^{2+} penetrated through these channels is small and incapable to trigger muscle fiber contraction. However, activation of these dependent Ca^{2+} DHP channels is mandatory in triggering the contraction. Activation of Ca^{2+} L-type-dependent channels (DHP dependent) drives two mechanisms:

- The flow of Ca^{2+} through the channel produces conformational changes in the subunits that compose it. Through the proximity of the T tubule with the sarcoplasmic reticulum within the triad, intimate contact is allowed between the dependent DHP channels and the Ca^{2+} channels of the sarcoplasmic reticulum and the RyRs-dependent channels. Activating dependent Ca^{2+} DHP channels activates RyRs-dependent channels [45].
- The release of Ca^{2+} from the sarcoplasmic reticulum increases the concentration of Ca^{2+} approximately 10^{-7} to 10^{-5} M. The bond between troponin-tropomyosin complex and actin becomes weak. The action potential causes a short-lived conformational change in DHP receptors that is transmitted directly to the associated RyRs Ca^{2+} release channels. Cytoplasmic Ca^{2+} binds to troponin C. Troponin changes position, pulling tropomyosin away from the active sites. This shift increases the probability that myosin-ADP-Pi heads will bind to the thin filament, dissociating their bound Pi and producing force. Ca^{2+} binds to troponin C rapidly (milliseconds) but dissociates slowly (tens of milliseconds) [46].

2.2.3 The Main Steps Involved in Muscle Contraction

The interaction between myofibrillar proteins myosin (the thick filament) and actin (the thin filament) allows muscles to contract. This fact was demonstrated long before the fine structure of the myofibril became known. In 1954, the mechanism of muscle contraction, based on muscle proteins that slide past each other to generate movement, was suggested by Andrew F. Huxley and is known as the sliding

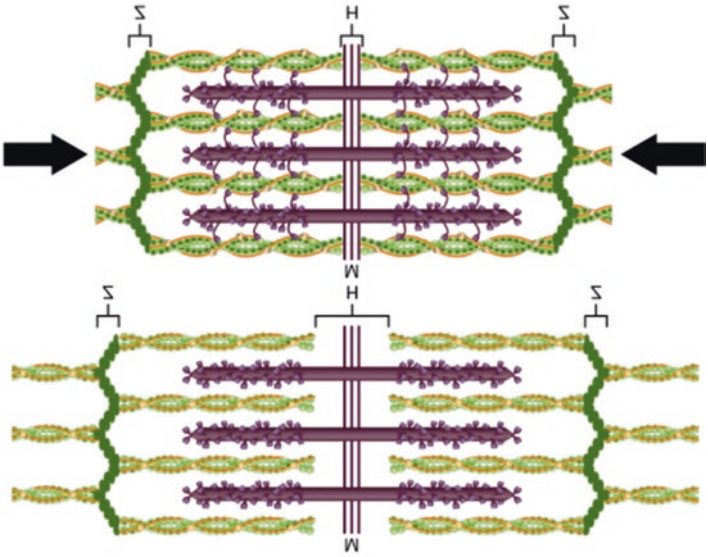

Fig. 2.5 The sliding filament model of muscle contraction. When a sarcomere contracts, the Z lines move closer together, and the I band becomes smaller. The A band stays the same width. At full contraction, the thin and thick filaments overlap completely. (Image credit: download for free at http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.119)

filament model of contraction [47–49] (Fig. 2.5). The movement of muscle in mammalian species is directly dependent on the hydrolysis of ATP as its source of energy [1]. The first step is represented by the exposure of actin active sites. In a second step, myosin crossbridges bind to actin active sites. ATP binds to myosin head and induces conformational changes of the actin-binding site. The third step is represented by cycles of the myosin heads. The light chain enzyme of the myosin head allows ATP cleavage in ADP and Pi. As a result of the dissociation of the macroergic bond, part of the energy is released, and the head of myosin bends from an angle of 90 degrees to an angle of 45 degrees with the advancement of the actin filaments by 11 nm [50]. After crossbridge attachment, the energy is released as the myosin head pivots toward the M line. This action is called the power stroke. When adenosine diphosphate (ADP) and Pi are released, both products remain bound to the myosin head. The fourth step consists of the detachment of crossbridges [51]. Another ATP binds to the myosin head, and the link between the actin active site and myosin head is broken. The active site is now exposed and able to interact with another crossbridge. When a muscle is stimulated to contract, the myosin heads start to walk along the actin filaments in repeated cycles of attachment and detachment. During each cycle, a myosin head binds and hydrolyzes one molecule of ATP. Myosin molecule moves the tip of the head along the actin filaments toward the plus end. This movement, repeated with each round of ATP hydrolysis, propels the myosin molecule unidirectionally along the actin filament. In the last step, the reactivation of myosin occurs when myosin heads split ATP and myosin head is in the resting position (Fig. 2.6). The contraction stops by Ca^{2+} returning to the sarcoplasmic

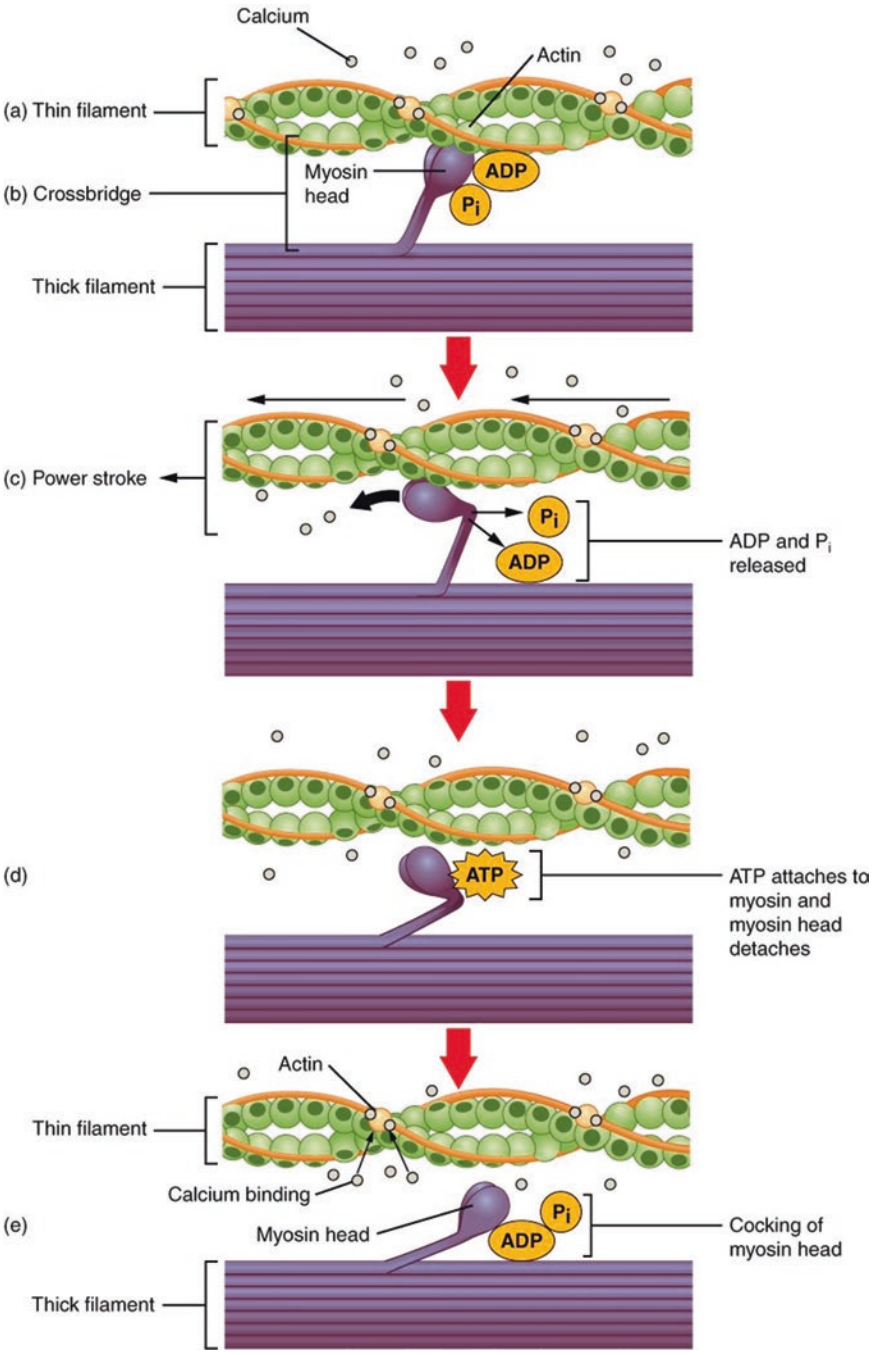

Fig. 2.6 (**a**) The active site on actin is exposed as calcium binds to troponin. (**b**) The myosin head is attracted to actin, and myosin binds actin at its actin-binding site, forming the crossbridge. (**c**) During the power stroke, the phosphate generated in the previous contraction cycle is released. This results in the myosin head pivoting toward the center of the sarcomere, after which the attached ADP and phosphate group are released. (**d**) A new molecule of ATP attaches to the myosin head, causing the crossbridge to detach. (**e**) The myosin head hydrolyzes ATP to ADP and phosphate, which returns the myosin to the cocked position. (Image credit: download for free at http://cnx.org/contents/14fb4ad7-39a1-4eee-ab6e-3ef2482e3e22@8.119)

reticulum via the SERCA pump. The SERCA pump is found in the membrane of the sarcoplasmic reticulum and plays a role in pumping Ca^{2+} against the concentration gradient. Pump activity is controlled by phospholamban, regulated in turn by β-adrenergic receptors. β-Adrenergic stimulation is followed by phosphorylation of phospholamban (activated form) followed by inhibition of Ca^{2+} pumps with increased concentration in the cytoplasm and increased contraction force.

Because all the sarcomeres contract together, the entire muscle shortens at the same rate. When a skeletal muscle fiber contracts the H bands and I bands get smaller, the overlapping zones get larger, the Z lines move closer together, and the width of the A bands remains constant. The contraction ends once the fiber has shortened by 30% (elimination of the I bands) [52, 53].

2.2.4 Types of Muscle Contractions

Single direct electrical stimulation of a muscle, or indirect through the motor nerve, with a constant current of a certain intensity and duration, causes a muscular twitch (rapid shortening followed by a return). Twitch is an elemental, biologically active functional manifestation of muscle contractility consisting of its shortening and tension development. Twitches can be experimentally produced by applying an electric current to a motor nerve. Under physiological conditions, there are no twitches. Shiver, contraction of extraocular muscles, and other types of contractions, even if they are short-duration contractions, require a short-term discharge of a large number of nerve impulses [54].

During twitch, a series of steps are described that follow the unique stimulation of the fiber muscle:

- There is a latency phase of approximately 5 ms from the initiation of the process to the beginning of the contraction. This is given by the time required to propagate the action potential and the time required to mobilize Ca^{2+} from the sarcoplasmic reticulum.
- There is a contraction phase of about 15 ms when the increased concentration of Ca^{2+} in the cytosol allows actin-myosin coupling that corresponds to muscle shortening and muscular force generation.
- There is relaxation phase, longer than 25 ms, in which the Ca^{2+} concentration in the cell slowly decreases by pumping it into RS, followed by the decrease of the actin-myosin bridges.

Physiologically, all contractions of the skeletal muscles are done by tetanus contraction. Tetanus contraction is a summary of twitches. Strong, efficient, variable-duration contraction is achieved. The contraction of the heart muscle is a response to a single stimulus, but due to the long duration of the action potential, the cardiac twitch is entirely different from the skeletal muscle. Increasing the frequency of stimulation of the muscle fiber generates a continuous and stronger contraction than the twitch. When the stimulus frequency is low during the contraction period,

incomplete relaxation periods will occur, and muscle tension will be inconsistent. This type of contraction is called incomplete tetanus. If the stimulation frequency does not allow relaxation periods during muscle contraction, a plateau of muscle tension appears, and the contraction is called complete tetanus. The developed force is maximal, superior to both twitch and incomplete tetanus contraction [54].

Muscle fiber generates tension through the action of actin and myosin cross-bridge cycling. While under tension, the muscle may lengthen, shorten, or remain the same. Muscle activity in the body is a combination of the isometric, isotonic, and auxotonic forms of contractions. An isometric contraction occurs when the contracting muscle is fixed to both extremities. Thus, the length of the fibers does not change during contraction, but the increase in muscle tension occurs [55]. The anti-gravity muscles, those which maintain the posture, and the masticatory muscles used in the process of crushing food perform isometric contractions. Isotonic contraction is performed by the muscle that raises a weight. During contraction, its length is reduced, but the tension is remaining unchanged. Isotonic contractions are characteristic of the movement of limbs in the process of walking or lifting of constant weight [56]. There are two types of isotonic muscle contraction: concentric and eccentric muscle contraction. In concentric muscle contraction, muscle fibers shorten as tension in the muscle increases, as when lifting a weight. In eccentric muscle contraction, although the actin and myosin filaments within the muscle fibers contract (to produce the force needed), the fibers themselves also slide alongside each other resulting in the overall lengthening of the muscle [57]. Muscle lengthens as tension in the muscle increases, as when slowly lowering a weight. Auxotonic contraction is an intermediate functional manifestation. During the contraction, the muscle shortens but with the progressive increase of the tension. Auxotonic contractions are combined with the previous ones in the work process when the superior muscular force defeats a growing external force [58].

2.3 Biochemical Diversity of Skeletal Muscle

In the last decade, the biochemical, structural, and functional properties of myofibers were intensively studied, but understanding molecular processes regulating fiber-type diversity is still poorly understood, due to the heterogeneity of cell types present in the skeletal muscle organ [2].

Skeletal muscle is a complex and versatile tissue composed of a variety of functionally diverse myofibers which reach their normal length at puberty (13–15 years). Regarding the mean fiber diameter in normal muscles, there are no significant differences between the three muscle fiber types which are less than 12% [59]. Gender difference shows larger myofibers in men than women for type I and type II. In women, type I fibers are larger than type II, while in men these dimensions are reversed. The muscle mass begins to decrease between 20 and 80 years by reducing the number of myofibers by 30–40% [60].

Skeletal muscle tissue is a very heterogeneous one, composed of a bundle of muscle cells which are implicated in a series of activities appropriate to each animal species. To deal with divergent activities, muscles are composed of muscle cells with large differences in metabolic profile and contractile properties, found under the influence of hormonal and neural systems. Moreover, it seems that nerve activity plays a major role in the determination of the fiber type [16]. Skeletal muscle fibers can be classified based on their color (red, high in myoglobin; white, low myoglobin), on their speed (slow, fast, intermediate), on their fatigability (fatigue resistant and fatigable), or on their myosin isoforms.

At the beginning of the nineteenth century, based on their *speeds of shortening*, muscle fibers were defined as slow or fast [61]. In the mid-twentieth century, by refining certain techniques for *myosin ATPase (mATPase) histochemistry* and electron microscopy and by advanced biochemical studies regarding oxidative and glycolytic enzymes, skeletal muscle cells were characterized in much more details. The combination of histochemical analysis for myofibrillar actomyosin ATPase (myosin ATPase) and for enzymes of energy metabolism gives rise to the fiber nomenclature. Also, the speed of contraction is dependent on how quickly the ATPase of myosin can hydrolyze ATP to produce crossbridge action. Based on these criteria, there are three main types of skeletal muscle fibers (cells): slow oxidative (type I), fast oxidative (type IIa), and fast glycolytic (type IIb) [62]. Fast fibers hydrolyze ATP approximately twice as quickly as slow fibers. The fast-twitch muscle fibers are known as the white muscle, while the slow-twitch muscle fibers are known as red muscle. Based on their fatigability, fast-twitch motor units can be categorized as fast-twitch fatigue resistant (type FR), fast-twitch fatigue intermediate (type FInt), and fast-twitch fatigable (type FF) [63].

Slow-contracting muscle fiber (type I) is characterized by (a) low myosin ATPase activity (compared with type II fibers), (b) high capacity for ATP production via oxidative phosphorylation (aerobic cellular respiration), (c) very dense capillary network, (d) high levels of intracellular myoglobin (predominant color is red), and (e) function for long periods without fatigue.

Fast-contracting muscle fiber (type IIa) is characterized by (a) higher myosin ATPase activity than type I fibers, (b) high capacity for ATP production via oxidative phosphorylation (aerobic cellular respiration), (c) dense capillary network, (d) high levels of intracellular myoglobin (predominant color is red), and (e) being more fatigue resistant than type IIb fibers.

Fast-contracting muscle fiber (type IIb) is characterized by:

(a) Higher myosin ATPase activity than type I fibers.
(b) Lower capacity for ATP production via oxidative phosphorylation than “red” fibers (anaerobic glycolysis); muscle fatigue occurs sooner.
(c) Sparser capillary network.
(d) No intracellular myoglobin (predominant color is white).
(e) These fibers fatigue quickly.

Type IIb fibers can be converted into type IIa fibers by resistance training.

Details about all these fibers can be found in Table 2.1.

Table 2.1 Comparison between the three main types of skeletal muscle fibers

Characteristic	Red/slow (type I) slow-twitch fibers	Red/fast (type IIa) fast oxidative fibers	White/fast (type IIb) fast glycolytic fibers
Color	Red	Red	White
Contraction speed	Slow	Fast	Very fast
Oxidative capacity	High	High	Low
Resistance to fatigue	High	Medium (intermediate)	Low
Diameter (of muscle fiber)	Small	Medium (intermediate)	Large
Capillary density	High	Medium (intermediate)	Low
Mitochondrial density	High	High	Low
Glycogen reserves	Low	Intermediate	High
Myosin ATPase activity	Low	High	High
Main (metabolic) pathway for production of ATP	Aerobic cellular respiration – final stage: oxidative phosphorylation	Both aerobic and anaerobic metabolic pathways	*Only* anaerobic metabolism, esp. anaerobic glycolysis
Anaerobic enzyme content	Low	Medium	*High*
Force production (i.e., force produced by muscle)	Low	Medium-high	Very high
Example of typical use	Repeated low-level contractions, e.g., walking or low-intensity cycling for 30 mins.	Used primarily for movements, such as walking (require more energy than postural control but less energy than sprinting). Activities involving speed, strength, and power	Used to produce rapid, forceful contractions to make quick, powerful movements. Short, fast, bursts of power such as heavy weight training, power lifting, and sprints
Examples of skeletal muscles with this type of fiber	Postural muscles of the neck and spine, leg muscles (type I and type IIa fibers)	Leg muscles (large quantities of both type I and type IIa fibers)	Arm muscles

Table 2.2 Panel of sarcomeric MHC genes with the corresponding protein products and their location

Gene	Proteins	Expression
MYH13	MyHC-EO	Extraocular muscle
MYH8	MyHC-neo	Developing muscle
MYH4	MyHC-2B	Fast 2B fibers
MYH1	MyHC-2X	Fast 2X fibers
MYH2	MyHC-2A	Fast 2A fibers
MYH3	MyHC-emb	Developing muscle
MYH6	MyHC-α	Jaw muscle and heart
MYH7	MyHC-β/slow	Slow muscle and heart
MYH7b	MyHC slow/tonic	Extraocular muscle
MYH15	MyHC-15	Extraocular muscle
MYH16	MyHC-M	Jaw muscle

Another classification system is based on *myosin heavy chain (MHC) isoforms*, and the heterogeneity of myosin isoform expression dates back to 30 years ago [64, 65]. Originally, four major myosin isoforms were identified: MHCI, MHCIIa, MCHIIx, and MHCIIb [66–68]. Recently, myosin ATPase histochemical staining allows the description of some other types, such as Ic, IIc, IIac, and IIab, based on the intensity of staining at different pH levels [69, 70]. Several isoforms of MHC are known to exist in mammalian skeletal muscle including IIm, alpha, neonatal, embryonic, and extraocular. These isoforms can be determined using anti-myosin antibodies or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [71]. Nowadays, one knows that these MHC isoforms are first established by intrinsic myogenic control mechanisms during embryonic development and are later modulated by neural and hormonal factors [9]. According to a study conducted by Schiaffino, in any muscle, different fiber types coexist. One can observe in Table 2.2 the complete panel of sarcomeric MHC genes with the corresponding protein products proposed by Schiaffino in mammalian species extrafusal muscle fibers [16].

2.4 Conclusion

Skeletal muscle physiology is complex, and there are many functional differences between fiber types starting with neuromuscular transmission, excitation-contraction coupling, and cycling of crossbridges and finishing with ATP consumption. Gene and protein expressions depending on the type of fiber are still at the beginning regarding their importance in several conditions leading to muscle atrophy.

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Chapter 3
Muscle Mass, Quality, and Composition Changes During Atrophy and Sarcopenia

Yosuke Yamada

Abstract Skeletal muscle mass (SMM) and muscle strengh reach their peak in 20s to 40s of age in human life and then decrease with advancing age. The decrease rate of muscle strength or power was twice to four times as large as that of the SMM. Thus, the normalized muscle force (muscle strength divided by SMM) also decreases in aging. It depends on the number of factors in skeletal muscle tissues and neuromuscular system. In human study, SMM cannot be measured directly without dissection so that all of the methodologies are indirect methods to assess SMM, even computing tomography or magnetic resonance imaging. Dual-energy X-ray absorptiometry, ultrasonography, anthropometry, and bioelectrical impedance analysis (BIA) are used as secondary indirect methods to estimate SMM. Recent researches show muscle composition changes in aging, and in particular, the ratio of muscle cell mass (MCM) against SMM decrease and relative expansion of extracellular water (ECW) and extracellular space is observed with advancing age and/or decrease of physical function. The intracellular water (ICW) and ECW estimated by segmental bioelectrical impedance spectroscopy or multifrequency BIA are good biomarkers of the ratio of MCM against SMM in limbs. The BIS and other state-of-the-art technology for assessment of muscle mass, quality, and composition are useful to fully understand the muscle atrophy in a living organism.

Keywords CT · MRI · DXA · BIS · BIA · Frailty · Cachexia · Muscle cell mass · Lateral force transmission

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3.1 Introduction

Muscle strength generally reaches its peak in 20s to 40s of age in human life and then decreases with age. Skeletal muscle mass (SMM) also decreases with age (Figs. 3.1 and 3.2). The study of Allen et al. (1960) was probably the first scientific report about SMM decrease with age [1]. Allen et al. reported that muscle mass is decreasing with age by calculating total body potassium (TBK) via whole body counter, using the fact that a small amount of radioisotope ^{40}K exists naturally. In this method, based on the hypothesis that the potassium volume (concentration) in body cell mass (BCM) is constant, the BCM was estimated from the TBK, and then the BCM was used as an index for skeletal muscle mass [2, 3].

Since then, various methods such as X-ray computed tomography (CT) and magnetic resonance imaging (MRI) have been invented (Figs. 3.1 and 3.2). Using these methods, the SMM change with age in the human body has been examined in many researches. In the systemic review for the SMM change with age by various measurement methods [4], the SMM decreased only 0.37% per year in female and 0.47% per year in male when compared with the young adult (18 to 45 years old) to the elderly (65 years old or over). The decrease rate of muscle mass per 10 years drops more steeply after a certain age (i.e., 50 to 65 years old) than younger age; the longitudinal study that assessed in older adults (65 years old or over) over 5 to 12.2 years showed that the decrease rate was approximately 0.51% [4]. The decrease rate is much lower than muscle strength.

The longitudinal study with the elderly showed the muscle strength decreased 2.5 to 3% in female and 3 to 4% in male in a year. In the cohort that muscle mass and muscle strength were measured at the same time (e.g., Baltimore Longitudinal Study and Health ABC study), the decrease rate of muscle strength was twice to four times as large as that of the SMM [5, 6] (Fig. 3.3). Furthermore, it is clear that low muscle strength rather than low SMM is a risk factor for mobility disability and mortality [7–9]. In consideration of the above, the meaning of muscle mass or strength measurement has become a controversial topic; it has been discussed that "dynapenia," which focuses on age-related loss of muscle function, is probably more useful than "sarcopenia" which is mainly considered on age-related loss of SMM [10, 11].

The term "sarcopenia" was originally created by Rosenberg at a meeting summary (1989) [12] of "Epidemiologic and methodologic problems in determining nutritional status of older persons (Albuquerque, New Mexico, USA, October 19–21)" in 1988. In its proceedings, Rosenberg mentioned that "the prevention and/or attenuation of decreasing lean mass with age" is one of the most important public health issues for exercise and nutrition for older adults and coined sarcopenia from Greek words σάρξ sarx, "flesh," and πενία penia, "poverty." Rosenberg summarized the meeting to introduce what the meeting was like and what the sentence meant [12].

One out of 25 persons was the elderly population (65 years old or over) in 1900, 1 out of 9 in 1989, and then 1 out of 5 in the twenty-first century. Drs. Samet, Rhyne,

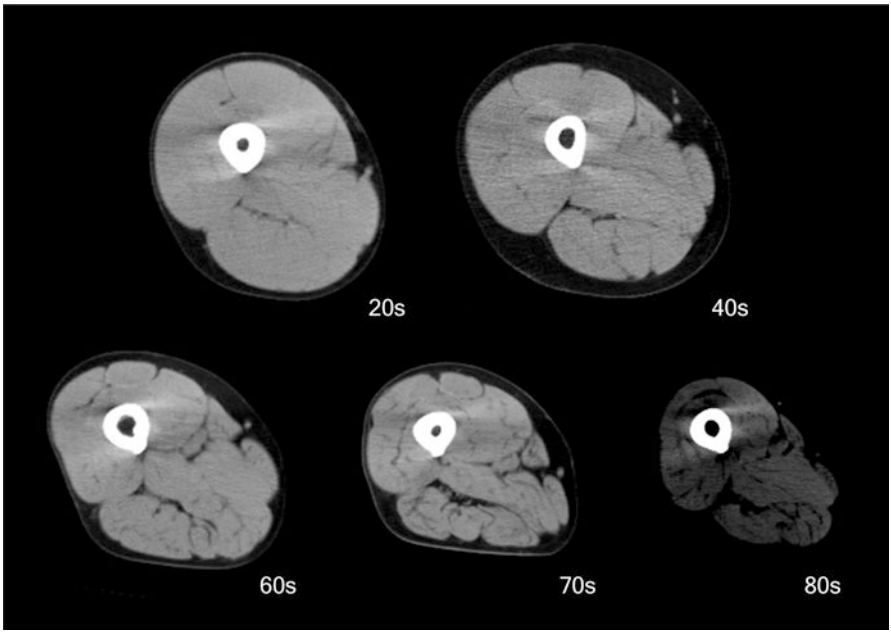

Fig. 3.1 Typical example of mid-thigh cross-sectional area (CSA) obtained by X-ray computed tomography (CT) in each age individual. Skeletal muscle CSA (gray area) is decreased with advancing age. In addition, the signal intensity of muscle area became low with advancing age. (The figure is reprinted from Yamada 2015 [2] with permission (see detail in Sect. 6 in this chapter))

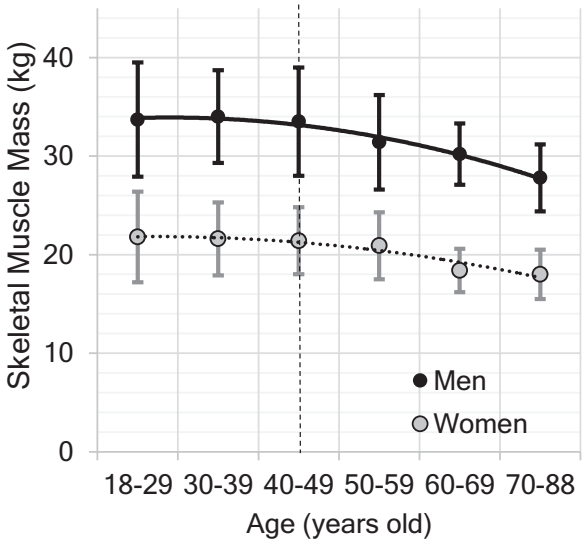

Fig. 3.2 Relationship between age and whole-body skeletal muscle mass assessed by magnetic resonance imaging (MRI). (The figure was created based upon Table 1 of Janssen et al. 2000 [3] for the present article by Yamada)

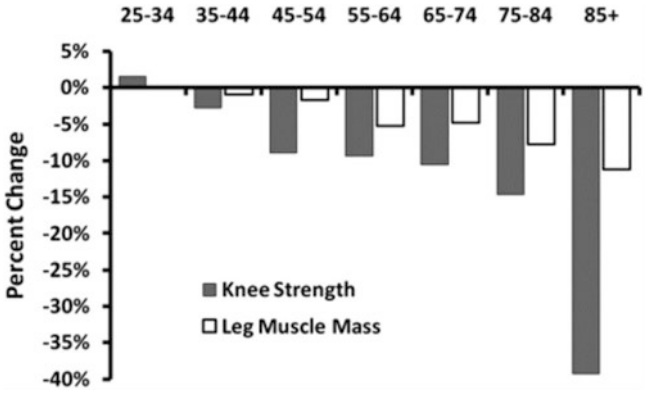

Fig. 3.3 Changes of knee extension strength (KES) and leg muscle mass (LMM) in Baltimore Longitudinal Study of Aging. KES was measured by isokinetic dynamometry, and LMM was assessed by dual-energy X-ray absorptiometry (DXA). The rate of decline for both parameters is steeper with older age (in particular, 45+ and 75+); the decrease rate of muscle strength was twice to four times as large as that of the muscle mass. (The figure is reprinted from Ferrucci et al. 2012 [5] with permission)

Harris, Hegsted, and Goodwin et al. [13–17] emphasized the diversity of elderly in the meeting; there is not only non-negligible differences between a 65-year-old and an 80-year-old person (chronological age) but also inter-individual variation of aging (biological age) which is different from chronological age. There are also difference in races, ethnicity, and sex. Furthermore, the activity level of elderly varies: some are independent and active, some cannot leave home, and others stay in the nursing home. Some uses multiple medications, which affects to the body and mental functions. We must conduct research for all those elderly since we cannot evaluate the populations of "normal aging" or "normal nutritional status" if we use the cohort of only elderly who visit a hospital, excluding active healthy elderly, or the cohort of elderly excluding persons who are charged in the nursing home or cannot leave home. Therefore, the method we should use is to evaluate various old population including a marathon runner and a person who needs nursing care, to clarify the effect of decreased function of each organ with age to food and nutritional conditions, and to have better understanding for the influence of food and nutrition to the maintenance or decreased function of each organ. From the NHANES, National Health and Nutrition Examination Survey, III (from 1988 to 1994), Harris and Kuczmarski et al. [15, 18] revealed these problems applying oversampling technique for 5000 elderly including 1300 who were older than 80.

Drs. Kuczmarski, Chumlea, Heymsfield, and Schoeller [18–21] lectured about body composition assessment method in the meeting, which is essential for nutritional status assessment. Each method has both advantages and disadvantages. Because of recent drastic progress of body composition assessment method, it is possible to evaluate various compositions instead of using a traditional two-composition model (fat and lean mass). Thus, using these methods, it is necessary

to have a wide variety of data including the abovementioned race and ethnic differences. Rosenberg asseverated that there is no important dramatic functional change with age other than lean mass change. Decreased lean body mass influences on various aspects such as mobility ability, physical functions, energy (calorie) intake and expenditure, nutrient consumption, nutritional condition, independence (nursing care requirement), cardiovascular function and/or respiratory function. To pay more attention to lean mass decrease, Rosenberg proposed the term sarcomalacia/sarcopenia and suggested that more research should be conducted for the relationship lean mass decrease and exercise. Muscle mass would be increased even in the elderly, and the elderly with frailty would drastically improve physical function.

In summary, Rosenberg [12] picked up Dr. Hegsted's topic related to recommended dietary allowance (RDA) [16]. What is the role of RDA for elderly with wide variety of characteristics? When it comes to the recommended food to maximize one's healthy living and to maintain activities in one's life cycle, it is necessary to understand the diversity and variability in young and old women and men.

Sarcopenia was originally the proposed term to proceed the research about loss of lean mass during age considering appropriate nutrition and exercise for each old person with understanding of variety of old people in the meeting summary comment. However, as it is mentioned above, from the results that many researches had proceeded focusing on muscle mass and strength since 1990, the risk for mortality and/or loss of physical function and independence cannot be fully explained by only muscle mass.

Therefore, the European Working Group on Sarcopenia in Older People (EWGSOP) in 2010 [22], the International Working Group on Sarcopenia (IWGS) in 2011 [23], the Asian Working Group for Sarcopenia (AWGS) [24], and the Foundation for the National Institutes of Health (FNIH) Biomarkers Consortium Sarcopenia Project [25] in 2014 defined sarcopenia as low muscle strength and/or low physical function in addition to SMM.

In those consensus, muscle strength and physical function are important components of sarcopenia, but the assessment of muscle strength and/or physical function is not sufficient to apply a medical diagnosis under the precedent of the medical diagnosis of osteoporosis or metabolic syndrome. The SMM is still used as a primary marker, which is a more objective parameter than voluntary force production or conducting physical function test [26–31].

It is, however, not easy to assess human's SMM *in vivo* accurately, and its definition is needed to be reconsidered. Especially, I would like to explain the concept of in vivo SMM is different from that of "muscle cell mass" (MCM). The ratio of MCM against SMM (MCM/SMM) changes with advancing age.

All methods of assessing SMM are indirect methodology since human body composition cannot be measured directly except for cadaver. As they are indirect methods, there are always hypotheses. The results of any indirect methods have systematic and/or random bias from those of direct measurement [32]. Therefore, when body composition is mentioned, the term "estimate, assess, or calculate" is used; avoid using the term "measure" in this article.

3.2 Estimate of Skeletal Muscle Mass (SMM) in Human Body

It has been tried to estimate SMM as one of the body compositions along with the fat and bone mass [1, 33]. In relation with obesity, the amount of body fat or percent body fat against body mass has been focused along with visceral fat, ectopic fat, hyperglycemia, hypertension, and hyperlipidemia. Bone mass and bone mineral content has been given attention with bone density, bone metabolism markers, and spine morphology because of its relationship with osteoporosis and risk of fracture. The SMM has been given importance in complex metabolic disorder syndrome (cachexia) that is characterized by the loss of muscle mass observed with drastic weight decrease in patients with chronic disease and myopathy such as muscular dystrophy and amyotrophic lateral sclerosis (ALS); however, the establishment of its clinical meaning in non-disease adult is delayed in comparison with body fat amount (obesity) and bone mass (osteoporosis).

On the other hand, in sports science area or exercise physiology, skeletal muscle mass assessment has been conducted relatively early because skeletal muscle mass has strong correlation with muscle strength or power which is one of the essential sport performance factors [34]. After various imaging methods and other estimation methods are invented, the research using assessment of muscle mass or muscle mass distribution has been performed strenuously [3, 34–44]. Especially, CT and MRI are currently considered as standard methods to estimate whole-body skeletal muscle volume or mass (e.g., skeletal muscle tissue density, 1.041 g/cm^3 [45]) since they can estimate the total volume of whole-body skeletal muscle tissue by filming the whole body and extracting signal from skeletal muscle tissue. Dual-energy X-ray absorptiometry (DXA) is considered an alternative method to separate bone mass, adipose mass, and other soft lean tissues. It does not estimate whole-body SMM itself that is different from MRI and CT; however, appendicular lean soft tissue (ALST) estimated by DXA can be converted to SMM measured by MRI (at least in American) using the equation by Kim et al. [46].

3.3 The Difference of Age-Related Decreases Between Muscle Mass and Strength

In consideration with the above, muscle strength decreases 2.5 to 4% in a year, but SMM decreases only 0.5 to 1% [4]. To scrutinize Janssen et al. [3] research which measured skeletal muscle mass by MRI in 468 females and males with age from 18 to 88, the SMM difference of 20s to 70s in the upper body is approximately 8%. The SMM difference of 20s to 70s in the lower body is ~26% in male and ~23% in female; the decrease rate of lower body is about three times as high as that of the

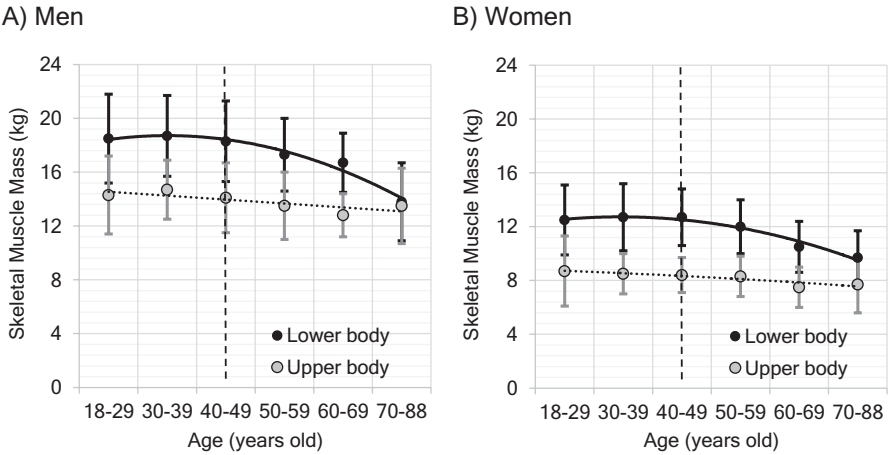

Fig. 3.4 Relationship between age and skeletal muscle mass (SMM) in the lower body and upper body in 268 men (**a**) and 200 women (**b**) aged 1888 years old. The SMM was assessed by MRI, and its difference of 20s to 70s in the upper body is approximately 8%. The SMM difference of 20s to 70s in the lower body is ~26% in male and ~23% in female; the decrease rate of the lower body is about three times as high as that of the upper body, but it is still only about 0.5% decrease in a year. (The figure was created based upon Table 1 of Janssen et al. 2000 [3])

upper body, but it is still only about 0.5% decrease in a year. It is worth noting that there is a significant difference in decrease rate between muscle groups even in the lower body muscles. Assessing for muscle thickness change of each body part with age, ultrasound imaging device has been especially used for many previous researches [34, 44, 47–53]. For example, when it is measured by ultrasound imaging, the decrease rate of the front thigh is greater than that of the back thigh [42, 43, 54, 55]; the decrease ratio of 20s to 70s in the front thigh muscle thickness is ~30%. These values are very similar to the direct measurement of cross-sectional area (CSA) of the vastus lateralis muscle in the cadavers by Lexell et al. [56]; the decrease ratio of 20s to 70s was ~26% (Fig. 3.4).

With all the above considered, the measurement sensitivity of muscle mass change is higher in using MRI or CT than in using traditional two-component method of lean mass estimation. Furthermore, the measurement of muscle groups, which atrophy rate is large, such as muscle mass in the lower body, is seemingly more useful than that of the whole-body muscle mass for the relationship with physical function. However, this explains only 20 to 50% of muscle force or its decrease rate, and the rest of 50 to 80% can be explained by, what we call, "factors other than SMM decrease" [4]. For these "factors other than SMM decrease," "neural factors" that include from central nerve to neuromuscular junction have been considered as major factors. Various researches have been proceeded, however, and other potential factors of neural factors are also discussed recently as described in the following sessions.

3.4 Concept About Skeletal Muscle Cell Mass (MCM)

In the abovementioned cadaver research by Lexell et al. [56], in addition to measurement of vastus lateralis CSA, myofiber number, myofiber size, and the ratio of fast muscle fiber to slow-twitch fiber were also measured under the microscope (Fig. 3.5a and b). Scrutinizing this research data brings about significant meanings. The CSA decrease rate of 20s to 70s was ~26%, but the myofiber number decrease ratio was up to 41%. The decrease rate of mean CSA of one myofiber was ~11% (Type I myofiber, ~0% decrease; Type II myofiber, ~25% decrease). Thus, from the values in literature, when I calculate "total myofiber CSA" using the equation of myofiber number multiplied by mean one myofiber CSA, the decrease rate of 20s to 70s is ~48% [57, 58]. This shows that the proportion of myofiber (cell) area to whole-muscle CSA is decreased with advancing age. SMM decrease rate with age is different from MCM decrease rate (Fig. 3.5c). As implied by Fig. 3.1a, this is because intercellular gap becomes large. Intercellular gap includes connective tissue, adipose outside of muscle cell, and extracellular water (ECW) (Fig. 3.5).

Normal imaging methods, like MRI, CT, or DXA, cannot evaluate this intercellular gap, and this results in overestimating muscle cell mass. Skeletal muscle is not a homogeneous tissue and composed of MCM, extracellular space (ECS), and adipose tissue mass (ATM) in its cell level (Fig. 3.2) [59]. Since the MCM gives tension, the assessment of MCM and/or the ratio of MCM/SMM is essential. It is well known that the proportion of ATM to SMM increases with advancing age; except for this, the MCM/SMM changes if ECS and MCM ratio changes. The ratio of solid to liquid in the MCM (intracellular water, ICW), the ratio of solid to liquid in the ECS (extracellular water, ECW), and the ratio of water in the ATM (adipose tissue water, ATW) are not always constant but can be considered to be relatively stable as 0.72, 0.97, and 0.14 in normal hydration status of homeostasis, respectively. Therefore, in this case, the ratio of intracellular water to total water (TW) in the skeletal muscle tissue (ICW/TW) can be considered an index for the MCM/SMM (Fig. 3.6).

3.5 Estimation Method of MCM/SMM

Segmental bioelectrical impedance spectroscopy (BIS) or multifrequency bioelectrical impedance analysis (MF-BIA) is useful to assess the ratio of ICW/TW that is related to the MCM/SMM. The detailed explanation for BIS and MF-BIA was described in our previous articles [60, 61] (Fig. 3.8), which is briefly summarized below. Muscle cell membrane is composed of phospholipid bilayer and works as a capacitor on the alternating current circuit. The alternating current with low frequency (e.g., 5 kHz) cannot pass through inside of cells and mainly pass through extracellular space. On the other hand, the alternating current with high frequency (e.g., 250 kHz or 500 kHz) can pass through inside of cells [62] (Fig. 3.4a). Since

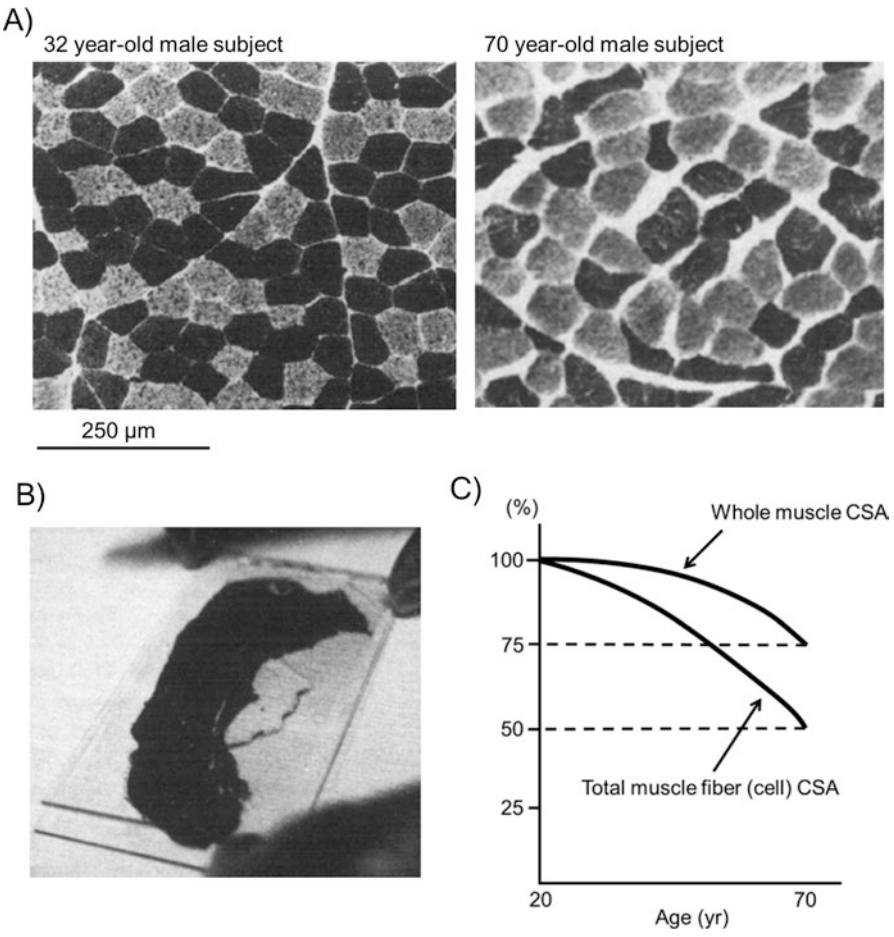

Fig. 3.5 (**a**) Micrographic picture of cross section of m. vastus lateralis from a young (left) and an old (right) individual. (Originally from Lexell et al. 1988. The scale of the picture from old individual was modified to match into the scale of the younger one by Yamada.) (**b**) The picture of prepared cross section of m. vastus lateralis for measurement of cross-sectional area (CSA). (**c**) The rate of loss of whole-muscle CSA and total muscle fiber (cell) CSA. Total muscle fiber CSA was calculated as muscle fiber number multiplied by mean fiber size by Yamada 2015. (Figures A and B are reprinted from Lexell et al. 1988 [56] and Fig. C is reprinted from Yamada 2015 [32] with permission)

the ICW/TW is relatively stable in normal young adults and there is strong correlation among TBW, ICW, and ECW [63, 64], single-frequency bioelectrical impedance analysis (SF-BIA) using 50 kHz is sufficient to evaluate skeletal muscle mass [65, 66]. For example, Miyatani et al. research [65] in young adults showed that, with impedance value at 50 kHz (Z_{50}), the impedance index (L^2/Z_{50}; L, segment length), which is an index related to muscle mass in the upper leg, lower leg, upper

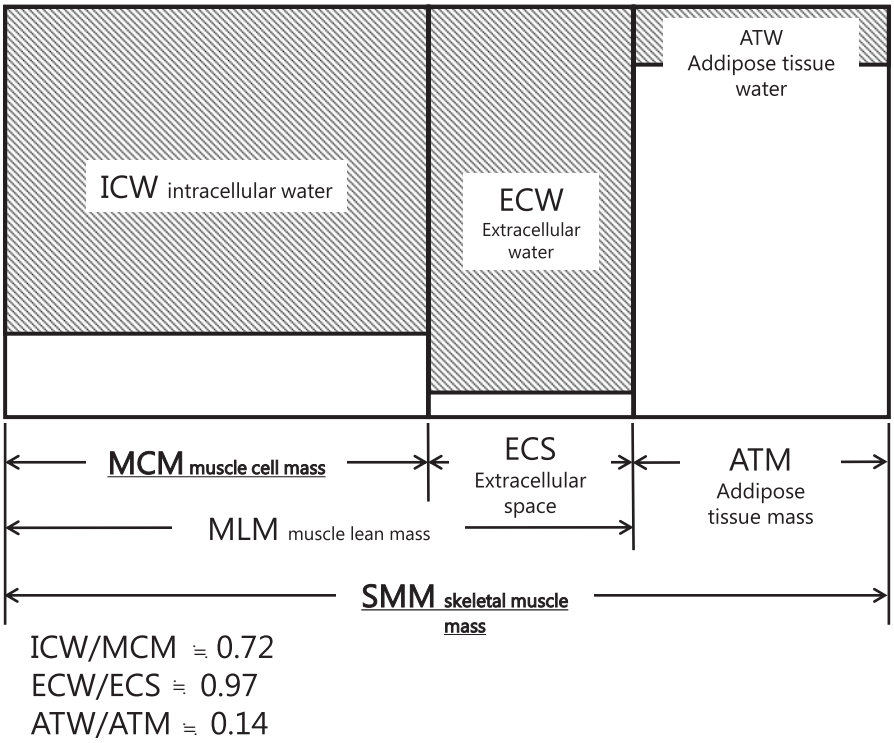

Fig. 3.6 Model of muscle composition (Mingrone et al. 2001). Skeletal muscle contains not only "contractile" tissue but also "non-contractile" tissue. Inter-muscular adipose tissue and intramuscular fat and extracellular water are "non-contractile" components in muscle tissue. (The figure is reprinted from Yamada 2015 [32] with permission)

arm, and forearm, was highly correlated to SMM obtained by MRI and maximal voluntary joint torques of corresponding muscle groups (Fig. 3.7).

On the other hand, in our research with 405 old female and male participants aged 65 to 90 years old [60], the impedance index of 50 kHz in the upper leg segments (L^2/Z_{50}) was just moderately correlated to maximal voluntary knee extension strength. This means the muscle mass must be evaluated in consideration with the ICW/TW change with age in the elderly [67]. Actually, the relative expansion of ECW and decrease of ICW/TW were observed in older adults compared with younger adults (Fig. 3.8). We, therefore, proposed to use the segmental MF-BIA for skeletal muscle mass evaluation and validated it against CT [68]. While the traditional method overestimates muscle mass in the people who have larger ECW/ICW ratio, the newly developed segmental MF-BIA can evaluate muscle mass properly in the elderly since the impedance value combination of 250 kHz and 5 kHz can discriminate ICW from ECW. In addition, this method shows more significant correlation in muscle strength in the elderly in comparison with the traditional method [60]. This index is also correlated to walking speed in the elderly [69] (Fig. 3.9).

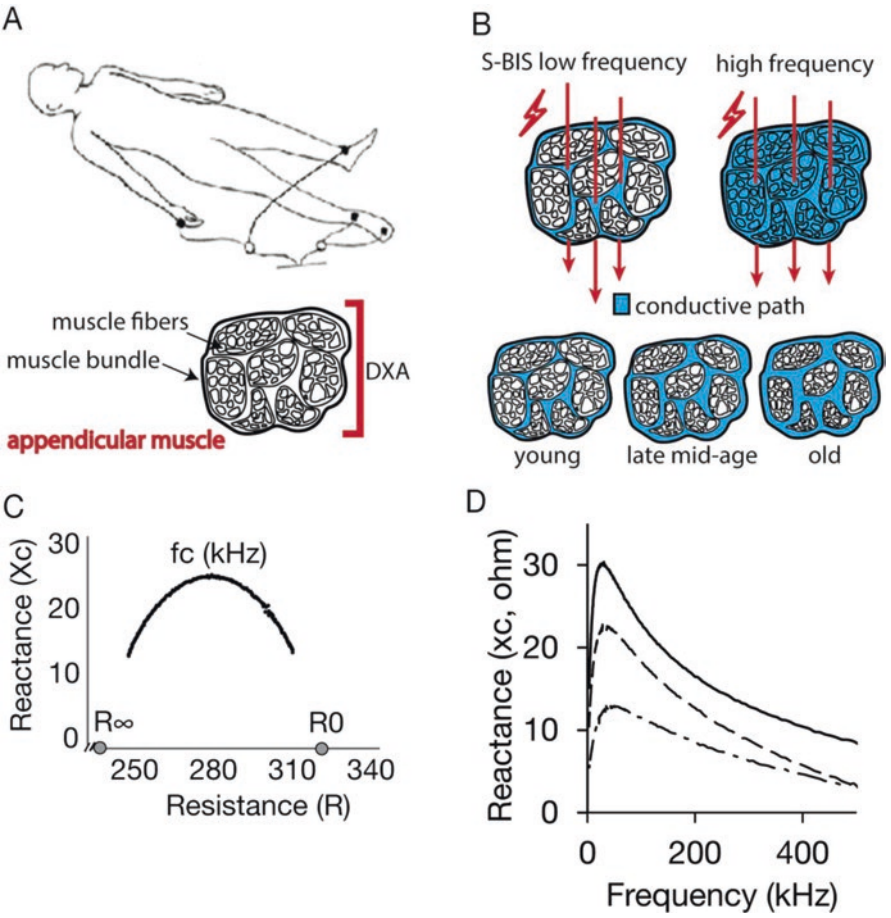

Fig. 3.7 (**a**) Upper panel: electrode placements of segmental bioelectrical impedance spectroscopy (S-BIS) measurement for a single leg. Lower panel: schematic representation showing muscle mass detection by dual-energy X-ray absorptiometry (DXA) and S-BIS. DXA measures appendicular lean mass and cannot inform about lean mass composition. (**b**) S-BIS takes advantage of the partitioning of contents in appendicular skeletal muscle between intracellular and extracellular pools. (**c**) Representative Cole-Cole plot of resistance versus reactance measures obtained by leg S-BIS from one individual from the study cohort. The intracellular resistance (R_I) was calculated using $1/[(1/R_\infty) - (1/R_0)]$. (**d**) Representative frequency versus reactance measures obtained by leg S-BIS from 29-, 56-, and 76-year-old female adults (solid line, dashed line, and chain line, respectively). Older adults tended to have lower reactance. (The figure is reprinted from Yamada et al. 2017 [61] with permission)

While this method used fixed frequencies of 250 kHz (or 500 kHz) and 5 kHz [63, 70], various frequency currents ranging from 1 to 1000 kHz (BIS; Fig. 3.4b) were used in the other method [71, 72]. Resistance values (R_0 and R_∞) at 0 kHz (direct current) and infinite frequency (∞ kHz) obtaining from Cole-Cole plot of resistance (R) vs. reactance (Xc) resulting in a semicircular arc, BIS characterizes

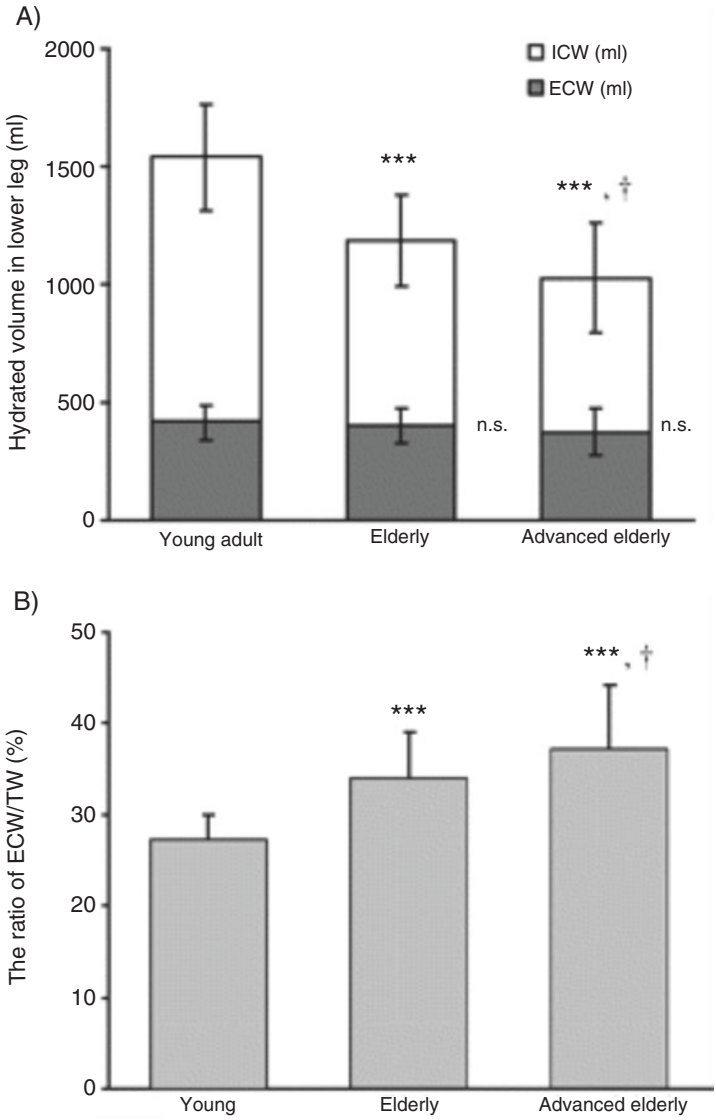

Fig. 3.8 Water distribution in the lower leg estimated by S-BIS (mean ± SD). (**a**) ***significantly lower intracellular water (ICW) than young adult ($p < 0.001$); †significantly lower ICW than elderly adults. No significant main effect was observed in extracellular water (ECW). The total bar shows the sum of ICW and ECW (total water [TW]). (**b**) The ECW/TW ratio increased significantly with aging. ***significantly higher than young adult ($p < 0.001$); †significantly higher than elderly adults. (The figure is reprinted from Yamada et al. 2010 [67] with permission)

the measurement segment for ECW and ICW. There is another model that is the combination of this model with the emulsion electrochemical model [64, 72] by Dr. Tetsuya Hanai (Hanai mixing theory) [73]; this is beyond scope of this article.

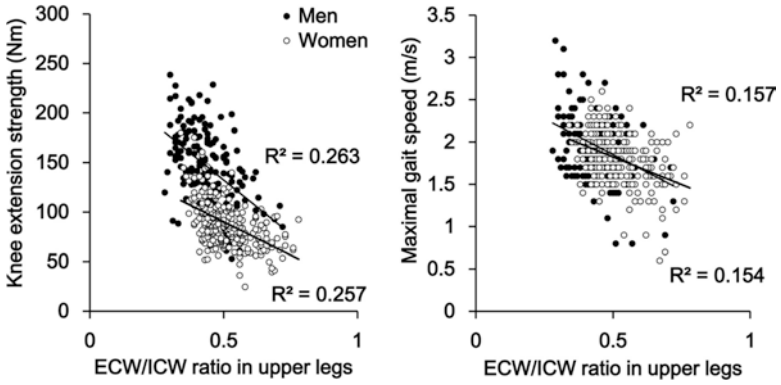

Fig. 3.9 The relationships between the ratio of extra- and intracellular water (ECW/ICW) in the upper legs as assessed by segmental bioelectrical impedance spectroscopy (S-BIS) and isometric knee extension strength (**a**) and maximal gait speed (**b**). ○ women and ● men. (The figure is reprinted from Yamada et al. 2017 [69] with permission)

The BIS method is theoretically reflected to ECW and ICW more precisely [64]. But when there is correlation coefficients with the muscle strength or power were compared between MF-BIA and BIS, there is no significant difference between MF-BIA and BIS. Although BIS is more strictly stick to the theory but reactance measurement is difficult especially at lower or higher frequency, R_0 and R_∞ that are calculated by extrapolation method of curve regression may have a large margin of error. It is, therefore, meaningful to directly use impedance at 250 kHz or 5 kHz that has less error cause [60]. Note that there is an alternative way for SF-BIA to use Xc and phase angle information to obtain body compositions [74, 75] and electrical characteristics of BIS are related to muscle function [61]. Additionally, most recent study shows that appendicular ICW estimating BIS have interesting information for sarcopenia [76].

Impedance is influenced not only by the amount of water but ion concentration in the fluid; thus, it is required to use assumption for the specific resistance of ICW and ECW. In relationship K^+ ion and BCM or ICW in the elderly [77], TBK/FFM or TBK/TBW decreases with age in the whole-body measurement, but TBK/BCM and TBK/ICW are constant [78]; this is supported by the data in rat exenterate skeletal muscle [79]. Therefore, ICW can be considered the index to reflect MCM. As another issue, the change of ICW/TW in the limbs with age obtained by BIS or MF-BIA is seemingly greater than that of ICW/TBW in the whole body in physiology field. This may be partly because few research has been conducted in elderly with age over 80; it is necessary to perform the investigation of skeletal muscle compositions in various ages. It is also necessary to evaluate edema, inflammation, body fluid shift after exercise or posture change, or the influence on various diseases [80–82].

3.6 Relationship Between Muscle Composition and Muscle Function

Whenever BIS or MF-BIA is used, ICW in the limbs, which is reflected to MCM, decreases with age [67], especially the elderly who require nursing care shows low ICW in the limbs [77]. In comparison with a traditional muscle mass index, ICW shows stronger correlation to muscle strength, muscle power, and ability to stand up from the chair; it is possible to discriminate the requirement of nursing care with good sensitivity and specificity. In addition, ICW/TW, which is a biomarker of MCM/SMM, also decreases with age and especially shows low value in the elderly who require nursing care. Interestingly, ICW/TW, being independent of skeletal muscle index of ICW, is also statistically significantly correlated to muscle strength, muscle power, and ability to stand up from the chair. ICW/TW decrease reflects the decrease of the ratio of muscle cells per unit volume; it is also the index for relative expansion of ECW or dilatation of extracellular matrix, connective tissue, or adipose tissue between muscle cells. The relationship between this index and the increase of adipose tissue mass and connective tissue must be scrutinized; if the density of muscle fiber is low (low muscle density), the decrease of lateral transmission of force can happen [83].

It is possible to evaluate muscle composition or muscle quality by not only relative increase of ECW by BIS but CT, MRI, diffusion tensor MRI (DT-MRI), Dixon MRI, or ultrasonic image echo intensity [84]. For example, Hounsfield unit (HU), signal strength of CT, is the degree of X-ray attenuation with the following conditions: distilled water at standard pressure (1000 hPa) (STP defined by IUPAC) and standard temperature (0 °C) is defined as 0 HU; the radiodensity of air is defined as −1000 HU. The HU value of the fat tissue is negative (approximately -100 to -50HU) while that of the muscle tissue is positive (approximately 0 to 100HU). Mean HU value of muscle tissue area decreases with age; the proportion of normal-density muscle area (30 to 100HU) decreases, and that of low-density muscle area (0 to 30HU) increases. This fact especially reflects to adipose tissue mass [85, 86]. However, since HU value of water is 0 HU and that of solid mass in the skeletal muscle shows high, mean HU value decreases even if the MCM/SMM decreases. Thus, the low HU value in the elderly possibly also reflects relative ECW increase in addition to adipose tissue mass increase. It is known that adipose tissue mass measured by MRI or a non-contraction factor is high in the elderly [87], the λ value of diffusion tensor MRI changes with age [88], and T2 value of the skeletal muscle at rest is high in the elderly [89]. In addition, in recent years, it is clear that ultrasonic image shows brighter in the elderly than in the young, and its echo intensity is negatively correlated to muscle force [52, 90–92]. Most recent study suggests that ultrasonic image echo intensity is correlated to muscle strength independent of the ratio of intracellular fluid to extracellular by BIS in the elderly [93] (Fig. 3.10).

As it is mentioned above, while muscle force decreases 2.5 to 4% in a year, the SMM decreases only 0.5 to 1% in a year [4]. In contrast, the actual decrease rate of

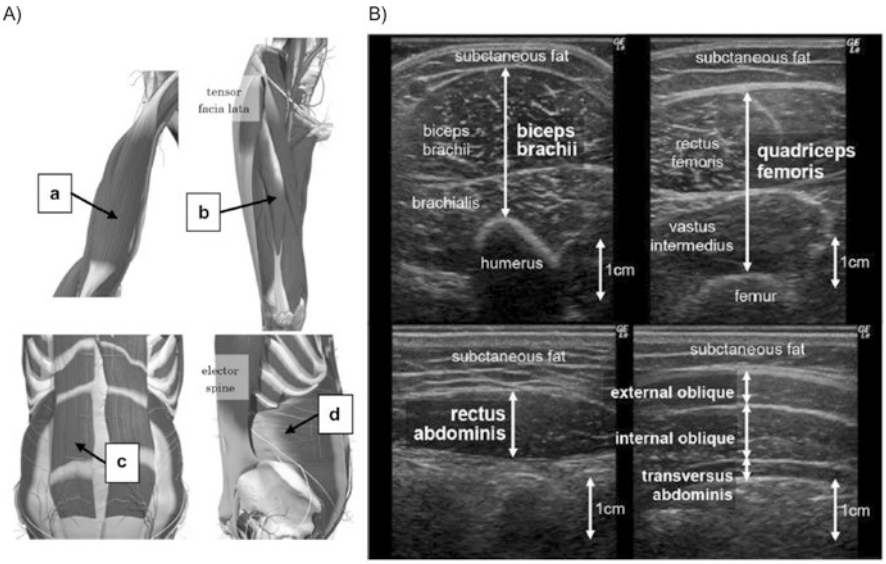

Fig. 3.10 (**a**) Ultrasound sites for each muscle. *a*.. Biceps brachii, two-thirds of the way between the acromion and the antecubital crease. *b*. Quadriceps femoris, midway between the anterior superior iliac spine and the proximal end of the patella. *c*. Rectus abdominis, 3 cm lateral to the umbilicus. *d*. External oblique, internal oblique, and transversus abdominis, 2.5 cm anterior to the midaxillary line, at the midpoint between the inferior rib and the iliac crest. (**b**) Representative ultrasound images. Echo intensity (EI) can be assessed by computer-assisted 8-bit gray-scale analysis using the standard histogram function in Adobe Photoshop Elements (Adobe Systems, San Jose, CA, USA) or other image software as an index of muscle quality. (The figure is reprinted from Fukumoto et al. 2015 [52] with permission)

MCM is thought to be as twice as that of SMM since the composition of muscle changes drastically. However, MCM decrease rate does not explain fully about the muscle force decrease. For this part, as it is mentioned above, ICW/TW (or MCM/SMM) is the factor to explain muscle force independent of ICW; the decrease of myofiber density is probably related to the decrease of lateral transmission of force [83]. But, in addition to this, various changes happen to the muscle tissue and the neuromuscular system [4]. Muscle tissue factors are as follows: the decrease of pennation angle and muscle fascicle length with age [94], selective atrophy of fast muscle fiber with change of its cross-sectional shape (e.g., a crushed shape) [56], qualitative and quantitative changes of extracellular matrix (ECM) [83, 95], decrease in the number of satellite cells relative to the total number of nuclei of muscle fibers [96], increased occurrence of coexistence of myosin heavy chain isoforms in single fibers [97], increased myonuclear domain (MND) size variability [98], and the decrease of Ca^{2+} sensitivity and the reduction of Ca^{2+} release [99]. Age-related change in the tendon tissue also occurred [100]. Neuromuscular system factors are as follows: decrease in the number of motoneurons and the remaining intact motoneurons sprouting to innervate the denervated fibers [101], decrease in α-motoneuron excitability [102, 103], excitability of the motor cortex to the

spine [104, 105], decrease of nerve conduction velocity [106], co-contraction of the antagonistic muscle [107], and elaborated muscle synergy adjustment [108]. However, exhaustive research is required to determine how much degree these factors influence to the decrease of muscle force with aging since there is a literature stating antagonist torques cannot explain the observed torque declines at the knee joint, for example [109].

At any rate, skeletal muscle cell mass in the body may change more drastically than it used to be considered. Ikenaga et al. reported that ICW at the thigh increased when slightly weighted (+200 g) shoe interventions were given to the elderly and the lower and long-term low degree burden (average 10,000 step walking for 100 min a day) was given to the lower limbs [110]. Also, ICW increase in the thigh was observed when weekly 90- to 180-min/wk moderate intermittent slow jogging interventions for 12 weeks were given, although the total muscle CSA obtained by CT was not changed [111].

3.7 Frailty, Sarcopenia, Skeletal Muscle Cell Mass, and Muscle Composition

World turns into the aging, aged or super-aged society, and life expectancy is increasing worldwide. The population of elderly over 75 is drastically increasing. The elderly gradually decreases physical function, daily activity level, and independence with advancing age [112]. This process is called frailty [113, 114]. According to Fried et al. criteria, if one has the presence of three or more of the following five components, one is frail: "shrinking: weight loss, unintentional," "grip strength weakness," "poor endurance and energy," "slowness," and "low physical activity level." "Poor endurance and energy" is included because it is a good indicator of VO_2max and is a predictive indicator of cardiovascular disease. Depending on cohort design, it is possible to determine frailty by just asking all questions, but basic concept of Fried criteria is to use actual measurement values since it consists of "weight (muscle mass) decrease," "grip strength," "aerobic capacity," "walking speed," and "daily physical activity." The concept of this type of frailty seems to be based on factors measured in exercise physiology area [113, 114]. Other several types of frailty indices were also proposed [115–117]. The frailty with or without muscle atrophy is a research topic for healthy life span from rodents [118–122], nonhuman primates [123], and human [114, 115, 124, 125].

The concept of frailty and sarcopenia is overlapped currently, and central component of frailty is considered to be sarcopenia. Since EWGSOP proposed the definition of sarcopenia with advancing age and its diagnosis criteria in 2010 [22, 26], active discussion is ongoing like IWGS [23, 27], FNIH sarcopenia project [28–31, 126], and AWGS [24]. In addition, the concept between sarcopenia, cachexia, and muscle wasting disorders is complex and sometimes confused in research or clinical settings [127–130]. One of the current important issues is that it is difficult to reach international consensus because the prevalence of sarcopenia is different depending

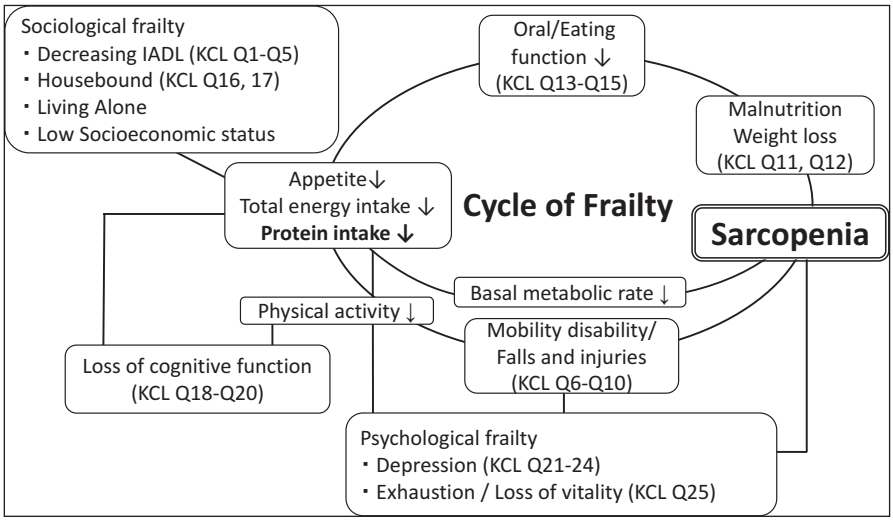

Fig. 3.11 Schematic diagram of the cycle of frailty by the Kihon Checklist (KCL) and its relationship to protein intake. *IADL* Instrumental activities of daily living, *KCL Q* question number of KCL. (This figure is reprinted from Nanri et al. [137] with permission)

on what guidelines and which SMM assessment techniques are used [131–133]. One of the biggest problems is that there is no consensus about how to assess "skeletal muscle mass (SMM)" quickly and easily in clinical settings [134]. For example, since it is not feasible to measure skeletal muscle mass in the whole body by CT or MRI in clinical environments and the measurement by DXA or BIA is device-dependent, there is no absolute method [135, 136]. Furthermore, SMM or CSA by CT that is estimated by ALST via DXA is moderately or poorly correlated to physical function decrease or total death risk [7–9]. To solve this, it is necessary to reconstruct the definition of "skeletal muscle mass." Most recent 4-year longitudinal study found that association of physical activity with age-related changes in quadriceps femoris muscle thickness and echo intensity in older adults [137]. As it is mentioned above, it is necessary to reconsider skeletal MCM and muscle compositions by paying attention to SMM compositions and their quality. In addition, the researches about effects of exercise, physical activity, nutritional status on MCM or SMM and complex frailty cycle are needed for future direction (Fig. 3.11) [138].

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Chapter 4
Muscle Changes During Atrophy

Adrian Dumitru, Beatrice Mihaela Radu, Mihai Radu, and Sanda Maria Cretoiu

Abstract Muscle atrophy typically is a direct effect of protein degradation induced by a diversity of pathophysiologic states such as disuse, immobilization, denervation, aging, sepsis, cachexia, glucocorticoid treatment, hereditary muscular disorders, cancer, diabetes and obesity, kidney and heart failure, and others. Muscle atrophy is defined by changes in the muscles, consisting in shrinkage of myofibers, changes in the types of fiber and myosin isoforms, and a net loss of cytoplasm, organelles and overall a protein loss. Although in the literature there are extensive studies in a range of animal models, the paucity of human data is a reality. This chapter is focused on various aspects of muscle wasting and describes the transitions of myofiber types during the progression of muscle atrophy in several pathological states. Clinical conditions associated with muscle atrophy have been grouped based on the fast-to-slow or slow-to-fast fiber-type shifts. We have also summarized the ultrastructural and histochemical features characteristic for muscle atrophy in clinical and experimental models for aging, cancer, diabetes and obesity, and heart failure and arrhythmia.

Keywords Muscle atrophy · Clinical conditions · Fiber-type shift · Immunohistochemistry markers · Molecular alterations

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4.1 Introduction

Muscle atrophy is also known as muscle wasting and represents a debilitating condition when muscle mass decreases due to several factors. Atrophy of a muscle can occur mainly in two ways, due to disuse or denervation, and it occurs in a variety of pathologies (Fig. 4.1). Malnutrition, alcohol-associated myopathy, aging, obesity, and diabetes can lead to different degrees of muscle atrophy [1–3].

It can also be present in debilitating diseases such as cancer, AIDS, liver cirrhosis, chronic obstructive pulmonary disease (COPD), kidney failure, heart failure, or sepsis [4]. Muscle atrophy can be confined to one muscle group if patients are bedridden or unable to move certain body parts or be more generalized in general pathological states. Muscle atrophy is characterized by the decrease in muscle mass due to the imbalance between protein synthesis and degradation. Denervation atrophy occurs when the muscle nerve is interrupted and the muscle tissue no longer receives stimulation signals from the nervous system. This type of atrophy may arise from damage to the central nervous system such as a spinal cord injury or peripheral nervous system such as a broken bone that destroys the surrounding nerve. Atrophies which usually reflect lower motor neuron deficiency can be found in Guillain-Barré syndrome, neuropathy, amyotrophic lateral sclerosis (ALS), multiple sclerosis, muscular dystrophy, spinal muscular atrophy, etc. [5–7].

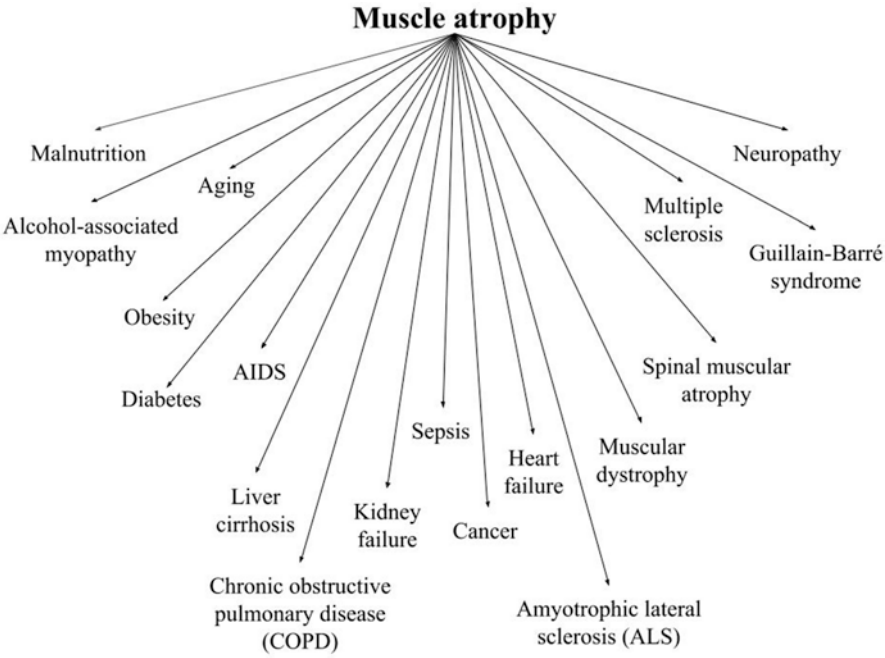

Fig. 4.1 Clinical conditions associated with muscle atrophy

4.2 Histochemical Changes of Myofibers in Muscle Atrophy

Muscle biopsy plays a crucial role as part of the diagnostic assessment for patients with neuromuscular conditions. Accurate histopathological diagnosis and identification of the major pathogenic defects lead to a better understanding of the disease and personalized patient management. Supported by ancillary tests and, if needed, by genetic counseling, the histopathological diagnosis contributes to the development of new therapies. Diagnosis of a muscle biopsy should always be based on proper clinical examination and family history in conjunction with such other useful investigations such as serum enzymes, molecular analysis, muscle imaging, and electromyography. The selection of the muscle should be based on the distribution of the muscle weakness, as judged by detailed clinical data.

Skeletal muscle atrophy can occur due to primary degenerative processes within the skeletal muscle fibers, in genetic or acquired myopathies or secondary to denervation and inflammation or spontaneously during aging. Neurogenic-type atrophy is a descriptive diagnosis with multiple different etiologies, and in such cases, the underlying etiology usually cannot be elucidated by muscle biopsy alone, and it needs correct clinicopathologic or radiologic correlation.

The hallmark histopathological feature of skeletal muscle atrophy is the loss or reduction of myofiber diameter. Affected myofibers are frequently smaller, rounded to angular with hypereosinophilic sarcoplasm. Denervation atrophy is characterized by characteristic histologic features such as compressed angular myofibers and crowded nuclei. Other histopathological features may be found: degenerated, necrotic, or hyalinized myofibers, split or fragmented myofibers, and myofibers with central nuclei. However, these changes are not specific for skeletal muscle atrophy and are more often associated with nonneurogenic causes and more traditional myopathies. Aging-related atrophy is characterized by decreased myofiber size and number, increased variation of myofiber size, and increased accumulation of degenerative inclusion bodies such as lipofuscin or lipid droplets. Also, replacement by connective tissue can be observed. Angulated myofibers are frequently observed, suggesting a possible role of spinal or nerve degeneration. Surrounding unaffected myofibers that are innervated differently may compensate by becoming hypertrophic. Atrophy can uniformly alter myofibers or selectively target specific muscle fiber types. For example, type II fibers are affected when atrophy is associated with cachexia or malnutrition, while type I fibers are selectively affected in thyrotoxicosis and several congenital myopathies and myotonic dystrophy [8]. However, atrophy of type II fibers is non-specific and occurs in a large number of myopathic disorders. It appears in almost any disease in which muscle strength is impaired secondary to problems remote from the muscle [9].

Muscle biopsies are interpreted based on the size of different types of muscle fibers in cross section. Among the most relevant parameters, one usually uses the perimeter of the myofiber, the cross-sectional area, and the smaller or the largest diameter [10]. However, these parameters are variable with age, sex, physical activity, dietary intake, and specific muscle. The concern to see the correlations between

these parameters and the hypo- or hypertrophy of the muscles has existed for a long time, in healthy subjects or pathology. In order to obtain the limits of normality of the cross-sectional areas, Pernus and Erzen analyzed the vastus lateralis muscle and found that the difference between type 1 and II fibers was not significant in size, whereas the differences between type 1 and 2b, type 1 and 2a, and type 2a and 2b fibers were significant [10].

Atrophy is a common occurrence when dealing with a muscle sample examined by conventional histopathological techniques or when using more elaborate ancillary tests. Changes in fiber number and size may specifically affect one or other fiber types or both of them. In healthy muscle, there is a checkerboard, mosaic-like pattern of type 1 and type 2 fibers in the same sample. In most myopathies, there is simultaneous occurrence of atrophic and hypertrophic fibers of both types. For example, in neurogenic disorders, such as spinal muscular atrophy, the groups of atrophic fibers are of both types, while hypertrophy is observed mainly in the groups of type 1 fibers. This is due to the reinnervation of the denervated fibers by surviving collateral nerves. Atrophy of type 2 muscle fibers is a non-specific event that can occur in many myopathic disorders. When dealing with type 2 muscle fiber subtypes, both 2A and 2B may be affected, but the specific involvement of type 2B fibers is more frequently seen. Selective type 2A muscle fiber atrophy is extremely rare and may occur in patients with a gene mutation for 2A myosin (MYH2) [11]. Selective type 1 atrophy occurs in several congenital myopathies and myotonic dystrophy.

The histochemical features of skeletal muscle fibers were used since early 1970 to differentiate between atrophic fibers and fibers with myopathic changes in non-denervated fascicles of juvenile and adult patients with benign spinal muscular atrophy [12]. They found that atrophic fibers contained no glycogen or RNA, acid phosphatase activity could not be demonstrated, and SDH activity was very low, being a mixture of lightly and deeply staining fibers. It has been shown that histochemical changes also appear in nerve root impairment leading to atrophy with a 6.4% decrease in size for type 1 and 9.8% for type 2 muscle fibers [13].

Immunohistochemical studies of developmental isoforms of myosin are very useful for assessing immaturity and helping distinguish between atrophic muscle fibers and regenerating ones. The presence of so-called fetal myosin is frequently rendered to reflect immaturity, but in some situations the presence of fetal myosin is misleading as there is abundant evidence from studies on animal models that immature myosin isoforms can be re-expressed in neurogenic myopathies (denervated muscle) as well as during aging [14]. In humans, nuclear clumps which are chronically atrophic fibers [15], some small muscle fibers in motor neuron disease and spinal muscular atrophy may express fetal myosin and sometimes additional developmentally regulated proteins.

It is generally accepted that human muscles are characterized by the capacity to increase their mass/strength (hypertrophy) and fatigue resistance (oxidative capacity) by training. Muscle fiber changes during different pathological conditions are essential to be studied before optimizing training and rehabilitation programs since

one needs to know the relative contribution of the signaling pathways to protein turnover in high and low oxidative fibers [16].

Several morphological factors may contribute to muscle atrophy, including muscle- and fiber-type heterogeneity, satellite cell diversity, and the susceptibility of different muscles and fiber types to muscle wasting (for review see [17]).

Muscle atrophy can be associated with slow-to-fast or fast-to-slow fiber shift. Depending on the pathological state, two types of fiber shifts have been described (Fig. 4.2): slow-to-fast fiber-type shift [18–23] and fast-to-slow fiber-type shift [24–38].

Skeletal muscle fiber subtypes are otherwise sensitive to specific pathological atrophic signals. Oxidative type I muscle fibers have a higher turnover of protein synthesis and degradation and are more resistant to fasting than type II glycolytic fibers [39]. Contrarily, type I muscle fibers are much sensitive to inactivity, microgravity, and denervation-induced atrophy [40].

In sarcopenia associated with aging was evidenced the fast-to-slow fiber transition in the myosin light chain population (e.g., MLC2 isoform), and this shift in aged skeletal muscles was explained by the tendency of slower-twitching fiber population to switch to a more aerobic-oxidative metabolism [37].

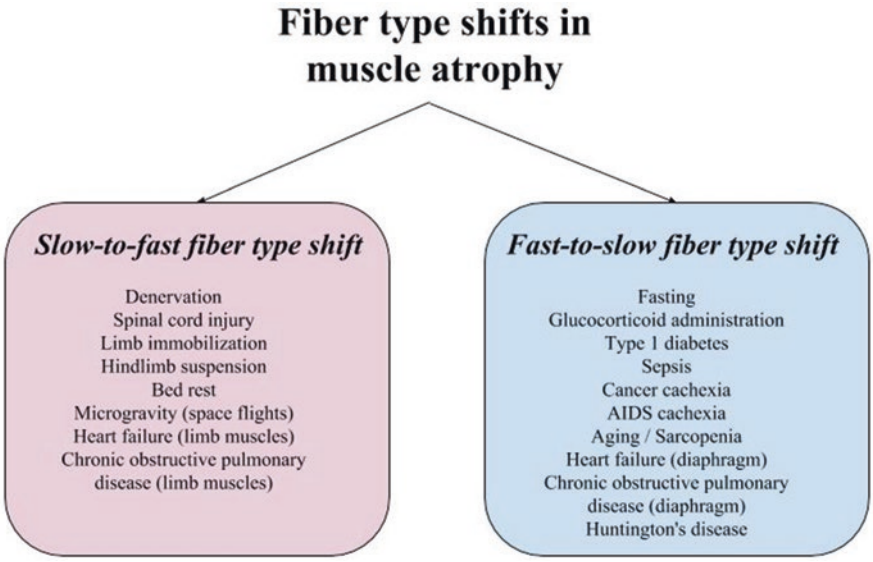

Fig. 4.2 Clinical conditions associated with muscle atrophy and the characteristic fiber-type shifts

4.3 Molecular Alterations in Muscle Atrophy

Muscle atrophy was described to be associated with major imbalances in the ubiquitin-proteasome system [41–59] and/or the autophagy-lysosome system [60–64] (Fig. 4.3).

Among the three components of the autophagy-lysosome system, e.g., macroautophagy, chaperone-mediated autophagy, and microautophagy, only macroautophagy was demonstrated to be involved in muscle atrophy. In animal knockout for lysosomal alpha-glucosidase, it was shown that macroautophagy is upregulated in both slow (type I) and fast (type II) fibers [60]. Downregulation of histone deacetylases was demonstrated to be associated with altered autophagy and consequent muscle atrophy [61, 62]. Abnormal mitophagy and consequent altered mitochondrial degradation of parkin, PINK1, Bnip3, and Bnip3L have been documented to play an important role in muscle atrophy [63, 65, 66].

Several signaling pathways have been described to be altered in muscle atrophy, including IGF1-Akt-FoxO signaling pathway, myostatin signaling pathway, NFκB signaling pathway, and glucocorticoid signaling pathway (for review see [67]).

The intimate mechanisms of muscle atrophy in pathological conditions have been demonstrated using animal models. For example, Forkhead box O (FoxO), a transcription factor which mediates nutrient and metabolic homeostasis using the pathway of protein kinase B, is upregulated under pathophysiologic catabolic conditions, such as denervation/immobilization, fasting, sepsis, and cancer cachexia [68]. FoxO1-related muscle atrophy primarily affects fast-twitch fibers [69]. Some factors are mainly involved in mitochondrial biogenesis, oxidative metabolism, and slow-twitch fiber formation such as peroxisome proliferator-activated receptor-γ coactivator-1 (PGC1α) which seems to play a dual role depending on its levels. It appears that a normal level of PGC1α has a protective effect against fiber

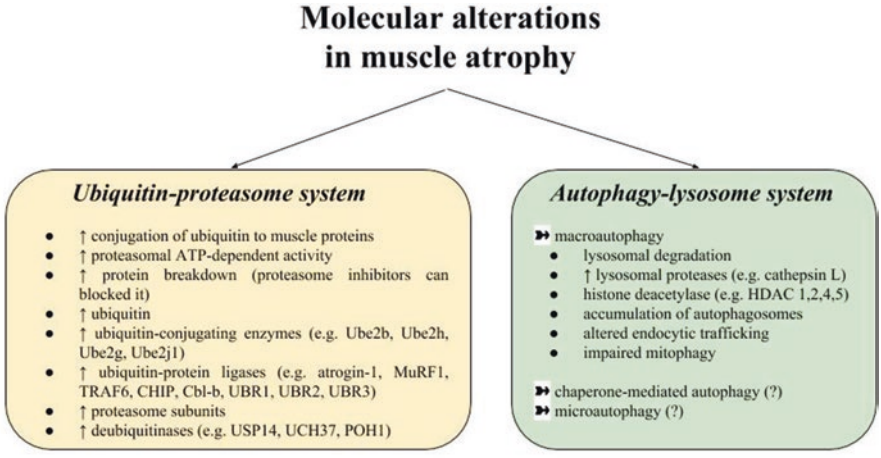

Fig. 4.3 Molecular alterations in muscle atrophy

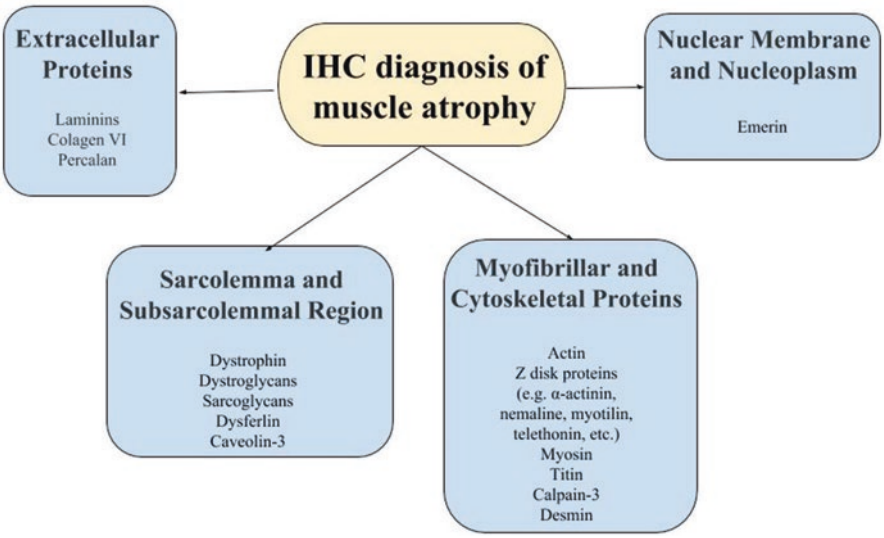

Fig. 4.4 Relevant markers in the immunohistochemical diagnosis of muscle atrophy classified based on their cellular localization

degradation, but excessive PGC1α levels will lead to muscle atrophy, especially for type IIb fibers [70].

Muscle atrophy can be identified based on immunohistochemistry (IHC) analysis in different muscle diseases like dystrophy and congenital/structural and inflammatory myopathy [71]. In clinical practice, diagnosis of muscle pathology associated with muscle loss is based on the IHC analysis of multiple proteins, including laminins, collagen VI, perlecan, dystrophin, dystroglycans, sarcoglycans, dysferlin, caveolin-3, actin, Z-disk proteins (e.g., α-actinin, nemaline, myotilin, telethonin, etc.), myosin, titin, calpain-3, desmin, emerin, etc. (Fig. 4.4) [71].

We have summarized the ultrastructural and histochemical features characteristic for muscle atrophy in clinical and experimental models for aging, cancer, diabetes and obesity, and heart failure (Fig. 4.5). However, it is difficult to distinguish specific muscle atrophy features that characterize each individual pathology due to the existent comorbidities. A detailed description of the muscle atrophy in different pathologies is done in the subsequent subsections of this chapter.

4.4 Muscle Atrophy in Aging

It is well known that human skeletal muscle fibers suffer age-related transformation. Lexell demonstrated that limb muscles of aging individuals are 25–35% smaller and have significantly more fat and connective tissue than those of younger persons. Type 2 fibers are smaller in old individuals, while type 1 fibers are less affected [30].

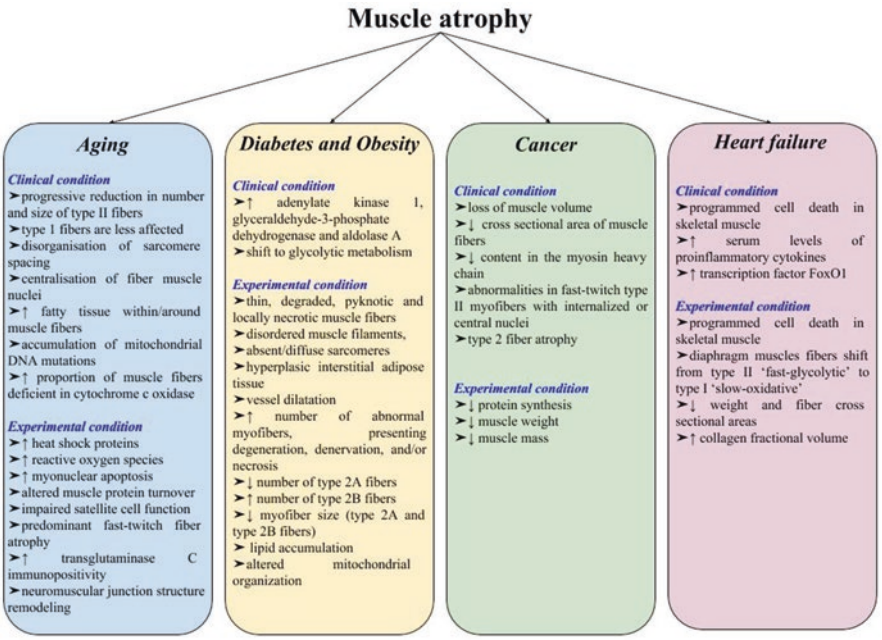

Fig. 4.5 Comparative ultrastructural, histochemical, and morphometric analysis in muscle atrophy associated with different clinical/experimental conditions

Progressive aging is followed by a reduction in number and size of type II fibers after 50 years of age, accompanied by a decline in the overall muscle cross-sectional area [72].

With aging, the skeletal muscle cross-sectional area decreases and ranges between 21% and 40%, compared to healthy young subjects [73], and is associated with poorer physical performance [74]. The cross-sectional area might also be affected by muscle disuse, these changes being more pronounced in the elderly [75]. We provided an example of histopathological analysis of fiber atrophy in sarcopenia (Fig. 4.6).

Muscle weakness and wasting are commonly seen in aging people, and, regarding histopathological examination, sarcopenia and cachexia are two of the most used terms to define the broad spectrum of microscopically aging-related changes in skeletal muscles. From a clinical point of view, it may be difficult to distinguish the two conditions [76]. Trying to establish some standards regarding the definition of the two terms, the European Working Group on Sarcopenia in Older People (EWGSOP) recommended using the presence of both low muscle mass and low muscle function for the diagnosis of sarcopenia [77]. By definition, sarcopenia affects typically more than half of people over 80 years old and is not related to a known condition or secondary causes of muscle loss, whereas cachexia is defined as a complex metabolic syndrome associated with underlying illness such as malignancy, chronic inflammation, insulin resistance, and others. Both processes imply

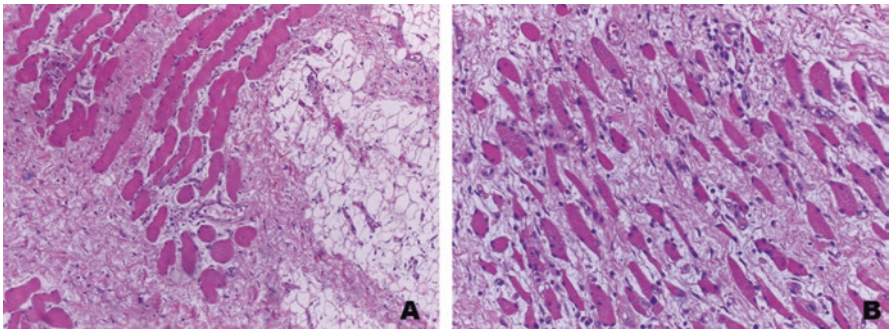

Fig. 4.6 Typical histopathological findings in sarcopenia: decrease in muscle fiber size (atrophy – **a**) and number (hypoplasia – **b**). The lost muscle fibers are replaced at first by fibrous, connective tissue and then by adipose tissue. Note the increased fiber size variability and increased interstitial fat and fibrotic connective tissue (magnification 100×, hematoxylin and eosin staining)

an involuntary loss of muscle mass, strength, and function and are a major contributor to disability in older people increasing the risks of falls and vulnerability consequently leading to functional dependence and disability [78].

The etiology of sarcopenia is not clearly understood, and the pathological mechanisms are not well documented. The decrease of muscle mass in aging people is a direct effect of muscle fiber atrophy but is also due to loss of skeletal muscle fibers. Microscopically, sarcomere spacing becomes disorganized, muscle nuclei tend to be centralized along the muscle fiber, and there is a significant increase of fatty tissue within and around the muscle fibers. Concomitant neuromuscular alterations have been observed in sarcopenia, including a decrease in the nervous firing rate to the muscle fibers, the plasma membrane which becomes less excitable, decreased number of motor neurons, and the deficient regenerative abilities of the nervous tissue. The number decline of muscle fibers may be site-specific and is well documented. Lexell et al. demonstrated that the number of muscle fibers in the vastus lateralis decline begins around age 25, and at age 80 there is approximately a 50% reduction in the number of these fibers [79]. There is an overall change in types of fiber proportion and composition with marked atrophy of type II fiber shift and a relatively higher proportion of type I fibers.

The reduction in the basal muscle protein synthesis does not seem to play a crucial role as originally thought. Recent data did not confirm the earlier reports and concluded that differences in basal muscle protein turnover between elderly and young men could not explain muscle loss with age [80].

Some ultrastructural changes also occur mainly due to age-related accumulation of mitochondrial DNA (mtDNA) mutations in postmitotic tissues. In aging muscles, there is an increased proportion of cytochrome c oxidase (COX)-deficient muscle fibers and occasional ragged-red fibers [81–84]. Mitochondrial dysfunction has been involved in apoptosis and may play a pivotal role in muscle fiber loss leading to sarcopenia [85]. However, sarcopenia is a multifactorial change and is related to a myriad of different pathological pathways such as increased heat shock pro-

teins, reactive oxygen species, myonuclear apoptosis, altered muscle protein turnover, and impaired satellite cell function with consequent muscle fiber-deficient regeneration [86]. Moreover, the immunohistochemical analysis in experimental aging indicated the predominance of fast-twitch muscle fiber atrophy (e.g., non-postural plantaris and extensor digitorum longus muscles), but not of the primarily slow-twitch fibers (e.g., postural soleus) [87]. Increased transglutaminase C immunopositivity in the soleus muscle was also reported in suspension-induced atrophy in the hind limb rat model [88]. The remodeling of the neuromuscular junction, e.g., reduction of axon terminal area, the absence of nerve terminals in some post-synaptic acetylcholine receptor areas, and variable end plate structure, was also evidenced in aging rats [89].

Besides the muscle-specific alterations pointed out above, there are other age-related changes in endocrine function, nutrition, or responsiveness to dietary factors as well as physical activity that may be responsible for the development or exacerbation of sarcopenia.

4.5 Muscle Atrophy in Obesity and Diabetes

Older adults can suffer muscle composition changes, due to fat accumulation in the excessive weight gain, leading to a two to five times decreased muscle strength comparative with age-related loss of muscle size in healthy older adults [90]. Muscle quality, interpreted as the ratio between some measure of muscle strength and power per unit of muscle mass, is important together with muscle mass to prevent functional decline. Body composition seems to contribute to muscle quality since ectopic fat depot found beneath the fascia and within the muscle and intramyocellular lipid storage is seen in persons with high risk of metabolic diseases, such as diabetes [91]. With age, fat deposits are redistributed in harmful ectopic locations such as intermuscular adipose tissue [92].

Muscle function is affected in obese patients with type 2 diabetes mellitus which have a 60% higher skeletal muscle expression of the atrophy transcription factor FoxO1 [93]. Protein degradation in muscles is due to the activation of the ubiquitin-proteasome, autophagy-lysosome, and caspase-3-mediated proteolytic pathways [63]. Furthermore, a range of proinflammatory pathways are upregulated, e.g., chemokine (c-c motif) ligand (CCL2) [94], signal transducer and activator of transcription 3 (STAT3), suppressor of cytokine signaling 3 (SOCS3), and nuclear factor κB (NF-κB) [95, 96].

Accumulation of advanced glycation end products is considered to be the main cause of skeletal muscle atrophy in diabetes, and several signaling pathways are involved, including the receptor for AGE (RAGE)-mediated pathway, the AMP-activated protein kinase (AMPK) pathway, and the Akt pathway [97]. Proteomics analysis of the skeletal muscle indicated an increase in adenylate kinase 1, glyceraldehyde-3-phosphate dehydrogenase, and aldolase A in obese/overweight and morbidly obese women compared to lean patients [98], and this shift to

glycolytic metabolism determined opposite muscle alterations in comparison to patients with drastic weight gain [99].

A clinical examination of 20 patients with type 1 or type 2 diabetes demonstrated that there is no direct correlation between the level of neuropathy and muscle fiber diameter/subtype distribution or the microvascularization [100]. However, the same study indicated an elevated number of type II fibers or capillaries in the striated musculature of patients with type 1 diabetes compared with the patients with type 2 diabetes [100]. In aged patients, comorbidity with type 2 diabetes determines an increased decline of the muscle mass and strength and diminished functional capacity with aging [101].

In experimental diabetes (e.g., streptozotocin-induced diabetes) was demonstrated that the fast muscles were more atrophied than slower ones due to the intense oxidative metabolism, while the fiber redistribution occurred for all fiber subtypes [102]. Based on the immunohistochemical analysis, in STZ-induced diabetes, muscle atrophy was characterized by thin, degraded, pyknotic, and locally necrotic muscle fibers, disordered muscle filaments, absent/diffuse sarcomeres, hyperplastic interstitial adipose tissue, and vessel dilatation [103]. Moreover, immunohistochemical analysis evidenced an increased number of abnormal myofibers, presenting degeneration, denervation, and/or necrosis, decreased number of type 2A fibers, increased number of type 2B fibers, decreased myofiber size (type 2A and type 2B fibers), lipid accumulation, and altered mitochondrial organization [104, 105]. Interestingly, exercise upregulates MuRF1 in STZ-diabetic animals compared to control animals [106].

4.6 Muscle Atrophy in Cancer

Debilitating and consumptive diseases such as cancer lead to muscle fiber shrinkage due to modifications of their cytoplasm organelles and protein loss. The more important the changes are, the more accurate the mortality prediction is. Skeletal muscle atrophy in cancer is conditioned by several factors: cancer type, cancer therapy, genetic predisposition, preexisting sarcopenia, reduced food intake, metabolic changes, and comorbidities [107].

Malignant tumors can also cause various neurological and musculoskeletal manifestations involving the central nervous system, peripheral nerve fibers, and neuromuscular junctions or muscles, mainly due to paraneoplastic syndromes. Sometimes these manifestations occur just prior to diagnosing the primary tumor [108]. Secondary aberrant immunological or endocrine mechanisms are believed to play a key role for paraneoplastic manifestations which can lead, among many others, to the degeneration or atrophy of muscle fibers.

For proper muscle functionality, the percentage and structural morphology and integrity of the slow-twitch, type I and fast-twitch, type II muscle fibers, is essential. During malignancies, muscle atrophy evolves through different stages such as precachexia, cachexia, and refractory cachexia [109]. Weber et al. showed that

cancer-related cachexia is associated with a loss of muscle volume, but not of muscle function and ability to generate force [110]. In general, studies on human subjects showed that cross-sectional area of muscle fibers is decreased, as well as content in the myosin heavy chain [111]. A study performed on patients newly diagnosed with colorectal cancer showed cancer-associated myopathy consisting in abnormalities in type II myofibers (fast type) which are with internalized or central nuclei and the presence of regenerating myofibers, suggesting a myogenic response to colorectal cancer [112].

The mechanism underlying the skeletal muscle atrophy in cancer is still under debate, and several theories have been proposed, including a cutback of protein synthesis, an elevation in protein in degradation, or a combined mechanism [107].

Type 2 fiber atrophy is a common but non-specific finding as a part of cachexia, due to various types of malignancies. Also, some inflammatory myopathies, such as dermatomyositis, are occasionally associated with malignancy, especially lung, gastrointestinal, ovarian, and nasopharyngeal carcinomas [113]. In a much more serious manner, skeletal muscles can be affected in paraneoplastic necrotizing myopathy. This rare and potentially fatal disease is a rapidly progressing proximal, symmetrical myopathy associated with end stages of various types of malignancies [114]. The main histopathological feature of this disorder is the widespread muscle fiber necrosis with minimal regeneration and with limited or absent inflammatory reaction. In some reported cases, focal or general capillary depletion was observed. In addition, the complement membrane attack complex (MAC) deposition was noticed in a significant proportion of endomysial capillaries [115]. Thickened hyalinized capillaries, sometimes called pipestem capillaries, are an ultrastructural feature related to paraneoplastic necrotizing myopathy [116].

In experimental cancer models, it was demonstrated that autophagy contributes to muscle wasting [117]. Several muscle changes have been evidenced in animal models of cancer, including increased proteolysis, decreased muscle weight and protein synthesis, increased expression of ubiquitin-dependent, calcium-dependent and lysosomal system, etc. [118]. However, due to the differences between cancer subtypes and the diversity of models of muscle wasting in cancer (e.g., Walker 256 carcinosarcoma, Morris hepatoma 7777, Yoshida ascites hepatoma 130, Lewis lung carcinoma, murine adenocarcinoma 16, MCG 101, C26 colorectal adenocarcinoma, human melanoma), it is difficult to find a subset of histological features that characterize experimental cancer. In some models have been proposed some therapeutic solutions that reverse/diminish muscle atrophy, including anti-myostatin drugs (e.g., soluble ActRIIB) [119] or the TRAF6 gene knockdown [58, 120, 121].

4.7 Skeletal Muscle Atrophy in Heart Failure and Arrhythmia

Among the comorbidities of chronic heart failure have been described skeletal muscle abnormalities, including abnormal energy metabolism, the transition of myofibers from type I to type II, mitochondrial dysfunction, reduction in muscular strength, and muscle atrophy [122]. Heart failure was demonstrated to upregulate the genes encoding atrogin-1 and MuRF1 in skeletal muscle with fiber type-specific atrophy [123]. Myostatin might represent an important link between skeletal and cardiac muscles, being able to promote distinct responses to protein metabolism in relationship with the fiber-type composition [124].

In experimental heart failure, the histological analysis indicated that diaphragm muscles fibers shift from type II "fast-glycolytic" to type I "slow-oxidative" [125]. Additionally, histological investigation of soleus muscle atrophy in hypertensive versus non-hypertensive rats indicated a decrease in weight and fiber cross-sectional areas and an increase of the collagen fractional volume [126].

In both clinical and experimental heart failure was identified the programmed cell death in skeletal muscle and interstitial cells that is triggered by cytokines [127]. Indeed, in patients with heart failure have been detected increased serum levels of proinflammatory cytokines [128]. Besides apoptosis, ubiquitin/proteasome and non-ubiquitin-dependent pathways have been documented to be involved in heart failure [127, 129].

In some genetic pathology, skeletal muscle atrophy was identified to be accompanied by abnormal cardiac rhythm. To date, peroneal muscular atrophy (i.e., Charcot-Marie-Tooth disease) was described to cause cardiac arrhythmias and conduction disturbances in association with peripheral muscle atrophy [130]. Often, patients with spinal and bulbar muscular atrophy develop a cardiac disease, manifesting as ST-segment abnormalities, Brugada syndrome (i.e., genetic disorder that results in abnormal ECG), dilative cardiomyopathy, or sudden cardiac death [131, 132]. Additionally, mutations in KCNH2 (the gene that encodes the human Ether-à-go-go-Related Gene type 1 (hERG1) K+ channels) determine long QT syndrome, a cardiac pathology characterized by ventricular arrhythmia, and upregulation of these channels was associated with skeletal muscle atrophy [133]. Interestingly, beta2-adrenoceptor agonists have been proposed to be potential pharmacological targets in skeletal muscle atrophy, but their chronic administration is prevented by cardiac side effects, including cardiac ischemia, arrhythmia, or heart failure [134].

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Chapter 5
Skeletal Muscle Damage in Intrauterine Growth Restriction

Leonard Năstase, Dragos Cretoiu, and Silvia Maria Stoicescu

Abstract Intrauterine growth restriction (IUGR) represents a rate of fetal growth that is less than average for the population and the growth potential of a specific infant. IUGR produces infants who are small for gestational age (SGA) but also appropriate for gestational age (AGA). It refers to growth less than expected for gestational age and is most often under 10th percentiles for age. It develops during the late second and third trimesters of gestation. The etiology of IUGR is multifactorial. One of the most important factors which leads to IUGR is a decrease of nutrients and oxygen delivered to the fetus by the placenta. The growth of adipose tissue and skeletal muscle is limited by the declined fetal nutrient supply later in gestation. IUGR affects about 24% of babies born in developing countries. Worldwide, IUGR is the second cause of perinatal morbidity and mortality behind the premature birth and a major predisposing factor to metabolic disorders throughout postnatal life, even at adult age. Skeletal muscle represents about 35–40% of the body mass and plays an essential role in metabolic homeostasis, being responsible for 65% of fetal glucose consumption. A reduction in skeletal muscle growth characterizes IUGR fetuses compared to normal weight neonates. The decrease in muscle mass is not compensated after birth and persists until adulthood. This is a review of the literature, a neonatological, clinical point of view on the effects of IUGR on striated muscles. The available studies on this subject are currently the results of experimental research on animals, and information about the human fetus and newborn are scarce.

Keywords Intrauterine growth restriction · Fetus · Muscle · Newborn · Glucose

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5.1 Introduction

Intrauterine growth restriction (IUGR) represents a rate of fetal growth that is less than normal for the population and for the growth potential of a specific infant. IUGR produces infants who are small for gestational age (SGA) but also appropriate for gestational age (AGA). It refers to growth less than expected for gestational age and is most often under 10th percentiles for age [1, 2].

IUGR may be categorized as symmetric (hypoplastic small for date), asymmetric (malnourished), and mixed [3]. The asymmetric form is more common than the symmetric one and specifies that body growth is limited to a much greater extent than head (brain) development. It grows during the late second and third trimesters (see Table 5.1).

The etiology of IUGR is multifactorial. It can result from maternal (age of mother, health, low inter-pregnancy interval, smoking, infections), fetal (congenital infections, congenital anomalies, genetic syndromes, or chromosomal abnormalities),

Table 5.1 Intrauterine growth restriction (IUGR type)

Characteristics	Asymmetric (disharmonic) malnourished IUGR	Disharmonic IUGR symmetric (harmonic) hypoplastic
Incidence	Most of IUGR (70–80%)	Uncommon (20–30%)
Birth weight (BW)	Any birth weight	≤ 1SD (percentiles 10)
Head circumference (HC)	> 1SD (percentiles 10) higher than BW (head sparing)	≤ 1SD or < 1 SD higher than BW (affect all growth parameters)
Factors	Extrinsic influences: Preeclampsia, chronic HTA, uterine anomalies	Intrinsic: Genetic, chromosomal, early gestational infection (TORCH), maternal alcohol use
Time when the fetus is affected	Later in gestation	Early gestation (under 16–20 gestation weeks)
Time when the fetus is affected	Early gestation (under 16–20 gestation weeks)	Later in gestation
Postnatal growth	Reductions in all parameters. Some studies observe gain in weight and height similar to IUGR symmetric but poorer long-term growth and poorer developmental outcome independent of HC at birth	Reduction in weight
		Length and head circumference – normal (brain-sparing growth)
Malnutrition	Less pronounced	More pronounced
Ponderal index	Normal (more than 2 SD)	Low (less than 2 SD)
Skeletal muscles	Cell number reduced	Cell number normal
	Cell size normal	Cell size reduced
Prognosis	Poor	Good

(continued)

Table 5.1 (continued)

Characteristics	Asymmetric (disharmonic) malnourished IUGR	Disharmonic IUGR symmetric (harmonic) hypoplastic
Long-term complication	Hypertension	
	Ischemic heart disease/stroke	
	Type 2 diabetes	
	Kidney disease	
	Liver disease	
	Hypercholesterolemia	
	Metabolic syndrome X	
	Obesity	
	Lung abnormalities – reactive airway disease	
	Cancer – breast, ovarian, colon, lung, blood	
	Schizophrenia/ Parkinsonism	
	Alzheimer disease	
	Polycystic ovarian syndrome, premature pubarche	
	Shortened life span	
	Depression, anxiety, bipolar disorder	
	Immune dysfunction	
	Osteoporosis	
	Social problems	
	Poor cognitive performance	

Data in this table are collected from the following Refs. [4–12]

and placental factors (multiple infarctions, chronic inflammatory lesions, abruptio placentae, velamentous cord, multiple gestation, placental weight less than 350 grams, abnormal uteroplacental vasculature) [3] or a combination of them [2]. One of the most critical pathways which lead to IUGR is a decrease of nutrients and oxygen delivered to the fetus by placenta [13].

IUGR affects about 24% of children born in developing countries. Worldwide, following the premature birth, the second cause of perinatal morbidity and mortality is represented by IUGR. Also, IUGR is a major cause for metabolic diseases throughout postnatal life, even at adult age [14, 15]. It seems that obesity [16, 17], insulin resistance, type 2 diabetes [18, 19], and cardiovascular disorders are more ordinary among adults who were smaller than normal at birth and very likely SGA secondary to IUGR [20–25].

IUGR seen as an adaptive physiologic process can determine adverse fetal, neonatal, and possibly adult consequences. There are studies which suggest that adult pathologies can be consequences of severe and prolonged fetal undernutrition. This condition may be defined as an example of "programming," in which the application of an insult in a critical or sensitive period of evolution may result in a long-life impact on the structure or function of the organism [26].

IUGR is due to reductions in energy supply to the fetus, limiting fat and glycogen storage and the growth of skeletal muscle. The fetus receiving less than the neces-

sary blood supply preferentially shunts blood to essential organs such as the brain at the expense of the liver, muscle, and fat. More extreme limitations of nutrients for more extended period affect both body weight and soft tissue mass [27]. Timing is crucial. Lower fetal nutrient supply later in gestation primarily restricts the growth of adipose tissue and skeletal muscle [3] (see Table 5.2).

Poor placental growth and function limit the placental supply of growth promoting hormones to the fetus, like steroid hormones and insulin-like growth factor-1 (IGF-1). More than this, reduced utilization of nutrients was observed in IUGR neonates than to those with normal birth weights [14, 28].

Skeletal muscle represents about 35–40% of the body mass and plays an essential role in metabolic homeostasis, being responsible for 65% of fetal glucose consumption [29, 30]. Metabolism and growth of skeletal muscle are influenced by growth factors, endocrine hormones (insulin, thyroid, adrenal, and pituitary hormones), oxygen, and nutrient availability [28, 31]. IUGR fetuses are characterized by a reduction in skeletal muscle growth compared to normal weight neonates (AGA – adequate gestational age). The decrease in muscle mass is not compensated after birth and persists until adulthood.

Fetal skeletal muscle growth is directly affected by placental insufficiency, one reason being that the essential nutrients and oxygen are redirected to vital organs during development. Some scientific researches using DXA measurement have demonstrated a correlation between lower adult muscle mass and low birth weight [32–34].

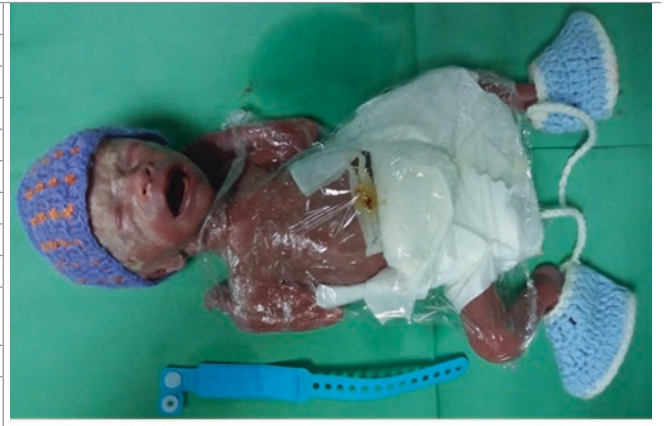
5.2 Endocrine Control Changes of IUGR

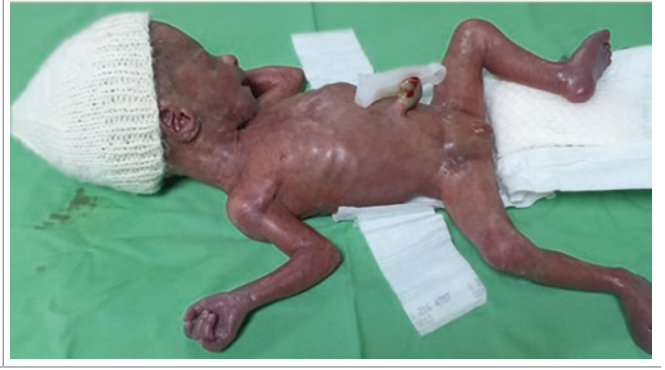
The fetal growth and development depend on insulin, thyroid, and adrenal hormones [35, 36]. Insulin has mitogenic effects on cellular growth, controls glucose uptake and consumption by tissues, and decreases protein breakdown. Insulin deficiency will lead to IUGR. Skeletal muscle is the primary location for insulin-stimulated glucose uptake accounting for about 70% of whole-body glucose disposal and is a crucial regulator of body energy metabolism [37]. The principal metabolic aim in the skeletal muscle is the synthesis of ATP for muscle contraction. In the same time, the skeletal muscle is liable for the generation and storage of glycogen, an insulin-dependent cycle. ATP is involved too, in the β-oxidation process which breaks down free fatty acids to supply muscle with carbon chain substrates [38]. In insulin resistance status, skeletal muscle is no longer responsive to the anabolic effects of insulin reducing insulin-stimulated glucose uptake [39]. Insulin also acts as a fetal skeletal growth factor. Scientific research experiments using fetal sheep showed that the lack of insulin is an effect of development restriction in cases of pancreatic agenesis [40]. Furthermore, insulin infusion into neonatal piglets promotes skeletal muscle protein synthesis [41, 42].

IUGR fetuses, experimental animal models or analysis of umbilical blood samples obtained by cordocentesis, have lower plasma glucose concentration compared with control fetuses. Fetal hypoglycemia limits glucose uptake by tissues, insulin secre-

Table 5.2 Clinical aspects of a newborn with IUGR

Large head when compared to the rest of the body (brain-sparing effect)
Large and wide anterior fontanelle (poor formation of membranous bones)
Absent buccal fat (old man aspect)
Small or scaphoid abdomen
Thin umbilical cord often stained with meconium
Decreased skeletal muscle mass and subcutaneous fat tissue
Loose, dry, and easy peelable skin
Long fingernails
Relatively large hands and feet compared to the body
Skin having a loose fold of skin in the nape of neck, axilla, interscapular area, and gluteal region (more than threefold)
Anxious and hyper-alert infant
Poor breast bud formation and immature female genitalia





tion, and the effect of insulin on glucose uptake by skeletal muscles. Insulin plays a key role as an anabolic hormone that enhances protein balance by inhibiting protein breakdown. Thus, a decreased plasma insulin concentration, associated with hypoglycemia, results in increased protein breakdown and lower protein accretion [14, 43].

IGF-I is modulated by glucose supply in the fetus. IGF-I has mitogenic effects inducing somatic cell development and proliferation. It affects the carriage of glucose and amino acids across the placenta. IGF-I deficiency causes a fall in fetal growth rate. IGF-II effect on human fetus is not well known although preclinical trials show that mutation in IGF-II gene determines smaller fetal size in mice. Insulin-like growth factor-binding protein-3 (IGFBP-3) is diminished in cord blood of IUGR [3, 44]. The cellular growth depends on the balance between the binding protein and IGF molecule itself. Vasoactive intestinal polypeptide (VIP) is a growth factor that affects the whole-body growth [3]. Hypothyroidism decreases circulatory and tissue concentrations of IGF-I, oxygen consumption, and glucose oxidation resulting in a deficiency of energy supply for growth [45].

5.3 Placental Insufficiency

One important factor modulating fetal development is nutrient delivery to the fetus via placental diffusion and transport [37], and one of the most frequent causes of IUGR is placental insufficiency [14]. Placental insufficiency is defined as a smaller than normal placenta with restriction of nutrient flow from mother to fetus [27, 37, 46]. Placental insufficiency affects around 8% of all pregnancies and is associated with chronic hypertension, pregnancy-induced hypertension, preeclampsia, infarcts, and idiopathic causes [28, 47].

The most elevated method used for characterizing placental insufficiency and for defining abnormalities in the umbilical artery is Doppler velocimetry [48]. Degradation of small muscle arteries due to placental condition results in a high pulsatility index [47, 49, 50]. When the umbilical blood flow and fetal oxygenation are lower, the fetal ductus venous dilated to provide enough nutrients and oxygen for the brain and heart [51]. Redistribution of blood flow to the vital organs occurs at the expense of nutrient and oxygen delivery to the periphery. This particular situation seems to contribute to 25–40% reduction in muscle mass of IUGR neonates [27]. As the fetus grows, the affected placenta cannot provide increased nutritional demands of the fetus, resulting in chronic fetal hypoglycemia and hypoxemia. Hypoxemia elevates plasma and amniotic fluid norepinephrine and epinephrine concentrations. Catecholamines act via the G-protein-coupled receptors, Adrα and Adrβ. Receptor expression patterns determine how tissues respond to catecholamines. Skeletal muscle predominantly expresses Adrβ1 and Adrβ2 subtypes [14]. Catecholamines affect skeletal muscle directly by selectively impairing insulin signaling and indirectly by suppressing insulin secretion from pancreatic β cells [14]. A chronic state of fetal hypoglycemia suppresses glucose oxidation. Consequently, an endocrine and metabolic adaptation develops to preserves fetal nutrients by

decreasing skeletal muscle energy requirements for protein synthesis and growth. Circulating concentration of IGF-1 is reduced too during fetal hypoglycemia which may contribute to increased fetal protein breakdown [14]. During gestation, muscle grows through a continued process of proliferation and fusion of myoblasts into determinated myofibers [52, 53]. Late gestation and postnatal muscle growth is generally produced by myofibers hypertrophy, as has been demonstrated in mice [54]. It is not known if the slow myofiber hypertrophy is an adaptation to reduced nutrients and growth factors or if it activates protein breakdown as a result of cellular stress. It is possible that the fetus develops a slower growth rate as a response to the redistribution of blood flow away from skeletal muscle, but as the placental insufficiency advances, with progressive hypoxia, catabolic pathways are activated, and the production of catecholamine and cortisol is increased [55]. The postnatal myogenesis involves maintenance of the satellite cells that reside around muscle fibers in a latency state and are activated during muscle growth, repair, and regeneration [56]. Insulin controls the cell number, has a direct mitogenic effect, and promotes myoblast proliferation and differentiation. It conducts the tissular glucose uptake and consumption and protein breakdown and increases protein synthesis in fetal skeletal muscle [57]. Therefore, insulin deficiency will lead to IUGR and impaired muscle growth.

5.4 Fetal Adaptation

Elsie Widdowson introduced over 40 years ago the idea that chronic fetal malnutrition (i.e., placental insufficiency and IUGR status) may disrupt normal myogenesis [58]. Placental insufficiency causes hypoxemia, hypoglycemia, hypercatecholaminemia, and suppression of glucose oxidation. Chronic hypoglycemia increases protein breakdown and rates of amino acid oxidation, lowering plasma insulin, glucose uptake, and fetal growth rate. These metabolic changes are correlated with placental oxygen supply and cannot be attenuated only by removing the nutrient deprivation [14]. IUGR is associated with slow rate and impaired growth and development of skeletal muscle [59]. Metabolic, physiological, and biochemical parameters of muscle fibers are influenced differently by the timing of the fetal injuries. In the third trimester, the myogenesis process is complete and the fiber amount is determined. Another factor which reduced fiber density and affects myotube development is represented by nutritional insult [14]. Severe fetal conditions like hypoxemia and hypoglycemia that can occur late in gestation period are correlated with reduced muscle mass by impairing fiber growth [14]. Several studies showed muscle fiber number to be set at birth [58, 60, 61]. Recently, this affirmation was supported by a study showing that this is true for several human muscles; however tibialis anterior and extensor digitorum longus muscles are able to increase their myofibers during the first postnatal week [62]. Following myogenesis, muscle growth by fiber hypertrophy requires myoblast incorporation to increase genomic DNA content. Human IUGR fetuses have reduced skeletal muscle DNA content but have normal

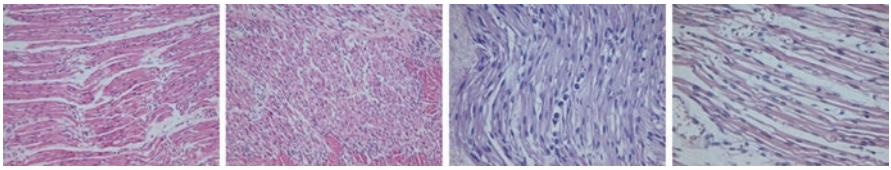


Fig. 5.1 Tight muscle biopsy (postmortem) from preterm newborn, 32-week gestational age, extremely low birth weight, 950 g, with severe symmetric IUGR, with single umbilical artery. Minimal to moderate variability of muscle fiber size together with a low fiber diameter can be seen; nuclei are large, located at the periphery. (Photo collection of the Alessandrescu-Rusescu National Institute)

protein-to-DNA ratios and normal molecular machinery needed for transcriptional control of proliferation [14, 63]. The mechanism that impaired proliferation and formation of myofibers is not entirely understood, though insulin and insulin-like growth factors which regulate fetal growth and myoblast proliferation are reduced. Composition and distribution of muscular fiber type are affected in IUGR, as well as fat and collagen concentrations [59, 64] (Fig. 5.1). Evidence of these findings are the changes detected in 12 proteins involved in processes like immune response, synthesis and degradation of proteins, cellular structure, and antioxidant function [28]. IUGR affects the proteomes of skeletal muscle in newborn piglets in a tissue-specific manner. Therefore it is assumed that altered expression of proteomes will enhance proteolysis, decrease polypeptide synthesis, cause oxidative stress, and affect health in IUGR newborns [28].

5.5 Postnatal Aspects of a Newborn with IUGR

Immediately after birth, newborns with IUGR bear the mark of chronic intrauterine pain in all tissues and, also, clinical evidence, particularly in the skeletal muscle and adipose tissue. The initial clinical appearance may highlight the type and degree of growth restriction (see Table 5.2). Their precise quantification can be achieved using several growth indices (see Table 5.3).

Growth and development in the postneonatal period are programmed by reactive hormonal changes in intrauterine life.

During embryonic myogenesis, only a small number of primary fibers are formed which then serve as a template for secondary fiber myogenesis in the fetal stage [65]. Due to the limited number of primary fibers formed during embryonic myogenesis, secondary myogenesis has a significant impact on muscle size and total fiber number [66]. The formation of secondary fibers is determined mainly by the fetal myoblasts number as well as their activity [67]. However, these cells are highly sensitive to nutrients which makes maternal nutrient supply a critical factor for muscle development at the fetal stage [52, 68]. In fact, many studies have shown that IUGR piglets have reduced muscle size and total fiber number (mainly second-

Table 5.3 Evaluation nutrition of newborn with IUGR

Indices	Formula	Interpretation
Ponderal index (PI)	[weight (in gram) × 100] ÷ [length (in cm)^3]	Less than 10 percentiles – Fetal malnutrition
Mid-arm circumference and mid-arm/head circumference ratios (Kanawati and McLaren's index) [80]	Mid-arm/head circumference ratios (MAC/HC)	Less than 0.27 – fetal malnutrition
Clinical assessment of nutrition score (CAN score) [81]	It includes nine parameters, namely, hair, cheeks, neck and chin, arms, legs, back, buttocks, chest, and abdomen. The maximum score is 36 with each parameter given a maximum score of 4 and a minimum score of 1, in which 4 denotes normal nutrition and 1 denotes malnutrition	CAN score of less than 25 is considered to be malnourished

ary fibers) which significantly affect postnatal muscle growth [28, 69–71]. The main contribution of nuclei for postnatal muscle growth is from muscle satellite cells [72]. Satellite cells were first discovered by Alexander Mauro in electron micrographs of frog skeletal muscle in 1961 [73]. These cells were found closely attached to muscle fiber, between fiber membrane and basal lamina, which were then named "satellite cell" [73]. Once activated, satellite cells undergo rapid proliferation, with a small portion of daughter cells renewing the original satellite cell pool, while the majority of these cells differentiate to myoblasts [74, 75]. These myoblasts fuse with existing muscle fibers and provide external nuclei, thereby increasing DNA content and protein synthetic capacity in each fiber [76]. The majority of adult muscle nuclei originate from the muscle satellite cell which suggests postnatal muscle growth potential is highly related to satellite cell number per muscle fiber, as well as their proliferation and differentiation [77–79].

5.6 Conclusions

IUGR is a major health problem of wide world with multiple determinant factors, such as maternal, fetal, placental, and genetic.

In order to minimize the risk of neonatal and perinatal mortality, early diagnosis and management of IUGR is needed.

There are primarily two types of IUGR, symmetrical and asymmetrical, depending on the onset of gestation and the IUGR etiology. These IUGR fetuses have both short-term and long-term complications, which make them high-risk neonates.

One of the major causes of IUGR is placental insufficiency that affects the supply of nutrients, oxygen, hormones, and growth factors. This affects the growth and fetal development and implicitly of skeletal muscles. Altering the structure and

growth of muscle fibers during gestation influences their growth and subsequent development.

Hormonal changes (e.g., hypoinsulinism) play an essential role in the determinism of muscle growth and development. Poor hormonal and oxygen supply affects the metabolism of skeletal muscles by programming deficient postnatal development and postnatal recovery throughout life.

IUGR fetuses may develop a long-term decrease in insulin-mediated growth that will also lead to insulin resistance in adulthood, being correlated with type 2 diabetes.

Improvement of postnatal weight loss is not associated with recovery of muscle mass to achieve similarity with AGA newborns.

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Part III
Molecular Mechanisms of Muscle Atrophy

Chapter 6
The Role of IGF-1 Signaling in Skeletal Muscle Atrophy

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Abstract Insulin-like growth factor 1 (IGF-1) is a key anabolic growth factor stimulating phosphatidylinositol 3-kinase (PI3K)/Akt signaling which is well known for regulating muscle hypertrophy. However, the role of IGF-1 in muscle atrophy is less clear. This review provides an overview of the mechanisms via which IGF-1 signaling is implicated in several conditions of muscle atrophy and via which mechanisms protein turnover is altered. IGF-1/PI3K/Akt signaling stimulates the rate of protein synthesis via p70S6Kinase and p90 ribosomal S6 kinase and negatively regulates protein degradation, predominantly by its inhibiting effect on proteasomal and lysosomal protein degradation. Caspase-dependent protein degradation is also attenuated by IGF/PI3K/Akt signaling, whereas evidence for an effect on calpain-dependent protein degradation is inconclusive. IGF-1/PI3K/Akt signaling reduces during denervation-, unloading-, and joint immobilization-induced muscle atrophy, whereas IGF-1/PI3K/Akt signaling seems unaltered during aging-associated muscle atrophy. During denervation and aging, IGF-1 overexpression or injection counteracts denervation- and aging-associated muscle atrophy, despite enhanced anabolic resistance with regard to IGF-1 signaling with aging. It remains unclear whether pharmacological stimulation of IGF-1/PI3K/Akt signaling attenuates immobilization- or unloading-induced muscle atrophy. Exploration of the possibilities to interfere with IGF-1/PI3K/Akt signaling reveals that microRNAs targeting IGF-1 signaling components are promising targets to counterbalance muscle atrophy. Overall, the findings summarized in this review show that in disuse conditions, but not with aging, IGF-1/PI3K/Akt signaling is attenuated and that in some conditions stimulation of this pathway may alleviate skeletal muscle atrophy.

Keywords Disuse · Aging · Hypertrophy · miRNA · Lysosome · Caspase · Calpain

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6.1 Introduction

Insulin-like growth factor 1 (IGF-1) is a key anabolic growth factor which is involved in tissue development during growth, as well as in adaptation and regeneration of mature tissues and cells. IGF-1 is expressed in multiple isoforms in almost all tissues and cells [1]. It is therefore not surprising that mice deficient in IGF-1 or its receptor show decreased viability, growth deficiency, and malformations in several tissue types [2]. IGF-1 is expressed in the liver, acts locally in an autocrine and paracrine manner on liver cells, but also has a strong endocrine function on other tissues like muscle. In muscle, IGF-1 isoforms that are most abundantly expressed are IGF-1Ea and mechano growth factor (MGF, also referred to as IGF-1Ec in humans or IGF-1Eb in rodents). In skeletal muscle, basal mRNA levels are higher for IGF-1Ea than MGF [3]. Paradoxically, the expression of IGF-1Ea is higher in relative small oxidative myofibers, expressing slow myosin heavy chains (MHCs) than in relative large, low oxidative myofibers expressing fast-type MHCs [4]. Expression of these main IGF-1 isoforms increases substantially in response to mechanical overload by stretching or increased contractile activity [1, 5, 6]. Moreover, IGF-1 expression is also enhanced biochemically by growth hormone (GH), and its half-life time and/or bioactivity is both negatively and positively regulated by several IGF-binding proteins (IGFBPs) as well as by albumin [1, 7, 8]. Since different IGFBPs can compensate for each other [7], single IGFBP measurements provide little evidence regarding the bioavailability of IGF-1.

Both IGF-1 isoforms are derived from the same gene which contains 6 exons. MGF is expressed by alternative splicing of exon 5 and 6 and differs from IGF-1Ea in its E peptide which contains exon 5 and 6 in stead of exon 6 in IGF-1Ea [6]. The IGF-1 domain of IGF-1Ea and MGF, which consists of exon 3 and 4, signals via the IGF-1 receptor (IGF-1R), which is a tyrosine kinase receptor expressed in both myofibers and muscle stem cells (also known as satellite cells). Also the E peptides of IGF-1Ea and MGF E are involved in signaling via the IGF-1R whereby the MGF E peptide is known for its stimulatory effect on satellite cell activation, proliferation, and migration [1, 9, 10]. Moreover, different IGF-1 isoforms exist also due to different promotor start regions upstream of exon 1 or 2 [11]. Transcripts including exon 1 are known as class 1 IGF-1 isoforms, whereas IGF-1 isoforms including exon 2 are referred to as class 2 [11]. Functional differences of the two classes remain however unclear [12]. IGF-1 and insulin share about 50% amino acid homology and can bind each other's receptors, albeit with lower affinity.

IGF-1 and MGF are well known for their autocrine and paracrine roles during muscle overload and myofiber hypertrophy, however, less is known about how IGF-1 is involved in the induction of muscle atrophy. An important signaling pathway in skeletal muscle atrophy is the IGF-1/phosphatidylinositol 3-kinase (PI3K)/Akt pathway, since this is involved in both protein synthesis and protein degradation [4, 13–22]. Here we provide an overview of the main signaling pathways via which IGF-1 and MGF modulate the rate of protein synthesis and degradation during

muscle atrophy, with particular emphasis on the IGF-1/PI3K/Akt pathway, and how IGF-1 signaling is altered.

6.2 The Role of IGF-1 in the Regulation of Protein Synthesis and Degradation

Changes in muscle size are the net effect of changes in the rate of protein synthesis and protein degradation. IGF-1 affects both processes, and as such, changes in its signaling have a strong effect on muscle size [4, 13–22]. In this paragraph, the role of IGF-1 in protein synthesis and different mechanisms of protein breakdown is reviewed.

Binding of IGF-1 to its receptor causes phosphorylation of the intracellular adaptor proteins Shc or insulin receptor substrate 1 (IRS-1), which results in the activation of two main pathways, RAS/RAF/MEK/ERK (also known as mitogen-activated protein kinase (MAPK) signaling) and PI3K/Akt, respectively [21, 23]. IGF-1-induced hypertrophy in rats is prevented by the inhibition of MEK [24], which indicates the requisite for MAPK signaling in hypertrophy in vivo. In myotubes however, inhibition of RAF has been shown to induce hypertrophy [25], suggesting an inhibitory effect of MAPK signaling on hypertrophy in vitro. These observations show that the role of MAPK in protein synthesis and degradation and the underlying mechanisms are not entirely understood. On the other hand, the IGF-1/PI3K/Akt pathway and its anabolic mechanisms underlying myofiber hypertrophy are well established. Translocation of PI3K to phosphorylated IRS-1 results in the phosphorylation of PI3K. Subsequently, this causes the phosphorylation of phosphoinositide-dependent kinase-1 (PDK1) which then phosphorylates the serine/threonine kinase Akt (also known as protein kinase B) [26]. Akt is involved in multiple cellular processes including proliferation, metabolism, and cell size regulation [27]. Because the IGF-1/PI3K/Akt pathway plays a major role in myofiber size, the main focus of this review will be on the role of this pathway during skeletal muscle atrophy.

6.2.1 Protein Synthesis

Changes in the rate of protein synthesis involve changes in the rate of mRNA transcription and translation, which in muscle are both enhanced by IGF-1 [see for review 13, 28]. IGF-1 increases protein levels of β-catenin (a transcription factor involved in skeletal muscle growth) by phosphorylation of glycogen synthase kinase 3 beta (GSK3β), which prevents atrophy and can even induce hypertrophy in dexamethasone-treated rats [29]. Moreover, IGF-1 has been shown to increase transcription rate of α-skeletal actin during differentiation and myosin heavy chain

(MHC) IIB in C2C12 myoblasts and myotubes, respectively [30, 31]. Increased transcription by IGF-1 may be regulated by myogenin and MyoD, which are both transcription factors involved in the expression of actin and myosin, since IGF-1 has been shown to induce myogenin and MyoD expression [32] and both transcription factors increase in the human vastus lateralis after resistance exercise [33]. Note, myogenin has been shown to be stimulated by IGF-1/PI3K/Akt signaling when simultaneously MAPK signaling is inhibited [24, 34]. Indeed, IGF-1 treatment has also been associated with a lack of increase in myogenin and MyoD [31, 35]. These observations show that IGF-1 enhances transcription, but the underlying mechanisms are not entirely clear.

In addition to transcription, the IGF-1/PI3K/Akt pathway stimulates translation by activation of a key anabolic target, the mammalian target of rapamycin (mTOR), which is a kinase that integrates multiple upstream signals, which are not solely derived from IGF-1/PI3K/Akt activation [36]. In addition to IGF-1, another important activator of mTOR is mechanical loading [37], and therefore disuse atrophy is likely to decrease mTOR activity even if IGF-1 signaling would be unaffected. Moreover, mTOR is affected by several other upstream mediators such as energy status or amino acids [36]. Activation of mTOR stimulates the rate of mRNA translation by phosphorylation of 4E-BP (also known as PHAS-1), which prevents its binding (i.e., inactivation) to the eukaryotic initiation factor (eIF) 4E (Fig. 6.1) [17, 38]. Furthermore, activated mTOR also activates p70S6Kinase (p70S6K) which stimulates mRNA translation by phosphorylating ribosomal protein S6 (rpS6) and activation of eukaryotic elongation factor (eEF) 2 [39–42].

Moreover, PDK1 which is phosphorylated by PI3K and subsequently phosphorylates Akt is also likely to be involved in enhancement of the rate of protein synthesis independent of Akt [26]. The role of PDK1 in skeletal muscle is not fully understood, but evidence from studies on several other cell types, including smooth muscle, suggests that PDK1 can phosphorylate Akt and has also the ability to directly activate p70S6K and p90 ribosomal S6 kinase (p90RSK), both increasing the rate of translation by regulation of rpS6 and eEF2 [26, 40, 41]. In smooth muscle cells, p90RSK is also activated by ERK [26]. In addition, mRNA translation rate is also increased by phosphorylation of Akt which then phosphorylates and inhibits GSK3β which is subsequently no longer able to suppress eIF2B activity [43]. GSK3β has been shown to be required for atrophy in C2C12 myotubes and is involved in both skeletal muscle hypertrophy and atrophy in humans [44, 45]. Moreover, GSK3β may also be inhibited by ERKs as it has been shown in cancer cells that ERKs facilitate the inhibition of GSK3β by [46].

The key regulatory kinases of which the activity is modulated by IGF-1 are p70S6K, p90RSK, and GSK3β, which are all involved in enhancement of the rate of mRNA translation (Fig. 6.1).

Fig. 6.1 An overview of the key signaling pathways underlying the hypertrophic effects of IGF-1. Stimulation is indicated by arrows, and inhibitory effects are indicated by lines capped by perpendicular lines. Solid lines represent established mechanisms; dashed lines represent mechanisms that have not been consistently proven in myofibers. Colors represent different pathways or downstream targets. Two important signaling pathways induced by IGF-1 are the IGF-1/PI3K/Akt pathway and the IGF-1/Ras/Raf/Mek/Erk pathway. Both pathways result in kinase activation or changes in binding proteins causing enhancement of mRNA translation by regulating ribosomal proteins, eukaryotic initiation factors (eIF), or eukaryotic elongation factors (eEF). Abbreviations: *IGF-1* insulin-like growth factor 1, *PI3K* phosphatidylinositol 3-kinase, *PDK1* phosphoinositide-dependent kinase-1, *GSK3β* glycogen synthase kinase 3 beta, *mTOR* mammalian target of rapamycin, *4E-BP* 4E-binding protein, *ERK* extracellular signal-regulated kinases

6.2.2 *Proteasomal Muscle Protein Degradation*

The prime system for muscle protein degradation is the ubiquitin-proteasome system [13, 14, 47, 48]. During protein degradation, contractile proteins are ubiquitinated by the consecutive actions of E1, E2, and E3 enzymes which can then be recognized and subsequently degraded by proteasomes. The gene expression as well as their function in muscle atrophy of two E3 ligases, Muscle Ring Finger 1 (MuRF1) and muscle atrophy F-box (MAFbx, also known as Atrogin-1), has extensively been examined [see for review 48]. Both E3 ligases are particularly involved

in the degradation of contractile proteins and eIF3f [49, 50]. During several atrophic conditions, MuRF1 and MAFbx expression levels are increased [48, 51], and these ligases are critical for the enhanced rate of protein degradation as MuRF1- or MAFbx-deficient mice showed a 36% and 56% reduction in denervation-induced muscle atrophy after 14 days, respectively [51]. Expression of MuRF1 and MAFbx is regulated by a group of Forkhead box O (FOXO) transcription factors which stimulate expression of several genes involved in diverse mechanisms of protein degradation, including proteasomal degradation [18, 19]. Transcriptional activation of MuRF-1 and MAFbx expression requires nuclear localization of FOXO transcription factors which is mediated by Akt. Active Akt phosphorylates FOXO transcription factors resulting in their cytoplasmic retention and inactivation of their function as transcription factors in the nucleus [52, 53]. FOXO1, 3, and 4 are the most important FOXO transcription factors involved in muscle atrophy and are all regulated by Akt [18]. Moreover, muscle atrophy induced by IGF-1R and insulin receptor knockout could completely be prevented by the combined knockout of FOXO1, 3, and 4, whereas knockout of single FOXO transcription factors had little or no effect [54], which indicates the importance of all three FOXO factors in muscle atrophy. In short, the IGF-1/PI3K/Akt pathway negatively regulates proteasomal degradation by inactivating FOXO transcription factors and hence the expression of the E3-ligases MAFbx and MuRF-1 (Fig. 6.2).

6.2.3 Lysosomal Muscle Protein Degradation

Autophagy is another key mechanism for muscle protein degradation [55]. Autophagy concerns the engulfment of cellular particles into autophagosomes which subsequently fuse with lysosomes to be degraded in the acid intralysosomal environment [55]. Several conditions like fasting and denervation result in the upregulation of expression of proteins involved in autophagy [56, 57]. Induced myofiber atrophy by constitutive active FOXO3 was attenuated by knockdown of *LC3*, a gene involved in autophagy [56]. An accumulation in ubiquitinated proteins was observed in autophagy-deficient mice [58], which suggest that some ubiquitinated proteins are specifically degraded by lysosomal degradation. These observations indicate the involvement of autophagy in muscle atrophy. In addition to its role in muscle atrophy, autophagy is also important for cell maintenance as this mechanism is also responsible for the clearance of misfolded proteins and dysfunctional organelles [55]; therefore both diminished and overactivity of autophagosomes could be harmful to myofibers. The first may affect the quality of myofibers, whereas the second affects the quantity of proteins within myofibers.

IGF-1 has been shown to regulate autophagy since deletion of insulin receptor and IGF-1R in mice increased an autophagic flux [54]. As in proteasomal degradation, deactivation of FOXO transcription factors by Akt is also a key mechanism in autophagy [56, 57]. FOXO3 is involved in the control of autophagosome formation by stimulating expression of two autophagy-related genes, i.e., *LC3* and *Bnip3* [56,

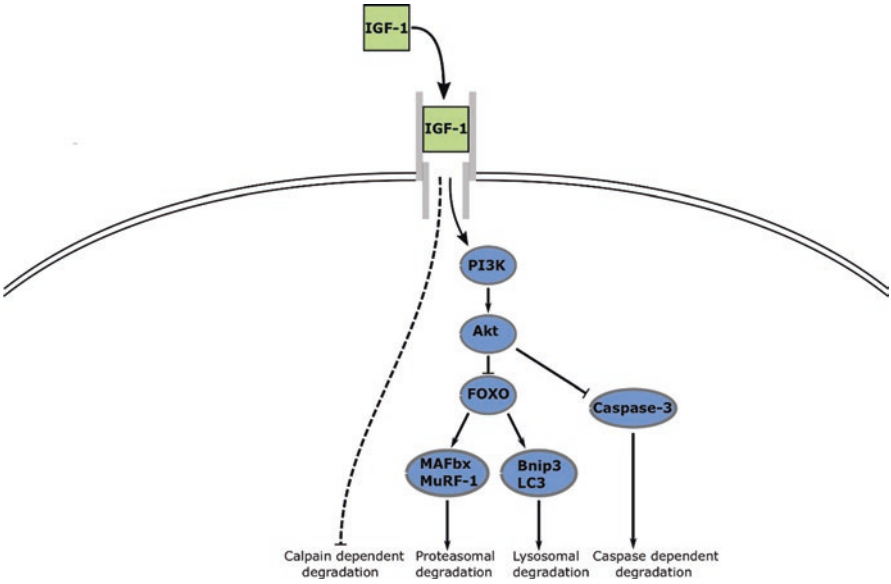

Fig. 6.2 An overview of the key signaling pathway underlying the anti-atrophic effects of IGF-1. Stimulation is indicated by arrows, and inhibitory effects are indicated by lines capped by perpendicular lines. Solid lines represent established mechanisms; dashed lines represent mechanisms that have not been consistently proven in myofibers. Abbreviations: *IGF-1* insulin-like growth factor 1, *PI3K* phosphatidylinositol 3-kinase, *FOXO* Forkhead box O transcription factors, *MuRF1* Muscle Ring Finger 1, *MAFbx* muscle atrophy F-box, *LC3* microtubule-associated protein 1A/1B-light chain 3, *Bnip3* BCL2 interacting protein 3

57]. In addition, the upregulation of several autophagic factors and autophagosome formation induced by fasting or denervation, was abolished by exogenous expression of constitutively active Akt, while inhibition of Akt increased lysosomal proteolysis [56, 57]. The inhibition of total mRNA synthesis while Akt was blocked largely suppressed the increased lysosomal proteolysis caused by Akt inhibition [57], which suggests that FOXO-induced transcription is largely responsible for increased lysosomal proteolysis. Although the effect was relatively small, mTOR inhibition also caused an increase in lysosomal proteolysis, while blocking of mRNA synthesis did not prevent this increase, which indicates that mTOR can also suppress autophagy independent of transcriptional control [57]. These observations are in line with results of a study on acute uremia whereby IGF-1/PI3K/Akt-independent stimulation of mTOR by leucine also suppressed autophagy [59]. These data suggest that an increase in IGF-1/PI3K/Akt signaling inhibits autophagy through activation of predominantly FOXO3 and also mTOR. In addition to fasting and denervation, 4 days of knee joint immobilization in young adult humans caused increased LC3B-II/LC3B-I protein ratios, an indication of increased autophagy, concomitant with decreased pAkt/tAkt levels [60]. This suggests that an increase in autophagy due to reduced IGF-1/PI3K/Akt signaling is associated with unloading. Overall, there is strong evidence that the IGF-1/PI3K/Akt pathway inhibits

autophagy during fasting, denervation, and potentially joint immobilization and that this is mediated predominantly through the inactivation of FOXO3 (Fig. 6.2).

However, the role of autophagy in different atrophic conditions is not unambiguous. In contrast to adults, in elderly LC3B-II/LC3B-I protein ratios were unaffected after 4 days of knee joint immobilization [60], which suggests that in aged muscle autophagy may not be induced by joint immobilization. In the long term, after 2 weeks of joint immobilization, no convincing increase in autophagy could be shown in both young adult and elderly. These observations indicate that in elderly joint immobilization does not increase muscle autophagy, whereas in adults autophagy is increased shortly after immobilization and does not occur in the long term [60]. The lack of a long-term effect of unloading is confirmed by a study in which mice were subjected to 91 days of unloading in the International Space Station, which showed no changes in autophagy-related gene expression [61]. In contrast, in adult and old rats undergoing hind limb suspension for 2 weeks, no clear increase in autophagy was observed suggesting that in this unloading model, autophagy may not play a role in the induction of muscle atrophy [62].

Autophagy seems also to be regulated independent of IGF-1/PI3K/Akt signaling, since mice showing aging-associated muscle atrophy, whereby IGF-1 signaling was unchanged, had an increase in autophagic vesicles [63]. This is in line with the effect of lipopolysaccharide (LPS) administration in rat skeletal muscle resulting in acute inflammation, which is associated with proteasomal and lysosomal proteolysis [64, 65]. LPS injection caused a decrease in IGF-1 mRNA expression and Akt phosphorylation [64, 65]. Although blocking of this LPS-induced inflammation restored Akt phosphorylation and autophagy-related protein expression [65], IGF-1 systemic or muscle-specific overexpression could not inhibit the LPS-induced increased autophagy-related gene expression [64]. This also suggests that autophagy is regulated independently of IGF-1 signaling. Indeed, p38 MAPK has been suggested to regulate autophagy [55, 66]. p38 can be stimulated by IGF-1 but also independent of IGF-1 by, for instance, oxidative stress [66].

Overall, there is strong evidence that autophagy is regulated by the IGF-1/PI3K/Akt pathway and is involved in fasting- and denervation-induced atrophy. However, the role of autophagy is not clear in all muscle atrophic conditions and seems to be transient and age dependent.

6.2.4 Caspase- and Calpain-Dependent Muscle Protein Degradation

6.2.4.1 Calpain-Dependent Protein Degradation

Calpains are cysteine proteases which are activated by free cytoplasmic calcium and degrade predominantly cytoskeletal proteins [see for review 67]. In skeletal muscle, three different calpain isoforms are mainly expressed, i.e., milli- and micromolar calpains (also referred to as calpain 1 and 2, respectively), which are named after their

sensitivity for calcium, and calpain 3, also known as p94 [67]. Although calpains are also able to degrade contractile proteins, they predominantly degrade Z-discs of sarcomeres which makes myofilaments available for degradation by the proteasome [67].

Calpain inhibition prevented immobilization-induced atrophy [68]. Moreover, calpain 3-deficient mice, which exhibit features of limb girdle muscular dystrophy type 2A, showed reduced muscle atrophy when subjected to unloading, suggesting calpain 3 requirement for muscle atrophy [69]. Because of their cooperation with the proteasome, it is conceivable that calpain expression is reduced by IGF-1, similar as E3 ligase expression. Indeed, both in vitro and in vivo studies on myotubes and mature myofibers show that IGF-1 inhibits calpain activity [70, 71]. Moreover, caloric restriction-induced muscle atrophy in neonatal calves was associated with an increase in calpain 1 activity and decrease in IGF-1 protein expression [72]. This observation is in line with that of another study showing that IGF-1 has an inhibitory effect on calpain-dependent proteolysis in dexamethasone-induced L6 myotube atrophy [73], which indicates that IGF-1 attenuates calpain activity. In contrast, another study investigating L6 myotube atrophy using the same calpain blocker in presence or absence of IGF-1 supplementation reported an increase instead of a decrease in myofibrillar protein degradation when calpain activity was blocked [74]. Although there are contrasting results regarding the effect of IGF-1 on calpain-induced proteolysis, the majority of these studies suggest an inhibitory effect of IGF-1 on calpain-dependent protein degradation (Fig. 6.2).

Although the role of IGF-1 in calpain activation is subject to controversy, a few studies have shown some insight in the interaction between calpain activity and Akt. In rat diaphragm muscle ex vivo, it has been shown that activation of calpains reduces Akt activity by lowering the binding of heat shock protein 90 (HSP90) to Akt which preserves Akt activity [67, 75]. Also, a reduction in pAkt in rat soleus muscle was prevented when unloading-induced calpain 1 activation was blocked. [76]. These results indicate that calpain activity reduces Akt phosphorylation. Note that Akt phosphorylation was not affected in calpain 3-deficient mice [69] which suggests calpain isoform specificity for the interaction with Akt activity.

The studies discussed above show that little is known regarding the role of the IGF-1/PI3K/Akt pathway in calpain-dependent protein degradation and to the best of our knowledge, a direct link between IGF-1/PI3K/Akt signaling and calpain activity in skeletal muscle has not been investigated. The data available suggest that calpain 1 but not 3 can inhibit Akt activity and that IGF-1 can inhibit calpain activity, but there is no evidence suggesting that an inhibitory effect of IGF-1 on calpain-dependent muscle protein degradation is mediated by IGF-1/PI3K/Akt signaling.

6.2.4.2 Caspase-Dependent Protein Degradation

Caspases are proteases, which in particular are involved in apoptosis and inflammation. Caspase-3 is activated in both angiotensin II-induced muscle wasting [77] and chronic kidney disease (associated with muscle wasting) [78]. Moreover, caspase-3 and caspase-9 activities increase during immobilization-induced muscle atrophy

[68, 79], and the inhibition of caspase-3 activity prevented immobilization-induced atrophy in the rat soleus [68]. In contrast, no increases in caspase-3, caspase-8, or caspase-9 activities have been observed following limb unloading in both rats and humans [62, 80]. These observations indicate that caspase-mediated protein degradation is involved in several but not all conditions of muscle atrophy. Although support for IGF-1/PI3K/Akt-induced calpain-dependent degradation is scarce, evidence for the IGF-1/PI3K/Akt involvement in the reduction of caspase-dependent protein degradation is more substantial.

Administration of recombinant active caspase-3 to cultured L6 myotubes or rat psoas muscle lysates causes cleavage of myofibrillar proteins resulting in a detectable 14kD actin fragment which is degraded by the proteasome [81]. Serum deprivation also results in enhanced myofibrillar fragmentation which is abolished after inhibition of caspase-3 activity by IGF-1 [81]. Moreover, the inhibitory effect of IGF-1 on caspase-3 activity in L6 myotubes has been shown to be PI3K dependent [81]. These results suggest involvement of caspase-3 in myofibrillar degradation and that this caspase-mediated protein degradation is counterbalanced by IGF-1/PI3K/Akt signaling. In addition to this in vitro evidence, during angiotensin II administration inducing muscle atrophy in mice, IGF-1 signaling reduced, which was indicated by decreased IRS-1 and Akt phosphorylation, while caspase-3-dependent actin degradation increased [77]. Moreover, transgenic mice overexpressing muscle-specific IGF-1 were prevented from caspase-3-mediated actin degradation after angiotensin II treatment [77]. These observations indicate that caspase-3 cleaves myofibrillar proteins resulting in actin fragments which are degraded by the proteasome and that activity of caspase-3 is negatively regulated by IGF-1/PI3K/Akt signaling. The results of these studies are in line with those of other studies suggesting an inhibitory effect of Akt on caspase-3 activation [c.f. 82, 83].

In contrast, rats subjected to hind limb suspension for 2 weeks showed no increases in caspase-3, caspase-8, or caspase-9 activity within their lower leg muscles, while IGF-1 serum levels were slightly decreased [62]. However, since a large fraction of circular IGF-1 is produced by the liver, serum levels do not accurately reflect muscle-specific levels. In addition, as phosphorylated Akt was not decreased during unloading, it cannot be concluded that decreased IGF1/PI3K/Akt signaling is concomitant with a lack in change of caspase activity. This is line with a study showing no changes in both IGF1/PI3K/Akt signaling and caspase-3 mRNA levels following unilateral leg unloading humans [80]. Taken together, IGF-1/PI3K/Akt signaling inhibits caspase-mediated protein degradation (Fig. 6.2). It seems that in atrophic conditions in which IGF-1/PI3K/Akt signaling is unaffected, caspase-dependent protein degradation remains unaffected as well, whereas caspase-mediated protein degradation decreases in atrophic conditions associated with reduced IGF-1/PI3K/Akt signaling.

6.3 The Role of IGF-1/PI3K/Akt in Skeletal Muscle Atrophy Models

Muscle atrophy is a hallmark of several conditions such as aging, disuse, space flight, and a variety of pathologies. These conditions have in common a reduction in contractile activity of myofibers as well as a reduction in intra- and extracellular mechanical stress and strains to which myofibers are subjected. Despite these similarities, the impact on IGF-1 signaling within muscles varies between different disuse models. Here we discuss the effects of several conditions associated with muscle atrophy on IGF-1 expression and signaling in an attempt to explain the muscle atrophy associated with the corresponding physicochemical conditions.

6.3.1 Muscle Denervation and IGF-1 Signaling

A widely used model for studying mechanisms underlying muscle atrophy in vivo is muscle denervation which is associated with severe atrophy. Denervation of muscles results in a tremendous loss of muscle activity, retaining little mechanical signaling, however fibrillations occur as side effect [84]. Here we will discuss effects of denervation on IGF-1/PI3K/Akt signaling and how alterations in IGF-1 signaling contribute to denervation-induced atrophy.

Denervation of skeletal muscle has revealed myofiber-type-dependent differences. Three days following denervation in rats, increased IGF-1 mRNA expression levels in fast, glycolytic extensor digitorum longus (EDL) muscle were observed, whereas in slow, oxidative soleus muscle, no changes in IGF-1 mRNA expression were observed [85]. Since calcium-calcineurin signaling regulates IGF-1 mRNA expression [86], the myofiber-type difference in IGF-1 mRNA expression following denervation could well be explained by more fibrillations in fast, glycolytic muscles than in slow, oxidative muscle in the first 3 days following denervation [84]. The increase in IGF-1 mRNA expression in the EDL following denervation was completely blunted at day 7 after denervation [85], suggesting that IGF-1 expression after denervation shows only a transient increase which decays during the first week. A lack of a long-term effect of denervation on IGF-1 mRNA expression has also been shown in rat gastrocnemius muscle 7 weeks after botulin toxin-induced denervation [87]. Moreover, during the first 2 weeks after spinal cord injury in rats, IGF-1 mRNA expression levels in the EDL and soleus muscle were unaltered [85], whereas increased IGF-1 mRNA levels in the plantaris and soleus muscle have been reported after 30 days of spinal cord injury [88]. It seems that IGF-1 mRNA expression is either unaffected or increased after denervation, which depends on muscle type, denervation model, and/or time of measurement.

Regarding the effects of denervation on IGF-1 protein levels, the literature is less ambiguous. IGF-1 protein levels in denervated muscle of rodents or upper leg muscles of humans with spinal cord injury are reduced [89, 90]. In line with these

observations, IGF-1R and Akt phosphorylation and protein levels of P13K and IRS-1 have been shown to be decreased after denervation in rodents [88, 89, 91, 92]. Although spinal cord injury is associated with reduced IGF-1 protein levels in human upper leg muscle, Akt phosphorylation was unaltered suggesting a difference between surgical denervation in animal models and human spinal cord injury [90]. Therefore, even though increases in IGF-1 mRNA have been reported following denervation, activity of IGF-1/PI3K/Akt signaling seems to be reduced, with a possible exception after human spinal cord injury.

Besides the observed denervation-related decrease in IGF-1/PI3K/Akt signaling, enhancement of this signaling pathway by either injection of IGF-1 into denervated muscle or transgenic muscle-specific overexpression of IGF-1 in mice has shown to diminish denervation-induced atrophy [19, 93–96]. Moreover, constitutive expression of activated P13K or Akt also inhibits denervation-induced atrophy in rodents [17, 97]. Similarly, several interventions counterbalancing denervation-induced atrophy are associated with increased Akt phosphorylation [98–101]. Taken together, IGF-1/PI3K/Akt activity reduces during denervation in adult skeletal muscle, and it is obvious that increasing IGF-1/PI3K/Akt signaling inhibits denervation-induced atrophy.

6.3.2 Muscle Unloading and IGF-1 Signaling

Unloading of muscles by limb suspension is a disuse model that causes substantial skeletal muscle atrophy. The obvious difference with denervation is the still intact neuronal innervation, but external and internal loads applied to the limbs remain low.

Hind limb suspension (HLS) for 1–2 weeks did not change IGF-1 mRNA levels in rodent soleus, gastrocnemius, or plantaris muscle [102–109]. In contrast to 1–2 weeks after HLS, IGF-1 mRNA expression levels in the soleus and tibialis anterior were decreased after 2 and 3 days of HLS [108, 110]. This suggests that IGF-1 mRNA expression is downregulated during the initial phase of HLS-induced atrophy but is not involved in the longer-term response. At the protein level, IGF-1 expression drops in rat soleus muscle after 2–4 weeks of unloading [111, 112]. In line with reduced IGF-1 protein levels, HLS in rodents for at least 14 days caused decreased phosphorylated Akt levels and/or IRS-1 protein concentrations in soleus and gastrocnemius muscle, indicating that HLS is a strong stimulus for atrophy which is accompanied by reduced IGF-1/PI3K/Akt signaling [17, 110, 111, 113, 114]. In addition to decreased IGF-1 protein levels, an explanation for the reduced Akt phosphorylation and muscle atrophy during unloading may be the increase in ubiquitin ligase Cbl-b expression which results in an elevated ubiquitination of IRS-1 complexes [114]. The contribution of Cbl-b to HLS-induced muscle atrophy is indicated by the observation that Cbl-b-deficient mice are protected from HLS-induced atrophy [114]. To summarize, IGF-1/PI3K/Akt signaling reduces during unloading in different rodent muscles, while IGF-1 mRNA expression is only decreased in the first days of HLS.

Regarding the effectiveness of pharmacological enhancement of IGF-1/PI3K/Akt signaling to counterbalance HLS-induced atrophy, the literature is contradicting. After a period of 1–2 weeks of HLS, muscle-specific overexpression of IGF-1 did not counteract muscle atrophy in mouse soleus, gastrocnemius, and tibialis anterior muscles [107, 115, 116]. These observations are in line with a study showing that systemic injection of both GH and IGF-1 does not attenuate HLS-induced atrophy in rats, however when combined with exercise, muscle atrophy was attenuated [117]. These studies suggest that stimulation of IGF-1 alone is not sufficient to blunt HLS-induced atrophy, which indicates that unloading-induced atrophy is induced by other mechanisms than by reduced IGF-1/PI3K/Akt signaling solely.

In contrast, several studies show that increasing IGF-1/PI3K/Akt signaling can counterbalance HLS-induced atrophy. Overexpression of IGF-1 by DNA electroporation into skeletal muscle or subcutaneous injection of a mixture of IGF-1 and its stabilizing binding protein IGFBP-3 attenuated HLS-induced atrophy in rodents [118, 119]. Also exercise associated with increased IGF-1 and MGF mRNA levels attenuated HLS-induced atrophy in rats [109]. In addition, injections with ghrelin, a growth hormone-releasing peptide, in mice during 2 weeks of HLS enhanced IGF-1/PI3K/Akt signaling in the plantaris but not in soleus muscle, which alleviated atrophy in the plantaris but not in soleus muscle [120].

It seems that HLS-induced muscle atrophy is accompanied by reduced IGF-1/PI3K/Akt signaling as a result of the degradation of IRS-1. Why pharmacological increasing IGF-1/PI3K/Akt signaling alleviates muscle atrophy in some studies but not all remains unsolved. Exercise, however, seems an effective intervention in attenuating unloading-induced muscle atrophy.

6.3.3 Immobilization and IGF-1 Signaling

Another frequently applied model for disuse and muscle atrophy is joint immobilization, using splints, casts, or surgical staples. The effect of joint immobilization-induced muscle atrophy on IGF-1 expression is however not clear. After ankle and knee immobilization in rodent, rabbit, dog, or human studies, levels of serum IGF-1, muscle protein, or mRNA were not affected [5, 121–124] or decreased [122, 125–127]. Moreover, in human muscle increased levels of IGF-1 mRNA in muscle have been reported upon immobilization [60, 127].

In humans, unilateral knee joint immobilization in 30° knee flexion for 2 weeks in young and old adults was surprisingly related to increased IGF-1 and MGF mRNA levels in m. vastus lateralis, while atrophy was less in old compared to young adults [60, 127]. In contrast, 2 weeks of unilateral knee immobilization in 50° flexion in young adults was associated with a lack of change in serum IGF-1 and mRNA expression levels of IGF-1 as well as MGF in m. vastus lateralis [123]. During immobilization in young adults, serum IGF-1, IGF-1, or MGF mRNA expression increased after administered growth hormone injections, however without attenuating muscle atrophy [123]. When the same protocols were applied to

elderly, results were quite similar, except that growth hormone injections and concomitant increases in serum IGF-1, IGF, and MGF mRNA prevented muscle atrophy [124]. These observations indicate that the angle of immobilization affects IGF-1 expression levels and that increased IGF-1 expression levels during immobilization (with or without growth hormone administration) can counterbalance immobilization-induced atrophy in old but not young adults. Since these results only report IGF-1 mRNA expression or serum levels, there is no certainty regarding the activity of the IGF-1/PI3K/Akt pathway. In accordance with the age effect in humans, attenuation of the reduction in Akt phosphorylation as observed during immobilization experiments by losartan supplementation could completely blunt muscle atrophy during 3 weeks of immobilization of the hind limb of old mice [128]. The protective effect of losartan was mainly by maintaining the number of myofibers, which decrease with aging. This might be an explanation for the age-related difference since IGF-1 is antiapoptotic and would therefore be able to inhibit a potential age-related loss of myofibers in immobilization-induced atrophy. Note that losartan treatment does not provide direct evidence for IGF-1/PI3K/Akt signaling since it affects other signaling pathways such as TGF-β signaling as well.

IGF-1R and Akt phosphorylation decreased during immobilization-induced muscle atrophy in young and old mice, which implies blunted IGF-1/PI3K/Akt signaling [122, 128, 129]. Akt phosphorylation also decreased in m. vastus lateralis of young but not adult humans after 2–4 days of knee joint immobilization [60]. In several models of atrophy including immobilization, miR-29b has been shown to be upregulated which downregulates IGF-1/PI3K/Akt signaling [89]. Subsequent in vitro overexpression of IGF-1 or PI3K concomitant with a miR-29b mimic attenuated miR-29b-induced atrophy [89]. Together these studies indicate that loss of IGF-1/PI3K/Akt signaling during joint immobilization contributes to immobilization-induced muscle atrophy although this may not be true for elder humans.

Increased IGF-1 receptor and Akt phosphorylation by angiotensin-(1-7) treatment alleviated immobilization-induced muscle atrophy in mice [129]. In contrast, in vivo overexpression of IGF-1 (viral mediated or induced by growth hormone) improved muscle morphology, indicated by less widened interstitial space, necrotic fibers, and inflammatory cells, but did not reduce myofiber diameter or muscle cross-sectional area during immobilization [125, 130, 131]. Moreover, mice with reduced mTOR activity show muscle atrophy to the same extent as control mice during immobilization [122]. Taken together, some studies on animal models successfully reduced muscle atrophy or morphology by increasing IGF-1 signaling or activation of downstream IGF-1 targets, while other studies did not show any reductions in immobilization-induced muscle atrophy.

From the above it is concluded that IGF-1/PI3K/Akt signaling reduces during joint immobilization. Whether stimulation of IGF-1 signaling plays a role in the maintenance of muscle mass during immobilization-induced muscle atrophy has not been unambiguously established, although in older subjects this may be the case.

6.3.4 *Muscle Aging and IGF-1 Signaling*

In addition to primary disuse models, aging is also associated with skeletal muscle atrophy. The loss of skeletal muscle mass and strength during aging, referred to as sarcopenia, is determined by combination of two processes, i.e., loss of myofibers and myofiber atrophy, which have different temporal distributions [132]. As a result of loss of motor units, remaining myofibers are possibly more active as compensation. Whereas under disuse conditions, predominantly type 1 fibers are affected, during aging type 2 myofibers are more susceptible to atrophy and necrosis compared to type 1 myofibers. In aging, the loss of muscle mass is likely due to a reduction in physical activity, oxidative stress, chronic low-grade inflammation, and changes in systemic serum proteins [133]. The chronic state of low-grade inflammation related to aging is associated with increased IL-6 and TNF-α plasma levels [134]. These cytokines can interfere with IGF-1 signaling (see 6.4 *Interference with IGF-1 Signaling*) and are therefore likely to play a role in aging-associated muscle wasting [135, 136].

IGF-1 serum levels decrease with age, but no differences in IGF-1 serum levels were shown between elderly females with and without sarcopenia [137]. Based on small effects of GH injections on muscle hypertrophy in elderly, while exercise is capable of inducing hypertrophy, several literature-based studies suggest that locally expressed IGF-1 is important in the maintenance of muscle mass, while there is no consistent evidence for a relationship between IGF-1 serum levels and age-related loss of muscle strength [138–140]. The role of the IGF-1/PI3K/Akt pathway is discussed below.

Cross-sectional analyses of a large cohort including over 100 human participants and different mouse models, suggest that IGF-1/PI3K/Akt signaling activity is unaffected during aging [141]. Whereas skeletal muscle mRNA levels of IGF-1Ea and MGF reduced with age in mice, this was not evident in skeletal muscle of human subjects. MuRF-1 knockout old mice showed a blunted atrophy but decrease in muscle force, which indicates that proteasomal degradation is essential for maintaining muscle quality during aging. In addition, MuRF-1 and MAFbx mRNA levels did not differ between old sedentary and young human participants [141]. These observations are in line with those of another study showing no change in IGF-1/PI3K/Akt signaling, indicated by unaffected IGF-1R and Akt phosphorylation, in skeletal muscle of klotho mutant mice, a mouse model with an aging-related phenotype showing muscle atrophy [63]. In addition, it was shown that MuRF-1 and MAFbx protein levels in skeletal muscle were not upregulated in klotho mutant mice compared to control mice [63]. Also, no differences in IRS1 phosphorylation did exist between old and young adult rats [142]. Together, these studies indicate that IGF-1/PI3K/Akt signaling is not downregulated with aging and sarcopenia is not the result of increased activation of the ubiquitin-proteasome system.

Note that in old rodent muscles, both similar [62, 63, 143] and lower [142, 144] pAkt/tAkt levels compared to young rodent muscles have been reported. In line with these observations, in biopsies of young and old human subjects, both similar

[141] and decreased [145] levels of pAkt/tAkt with age have been reported. The decrease in pAkt/tAkt in aged humans was likely due to increased levels of tAkt, while pAkt levels were not affected, which suggest that in old human muscle, IGF/PI3K/Akt signaling activity is not reduced, but Akt synthesis is upregulated [145]. Although some studies show decreased levels of pAkt/tAkt related to aging, there is not an obvious reduction in IGF-1/PI3K/Akt signaling.

In mice, virus-mediated or transgenic overexpression of IGF-1 can prevent aging-induced muscle atrophy and a decrease in type 2B fiber fraction [146, 147]. Despite elevated IGF-1 expression, sedentary transgenic IGF-1 old mice did not have larger myofiber diameters compared to their aged-matched controls, whereas sedentary transgenic IGF-1 adult mice did show larger myofiber diameters compared to their aged-matched controls [148]. This suggests a decreased anabolic response to IGF-1/PI3K/Akt signaling with age rather than the inability of IGF-1/PI3K/Akt signaling to prevent the aging-associated atrophy. Indeed, overload of hind limb muscles of young, mature, and old rats showed reduced hypertrophy and decreased upregulation of MGF and IGF-1 receptor mRNA with age [149]. This result is in accordance with those of other studies suggesting an impaired anabolic response of the IGF-1/PI3K/Akt pathway in aged rats [142, 150]. From this it can be concluded that the trophic response to IGF-1 decreases with age, but is not completely lost and overexpression of IGF-1 is capable of attenuating aging-related muscle atrophy. Moreover, decreased Akt phosphorylation but no changes in activity of downstream targets of mTOR upon a single bout of resistance exercise were observed in old compared to adult humans, suggesting that the synthesis machinery is not affected by age but rather the IGF-1/PI3K/Akt signaling [151]. A possible explanation is that exercise-induced IGF-1/PI3K/Akt signaling is inhibited by increased levels of IL-6 and TNF-α associated with the chronic low grade of systemic inflammation seen with aging [135, 136].

Regarding the effects of aging, IGF-1/PI3K/Akt signaling does not seem to be reduced during aging-associated muscle atrophy, while IGF-1 overexpression is able to inhibit aging-associated muscle atrophy. However, the anabolic potential of this pathway reduces with age, which might be due to increased interference of pro-inflammatory cytokines.

6.4 Interference with IGF-1 Signaling

Changes in IGF-1/PI3K/Akt signaling can be the result of decreased IGF-1 expression, bioactivity, receptor availability, or inhibition along its pathway. Insight into the mechanisms affecting IGF-1/PI3K/Akt signaling will reveal possible candidates for counterbalancing reduced IGF-1/PI3K/Akt signaling. Because IGF-1 is involved in many tissues and cell types, clinical interventions should be muscle specific or target a factor which interferes with IGF-1 and has a lesser general effect. Although it is outside the scope of this review to discuss all different interfering factors, a few important ones are pointed out.

AMP-activated kinase (AMPK) interferes with IGF-1 signaling by inhibiting and stimulating the downstream targets mTOR and FOXO3 [152–154]. Moreover, AMPK/FOXO3 signaling increased during HLS-induced muscle atrophy in rats [155], which could explain why not all studies report an effect of IGF-1 overexpression on HLS-induced muscle atrophy. Another negative regulator of myofiber size is myostatin, which is a member of the TGF-β family [156]. Myostatin inhibits Akt via Smad3 signaling and has therefore opposite effects compared to IGF-1 [157]. Several types of muscle atrophy are associated with increased myostatin expression (see Chap. 8).

As mentioned before, also pro-inflammatory cytokines like IL-6 and TNF-α can interfere with IGF-1 signaling and likely play a role in muscle atrophy associated with systemic inflammation, such as aging [135, 136]. IL-6 is able to inhibit mTOR, p70S6K, and p90RSK activation in muscle cells, without affecting Akt phosphorylation [158]. TNF-α impairs IGF-1R sensitivity [136] and increases MuRF-1 expression by activating a group of transcriptions factors known as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [13]. Reducing systemic inflammation could counterbalance inflammatory-associated muscle wasting by enhancing the effect of IGF-1. Regular exercise stimulates IGF-1 expression and has an anti-inflammatory effect [159] which attenuates the interference of cytokine signaling with IGF-1 and is therefore a safe and cheap intervention to counterbalance muscle atrophy associated with elevated levels of IL-6 and TNF-α.

Recent studies have shown that microRNAs (miRNAs) are capable of interfering with IGF-1 signaling and thereby play an important role in muscle atrophy [89, 160]. miR-29b negatively regulates IGF-1 and PI3K expression and has been shown to be upregulated in the tibialis anterior, soleus, and EDL muscle in denervation-induced muscle atrophy [89]. Moreover, miR-29b was also upregulated in immobilization, dexamethasone, fasting, cancer cachexia, and aging-induced muscle atrophy [89]. In addition to miR-29b, miR-18a also suppresses IGF-1/PI3K/Akt signaling, and its overexpression induces muscle atrophy [160]. Because of the general role of miR-29b in muscle atrophy (i.e., upregulation in several muscles and atrophic conditions) and the observation that many miRNAs have been shown to be tissue specific [161, 162], miRNAs are promising targets for counterbalancing muscle atrophy. Preclinical and clinical trials in which miRNAs are targeted are currently conducted, although, to the best of our knowledge, not aimed to prevent or restore muscle wasting.

6.5 Conclusions and Future Perspectives

Here we reviewed the role of IGF-1 signaling in the induction of muscle atrophy and show that in disuse conditions muscle atrophy is in part due to a decline in IGF-1 signaling, whereas with aging-associated muscle atrophy, IGF-1 signaling remains unaffected. Moreover, enhancement of IGF-1/PI3K/Akt in some conditions is an effective strategy to counterbalancing muscle atrophy, however this does not

apply to all disuse conditions. Under hypertrophic conditions by mechanical loading, IGF-1/PI3K/Akt signaling increases muscle mass by stimulating protein synthesis and inhibiting protein degradation. Protein synthesis is stimulated by mTOR, which activates p70S6K and p90RSK, which are downstream targets of Akt and PDK1. Akt also stimulates protein synthesis by inhibiting GSK3β activity. During atrophic conditions, protein synthesis is reduced and/or protein degradation is increased. The four main mechanisms in protein degradation are proteasomal-, lysosomal-, and caspase- and calpain-dependent protein degradation. Regarding the role of IGF-1 in protein degradation, it is clear that IGF-1 inhibits proteasomal-mediated muscle protein degradation by lowering the expression of E3-ligases, resulting in attenuated protein ubiquitination. Reductions in expression of E3 ligases are a result of inactivation of FOXO transcription factors by phosphorylated Akt. In addition, FOXO inactivation by phosphorylated Akt also reduces lysosomal degradation. When IGF-1/PI3K/Akt signaling decreases during atrophic conditions, caspase-dependent degradation seems to be reduced as well. Future research is required to obtain more detailed insight in the role of IGF-1/PI3K/Akt signaling on calpain-dependent degradation.

The role of the IGF-1/PI3K/Akt pathway differs between different models of skeletal muscle atrophy. During denervation-induced atrophy, IGF-1/PI3K/Akt signaling activity is reduced, and upregulation of IGF-1/PI3K/Akt signaling counterbalances denervation-induced muscle atrophy. In contrast, during unloading- and joint immobilization-induced atrophy, IGF-1/PI3K/Akt signaling activity is reduced as well, but it remains unclear whether upregulation of the IGF-1/PI3K/Akt pathway is sufficient to attenuate denervation- or joint immobilization-induced muscle atrophy, suggesting that other pathways are involved which cannot be compensated by IGF-1/PI3K/Akt signaling. No obvious downregulation of IGF-1/PI3K/Akt signaling is shown during aging-associated atrophy. Although the anabolic potential of the IGF-1/PI3K/Akt pathway reduces with age, activation of this pathway has the ability to achieve recovery of aging-associated muscle atrophy.

The role of miRNAs in regulation of myofiber size is a novel and promising area for further research. Many miRNAs are tissue specifically expressed and could target IGF-1 signaling components in muscle wasting without affecting its role in many tissues and cell types. Although there is substantial evidence showing that miRNAs can interfere with IGF-1/PI3K/Akt signaling, there remains a lack of knowledge regarding the possibilities to counterbalance muscle atrophy by targeting miRNAs. Because of the general effects of miRNAs in several conditions of muscle atrophy and muscle phenotypes, future studies should aim for more insight in knowledge regarding biological functions of miRNAs and clinical application of altering miRNA activity in prevention and recovery of muscle atrophy. Overall, IGF-1/PI3K/Akt is a key signaling pathway in protein synthesis and degradation, of which its activity is attenuated during several disuse models.

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Chapter 7
mTOR Signaling Pathway and Protein Synthesis: From Training to Aging and Muscle Autophagy

Jocemar Ilha, Caroline Cunha do Espírito-Santo, and Gabriel Ribeiro de Freitas

Abstract In muscle tissue there is a balance between the processes muscle synthesis and degradation. The mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in regulating protein synthesis in order to maintain muscular protein turnover and trophism. Studies have shown that both down- and upregulation mechanisms are involved in this process in a manner dependent on stimulus and cellular conditions. Additionally, mTOR signaling has recently been implicated in several physiological conditions related to cell survival, such as self-digestion (autophagy), energy production, and the preservation of cellular metabolic balance over the lifespan. Here we briefly describe the mTOR structure and its regulatory protein synthesis pathway. Furthermore, the role of mTOR protein in autophagy, aging, and mitochondrial function in muscle tissue is presented.

Keywords mTOR pathway · Muscular synthesis · Muscle trophism · Muscle autophagy

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7.1 Background

The abilities to get out of bed, stand up from a seat, walk, or reach for an object, such as a glass of water, are examples of the most common activities of daily life, which are fundamental to independence. The integrity of muscle mass and the capacity to generate muscular force are essential prerequisites for the performance of such activities. Moreover, maintaining and gaining muscle mass are critical features for the preservation of health and quality of life. Skeletal muscle is a highly adaptable human tissue with a known sensitivity to environmental factors, such as the mechanical overload imposed by muscle activity, as well as muscular disuse caused by inactivity in situations of trauma, chronic illness, or aging [1–4].

In muscle tissue, there is a balance between the processes of synthesis and degradation, with the continuous renewal of muscle proteins [5]. In healthy muscle, the mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in regulating protein synthesis in order to maintain muscular trophism. Notably, in muscle hypertrophy, the mTOR pathway is upregulated [5, 6]. By contrast, under hypotrophic conditions there is a reduction in mTOR pathway biomarkers [7], showing the direct role of this pathway in maintaining muscle fiber size.

Additionally, mTOR signaling has recently been implicated in several physiological conditions related to cell survival, such as self-digestion (autophagy), energy production, and the preservation of cellular metabolic balance [8]. Moreover, deregulation of this mechanism leads to pathologic alterations associated with several diseases, such as cancer, neurodegeneration, and infection, as well as alterations to muscle homeostasis in the aging process.

In this chapter, we summarize the structure and roles of mTOR and the mTORC1 complex in protein synthesis and during muscle hypotrophy. Below, we first describe the structure of the mTOR protein and its regulatory protein synthesis pathway. Afterward, we outline the role of mTOR in autophagy, aging, and its mitochondrial function in muscle tissue.

7.2 The Structure of TOR Signaling

The TOR protein was first identified in *Saccharomyces cerevisiae* yeasts. In these yeasts, rapamycin – a compound produced by bacteria originally isolated from the soil of Easter Island – was able to inhibit gene activity for eukaryotic cell growth and proliferation, while it remained bound to a highly conserved domain, called FK506-binding protein 1A (FKBP12) [9, 10]. Thus, it has been suggested that the protein products of these genes might be targets of rapamycin, designated the TOR – target of rapamycin [11]. In mammalian cells, the TOR ortholog was also identified and named mTOR, i.e., the mammalian target of rapamycin [12].

The mTOR is a serine/threonine kinase capable of integrating several stimuli from the medium, such as nutrients, growth factors, energy, and stress to regulate

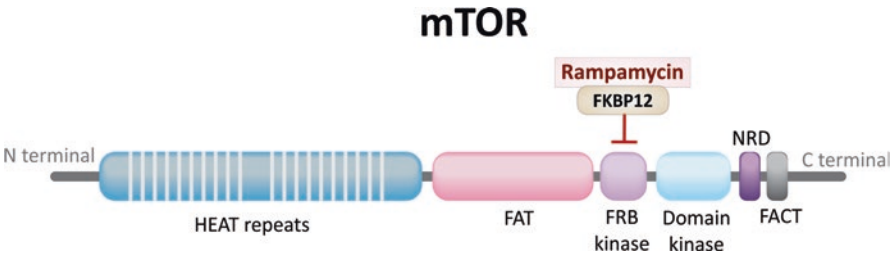

Fig. 7.1 Illustration of the structural composition of the mammalian target of rapamycin (mTOR) with its domains: HEAT, FAT, FRB, kinase, and FACT

cell growth, proliferation, and metabolism [13]. Structurally, mTOR contains 2549 amino acids; additionally, the HEAT component (responsible for inter-protein interaction), FAT, FRB (rapamycin-binding site), catalytic domain kinase, and FATC form the mTOR major domains (Fig. 7.1). The FAT and FATC domains are always found in combination and contribute to the catalytic activity of mTOR [14–16]. To date, only a few mTOR phosphorylation sites have been described, namely, Thr-2446, Ser-2448, Ser-2481, Ser-1261, and Ser-2481, being the self-phosphorylation site for regulating intrinsic mTOR activity [17].

In mammals, mTOR works by forming two multi-protein complexes – mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) – which are responsible for different physiological functions and have distinct levels of sensitivity to rapamycin [18]. The mTORC1 is a raptor-sensitive complex composed of mTOR associated with Raptor (regulatory protein of mTOR activity kinase) [19, 20]. In mTORC2, mTOR is associated with Rictor (an mTOR partner insensitive to rapamycin), mTOR-associated protein (mLST8), and SIN1. The functions of mTORC2 include the activation of the Akt protein for protein degradation [19, 21], among others. However, few studies are available on mTORC2 activity [22].

On the other hand, mTORC1 has been the subject of several studies because of its variety of functions, the best described being related to the initiation of protein translation and transcription mechanisms for cell growth [13]. To play this important role, mTORC1 activity is generally regulated by the phosphatidylinositol 3-kinase (PI3K)/Akt/tuberous sclerosis complex 1 and 2 (TSC1–2) pathway in the presence of insulin or other growth factors [23] (Fig. 7.2).

Thus, the responsiveness of mTORC1 to insulin and growth factors is provided through the activation of PI3K and Akt protein kinases. In the presence of the stimulus, the tyrosine residues of the p85-PI3K regulatory subunit is activated and provide subsidies for the p110-PI3K catalytic subunit transfer phosphate pools to the phosphatidylinositol-3,4,5-triphosphate (PIP-3) membrane phospholipids. Once activated, PIP-3 attracts several protein kinases, especially Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK-1), translocating them to the cell membrane [24]. Then, the PDK-1 and PDK-2 proteins activate the Thr-308 and Ser-473 residues, respectively, for activation of Akt. Under favorable conditions for protein synthesis, activation of Akt culminates with the phosphorylation and inhibition of the TSC1-2 complexes, which in turn convert the protein Ras homolog

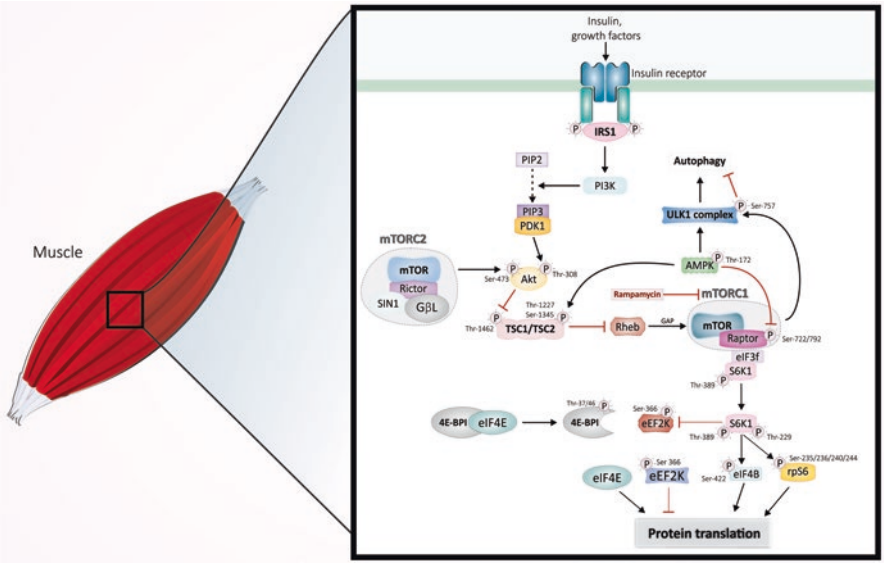

Fig. 7.2 The mammalian target of rapamycin (mTOR) pathway integrates signals from nutrients, energy status, and growth factors to regulate many processes, including protein translation, autophagy, and ribosome biogenesis and cell proliferation. In skeletal muscle, the binding of insulin-like growth factor-1 (IGF-1) and/or insulin to its receptor on the cell membrane leads to the phosphorylation of insulin receptor substrate 1 (IRS-1), an adapter protein that activates phosphatidylinositol 3-kinase (PI3K). Activated PI3K generates phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits (3- phosphoinositide-dependent protein kinase 1) PDK1 and phosphorylates the protein kinase B or Akt. The tuberous sclerosis complex (TSC1-TSC2) is a target downstream of Akt and inhibits the small G-protein, Ras homolog enriched in brain (Rheb) – a regulator of mTOR. The rapamycin-sensitive mTOR complex 1 (mTORC1) contains multiple proteins and phosphorylates the 70 kDa ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1(4EBP1) for protein translation. Once phosphorylated, the p70S6K acts on ribosomal protein S6 (S6 or rpS6), eukaryotic translation initiation factor 4B (eIF-4B), and eukaryotic elongation factor-2 kinase (eEF-2K). The phosphorylation of 4E-BP1 regulates eIF-4E availability, dissolving the 4E-BP1/eIF-4E complex. The inhibition of the mTORC1 activates autophagy by phosphorylation of Unc-51 like autophagy activating kinase 1 (Ulk1) in the presence of AMP-activated protein kinase (AMPK). In addition, the mTOR can also suppress protein degradation via mTOR complex 2 (mTORC2) interaction with Akt

enriched in brain (Rheb) to its inactive state, which allows the activation of mTORC1 [21] (Fig. 7.2).

At the same time, activation of mTORC2 via the PI3K/Akt/TSC1-2 pathway leads to the activation of Akt in order to control protein degradation [19, 21]. Once phosphorylated by mTORC2, Akt plays a role as a negative regulator of the transcription factors called forkhead box protein (FoxO), shifting it from the cell nucleus to the cytoplasm. The retention of FoxO in the cytoplasm impedes the regulation of two ubiquitin ligases: atrogin-1 or MAFbx and MuRF1, both related to ubiquitin-proteasome system signaling, considered the major pathway of proteolytic degradation of eukaryotic cells [21, 25].

7.2.1 The mTOR Signaling in Muscle Protein Synthesis

The mTOR is considered the major effector of proliferation and cell growth through the regulation of protein synthesis. In muscle tissue with preserved innervation, it was observed that in the presence of rapamycin, muscle growth was partially inhibited, showing that mTOR is an important pathway for trophic muscle regulation [26].

Stimulation of mTOR protein synthesis via mTORC1 is the most common biological response controlled by this pathway under favorable conditions, such as nutrient and oxygen availability [27]. In the presence of the appropriate stimulus, the mTORC1 mediates the signaling of two major substrates: the 70 kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [5, 21]. In general, both p70S6K and 4E-BP1 control the initiation of translation, favoring the attachment of messenger ribonucleic acid (mRNA) to the 40S ribosomal subunit (Fig. 7.2). This in turn binds to other eukaryotic initiation factors to perform the codon reading and consequently promote the synthesis of new proteins [23] (Box 7.1).

In skeletal muscle, growth factors [35], mechanical adaptive overload [6, 36], and resistance exercise [37, 38] are described as the main promoters of protein biosynthesis. Bodine et al. [6] and Goodman et al. [36] reported overload in *plantaris* muscle increased protein content, characterized by augmented tissue cross-sectional area (CSA) and weight. These morphological changes go along with the phosphorylation of p70S6K and release of the eIF-4E of 4E-BP1/eIF-4E complex. By contrast, the combination of muscle workload with the rapamycin – mTORC1 inhibitor – attenuates hypertrophy and prevents p70S6K phosphorylation and the release of eIF-4E, indicating that muscle trophism is closely linked to the mTOR/p70S6K/4E-BP1 pathway [6, 36].

Box 7.1: Target Downstream of mTOR

The p70S6k signaling pathway culminates in phosphorylation of multiple serine residues of 40S ribosomal protein S6 (RpS6) [28], which correlates with enhanced translation of mRNAs with a 5′-terminal oligopyrimidine (TOP) [29] and regulation of ribosomal protein synthesis [30]. In addition, p70S6K can alternatively phosphorylate and regulate the eukaryotic initiation factor (eIF) 4B (eIF-4B), responsible for facilitating mRNA binding to ribosomes [31] and the eukaryotic elongation factor (eEF) 2 kinase (eEF-2K), implicated in ribosomal translocation during the elongation stage of protein synthesis [32]. Another key modulator of protein biosynthesis is 4E-BP1, which is a natural inhibitor of translation started by repression of eIF-4E. When phosphorylated, 4E-BP1 releases eIF-4E, responsible for the recruitment of eIF-4G and eIF-4A and formation of the eIF-4F complex translation [33]. In eukaryotic cells, the initiation of translation requires the formation of the eIF-4F complex (eIF-4E, eIF-4A, and eIF-4G) to direct ribosomes to codon initiation [34].

Although both p70S6K and 4E-BP1 are implicated in the regulation of cell size, each has a distinct role in muscle protein biosynthesis. Studies have reported that p70S6K1 (an isoform of p70S6k which contains the Thr-389 threonine residue for mTOR phosphorylation) is crucial to initiating protein synthesis and preventing muscle hypotrophy [39]. In the temporal context, p70S6K1 is the first protein to be phosphorylated after resistance training, remaining in high concentrations for hours after training, and is associated with phosphorylation of S6 and increased muscle protein content [38]. In p70S6K1-deficient mice, genic deletion induced an atrophic phenotype marked by reduction in the CSA of soleus muscle, even in the presence of phosphorylated 4E-BP1 – another regulator of protein translation [39]. This indicates muscle trophism substantially depends on p70S6K1 (Box 7.2).

Box 7.2 Downregulation of p70S6K by Other Pathways

The MAPK/ERK pathway contributes to cell proliferation by direct phosphorylation of the S6 protein

Unlike p70S6K1, the p70S6K2 isoform is activated by mTOR at Thr-388 residue, but regulatory proteins composing MAPK/ERK pathway are indispensable for the complete activation of this isoform. However, the relevant role of MAPK/ERK pathway has been implicated in the direct phosphorylation of Ser-235/236 residue in ribosomal protein S6 (substrate of p70S6K1 and p70S6K2, involved in cell growth-cell proliferation) [45]. Pende et al. [46] showed that p70S6K1−/−;p70S6K2−/−mouse cells exhibit impairment of S6 phosphorylation, interfering in animal viability, but the proliferative responses of these cell types were not affected. At the same time, S6 phosphorylation persisted at Ser235 and 236 residues (residues not phosphorylated by p70S6K2), in response to mitogens, suggesting the involvement of MAPK/ERK in the maintenance of cell proliferation, in the absence of mTOR/p70S6K [46]. This means that the contribution of the MAPK/ERK pathway might involve the amplification of the p70S6K2 isoform, phosphorylated by mTOR in a distinct threonine residue (Thr-388) or in the direct phosphorylation of S6, to promote cell growth.

PKC is relevant for increased S6K2, but not S6K1, in cytoplasm cell

PKC is also a protein regulator of the p70S6K2 isoform (but not S6K1), which plays a role in the localization of p70S6K2 inside the cell [47]. This phospholipid-dependent serine/threonine kinase activates a domain nuclear localization sequence (NLS) binding at Ser486 residue of p70S6K2, in the nucleus, and promoting p70S6K2 nucleo-cytoplasmic shuttling, without affecting its activity [45]. This suggests PKC-mediated cell growth might be due to increased availability of p70S6K2 to the cytoplasm, which may be phosphorylated by mTOR at Thr-388 residue and other mitogenic factors, such as the MAPK/ERK pathway as mentioned above.

Phosphorylation of 4E-BP1 alone appears to be insufficient to promote increased muscle trophism. In fact, several studies have shown that in hypertrophic muscle, the upregulation of 4E-BP1 occurs concomitantly with p70S6K in an mTOR-sensitive manner [6, 40], indicating that the 4E-BP1 phosphorylation is a coadjutant in protein synthesis. However, during resistance exercise, 4E-BP1 plays an inverse role. In this context, it prevents the translation of new proteins [7]. In conditions involving energetic imbalance, such as exercise, metabolic modulators are activated to direct energy to cellular events indispensable for survival and reducing protein synthesis (see more in Vavvas et al. [41] and Musi et al. [42]). This suggests 4E-BP1 indirectly participates in energetic control and reducing muscle biosynthesis (saving energy), since the phosphorylation status of 4E-BP1 is temporarily reduced during resistance training [7].

Recently, researchers have found that resistance and endurance exercise programs can stimulate Akt, mTOR, and p70S6K, which are both involved in protein synthesis pathway [38, 43]. However, Akt/mTOR/p70S6K cascade signaling is transitory and only remains active during endurance exercise [43], being interrupted immediately after training. On the other hand, after resistance exercise, which promotes increases in strength generation capacity, morphological changes, and protein content, phosphorylation of mTOR/p70S6K/S6 remains active for up to 4 h after training [38].

Although the mTORC1 is preferentially activated in response to resistance exercise, it is possible that distinct pathways regulate the trophic state induction and maintenance mechanisms. Using an electrical stimulation protocol in the *tibialis anterior* combined or not with rapamycin, West et al. [44] found a reduction in muscle protein synthesis and the ribosomal RNA precursor in animals treated with rapamycin up to 6 h after training. These changes were associated with a reduction in p70S6K and S6K phosphorylation. After this interval, protein synthesis, but not ribosome biogenesis, increased in a rapamycin-insensitive manner and is not mediated by improvement in the translational capacity. Furthermore, the activation of mitogen-activated protein kinase (ERK 1–2) and dephosphorylated eEF-2 indicates a reduction in p70S6 protein phosphorylation – occurring concomitantly with increased protein synthesis – suggesting an alternative mTOR-independent mechanism for long-term cell size regulation in skeletal muscle [44].

Lastly, upstream targets of mTOR, such as Akt, have received considerable attention due to their capacity for upregulation after trophic stimulus. Léger et al. [5] reported that the activation of Akt occurs in parallel with the inhibition of FoxO protein. This transcription factor is required for the regulation of two types of ubiquitin ligases: atrogin-1 or MAFbx and MuRF1, both related to ubiquitin-proteasome system signaling – considered the major pathway of eukaryotic cell proteolytic degradation [21, 25]. For this reason, Léger et al. [5] believe the inhibition of FoxO – which consequently prevents muscle atrophy in healthy muscle – can partially regulate muscle trophism.

7.3 mTOR Signaling Autophagy, Aging, and Mitochondrial Function in Muscle Tissue

Autophagy is a mechanism of cellular self-degradation that plays an important role in cell survival. It is involved in promoting energy production and preservation of the cellular metabolic balance and removing damaged organelles and proteins that may be toxic to the body in some conditions [8]. Although this cellular mechanism is primarily protective, it can also play a role in cell death. Moreover, dysfunction of this mechanism is associated with several diseases, such as cancer, neurodegeneration, and infection, as well as with the cellular aging process.

With aging, there is a change in the balance of the regular autophagy, with a gradual reduction in this process. This results in the accumulation of severely deteriorated proteins and organelles, increasing oxidative stress and tissue damage, inducing a progressive loss of system integrity, damaging functions, and making the organism vulnerable – thus limiting its useful life [48–50]. This dysregulation is associated with several pathologies in humans, including neurodegenerative diseases; lysosomal disorders; cellular senescence and changes in muscular function, such as loss of myofiber number and protein content (hypotrophy); and reduction of muscle contractility, strength, and resistance [8, 48, 50–52]. The mTOR/mTORC1 pathway – by regulating Unc-51 like autophagy activating kinase 1 (Ulk1) – seems to play a crucial role in this process (Fig. 7.2). This pathway may trigger two cellular processes – protein synthesis and autophagy – depending on the nutrient content and energy available in the mTOR cascade targets, 4EBP1, p70S6K, AMPK, and Raptor [53].

Muscle biopsies, performed in humans after endurance exercise and high-intensity exercises, demonstrate that both physical exercises with the stimulation of insulin-like growth factor-1 (IGF-1) and insulin-related energy issues are able to control the autophagy flow via mTORC1 or AMPK by their interactions with the Ser/Thr Ulk1 kinase complex [54–56]. The balance between the TORC 1 and 2 signals is maintained by the release/inhibition of Akt activity and consequently a negative/positive regulation of autophagy, which is one of the key points in the regulation of the Akt pathway for the autophagy and aging process, depending on which TOR complex is active [54].

When insulin and IGF-1 growth factor signaling occurs via lipid and phosphatase and tensin homolog (PTEN) protein, which negatively regulates insulin/PI3-K activity, there are activation of Akt and a tendency for activation of the TORC1 complex, promoting the suppression of autophagy by Raptor-mediated phosphorylation of ULK1 at Ser-757 [53, 55]. Under unfavorable conditions, such as nutrient reduction or as demonstrated in the treatment with rapamycin in animal models, the mTORC1 pathway is blocked, thus inducing AMPK activation, which in turn interacts with Ulk1 and ATG13 promoting its phosphorylation at Ser-555 and blockade of the Raptor – triggering the onset of autophagy/phagocytosis [50, 57].

In addition to this relationship with autophagy/aging, the mTORC1 complex participates in energy regulation through mitochondrial activity, enhancing functional

capacity and stimulating its biogenesis through peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 alpha (PGC-1α) and YY-1 transcription factor [58, 59]. There is a potential mechanism based on redox activity of the mTOR pathway, detecting nutrients and mitochondrial activity, and its signaling may be active in the reciprocal direction regulating mitochondrial metabolism [60].

The relationship of the Raptor-mTORC1 complex with oxygen consumption in mitochondria and oxidative capacity has been shown in experiments involving blocking TORC1 complex activity by rapamycin, which results in decreased mitochondrial oxygen consumption levels [60–62]. The stimulation of the mTOR pathway promotes an increase in ATP production by phosphorylation and the regulation of the balance between glycolysis and mitochondrial metabolism [60–63]. These data show that the mTORC1 complex plays a role in the control of mitochondrial oxidative function, positively regulating PGC1-α activity and in turn modulating the mitochondrial gene and oxidative metabolism, contributing to cell growth and mitochondrial metabolism [58, 59].

In situations of prolonged immobilization or when there is a reduction in muscle activity, as in the case of the aging process, signs of skeletal muscle atrophy – reduction in trophism and muscle strength – are triggered. This process is closely related to the decline in mitochondrial function, reduction in protein synthesis, and higher protein degradation – ATP-dependent processes [64–66].

These regulatory mechanisms of atrophy, energy content, autophagy, and mitochondrial function via the mTOR pathway are complex and may decrease with age in most tissues. This promotes impaired homeostasis and reduced cellular respiration, leading to an increase in free radicals within cells, and may cause damage to a number of systems, including the heart and skeletal muscle, pancreas, and liver [49, 63, 66].

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Chapter 8
Past, Present, and Future Perspective of Targeting Myostatin and Related Signaling Pathways to Counteract Muscle Atrophy

Willem M. H. Hoogaars and Richard T. Jaspers

Abstract Myostatin was identified more than 20 years ago as a negative regulator of muscle mass in mice and cattle. Since then, a wealth of studies have uncovered the potential involvement of myostatin in muscle atrophy and sparked interest in myostatin as a promising therapeutic target to counteract decline of muscle mass in patients afflicted with different muscle-wasting conditions. Insight in the molecular mechanism of myostatin signaling and regulation of myostatin activity has resulted in the identification of specific treatments to inhibit myostatin signaling and related signaling pathways. Currently, several treatments that target myostatin and related proteins have been evaluated in preclinical animal models of muscle wasting, and some potential therapies have progressed to clinical trials. However, studies also revealed potential downsides of myostatin targeting in skeletal muscle and other tissues, which raises the question if myostatin is indeed a valuable target to counteract muscle atrophy. In this review we provide an updated overview of the molecular mechanisms of myostatin signaling, the preclinical evidence supporting a role for myostatin and related proteins in muscle atrophy, and the potential issues that arise when targeting myostatin. In addition, we evaluate the current clinical status of different treatments aimed at inhibiting myostatin and discuss future perspectives of targeting myostatin to counteract muscle atrophy.

Keywords Myostatin · Signaling pathways · Muscle atrophy · Therapy

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8.1 Background

Elucidation of the molecular mechanisms and signaling pathways involved in different forms of muscle atrophy is crucial for the identification of potential targets to counteract muscle wasting. One of the most promising potential therapeutic targets to counteract muscle atrophy that emerged in past years was myostatin. Myostatin was identified in mice over 20 years ago in 1997 as growth and differentiation factor 8 (GDF8), a new member of the TGF-β superfamily and specific regulator of muscle mass. Remarkably, genetic deletion of myostatin in male and female mice resulted in hypermuscularity caused by muscle fiber hyperplasia and to lesser extent muscle fiber hypertrophy [1]. In the same year, three groups independently identified mutations in the myostatin gene in double-muscled Belgian Blue and Piedmontese cattle, which show increased muscle mass compared to conventional cattle mainly due to muscle fiber hyperplasia [2–4]. In the following years, myostatin loss-of-function mutations were also identified in other species that display hypermuscular phenotype including Texel sheep [5], whippet racing dogs [6], and Thoroughbred horses [7, 8], showing that myostatin is evolutionary conserved in mammals. Importantly, the association of decreased myostatin levels with athletic performance was demonstrated in one study in whippets where haploinsufficiency of myostatin was associated with increased muscle mass and improved racing performance [6]. However, the advantage of this mutation in racing performance is lost in dogs homozygous for this allele, since these so-called bull whippets develop a double-muscled phenotype that hinders performance at the racing track [6]. Functional conservation of myostatin in humans was furthermore established in a study where the authors identified an intronic mutation in the *MSTN* gene of a German boy that resulted in missplicing of the mRNA and introduction of a premature stop codon causing pronounced muscle hypertrophy [9]. This homozygous mutation in the boy was furthermore associated with extraordinary muscle strength and was inherited via his mother, who was heterozygous for the mutation and a former professional athlete [9].

The identification of myostatin as a muscle-specific regulator of muscle mass sparked interest in myostatin as a potential novel therapeutic target to counteract muscle atrophy. The discovery of myostatin was especially exciting since loss of function did not seem to result in pronounced side effects and resulted in specific increase of muscle mass. In this review we will describe the preclinical evidence that targeting myostatin and related signaling pathways may counteract muscle atrophy in different muscle-wasting conditions and present an update on the clinical translation of different compounds that target these pathways. In addition, latest insight in the upstream and downstream molecular pathways involved in myostatin signaling will be discussed as well as cross-signaling of this pathway with other pathways involved in the regulation of muscle mass.

8.2 Molecular Mechanism of Myostatin Signaling in Skeletal Muscle

Myostatin, also known as growth and differentiation factor 8 (GDF8), is a member of the transforming growth factor beta (TGF-β) family of growth factors/cytokines. The TGF-β family consists of TGF-βs, activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) and can roughly be divided into secreted ligands that mediate downstream intracellular signaling via Smad1/5/8 proteins (BMPs/GDFs) or Smad2/3 proteins (activins, myostatin, GDF11, TGF-β). The active, mature domains of TGF-β, activins, and myostatin form dimers via cross-linking of conserved cysteine residues, and these dimers interact with the receptor domains of type II receptor kinases present on cell membranes. Subsequent recruitment and activation of type I receptor kinases result in activated ligand-receptor complexes that mediate downstream intracellular signaling via intracellular phosphorylation of the receptor-regulated R-Smad proteins Smad2 and Smad3 (Fig. 8.1). Co-Smad protein Smad4 interacts with phosphorylated R-Smad proteins, which results in the translocation of these heteromeric Smad complexes to the cell nucleus where they regulate transcription of target genes by interacting with other sequence-specific transcription factors and cofactors [10]. In addition, myostatin and related ligands also activate several intracellular noncanonical pathways such as

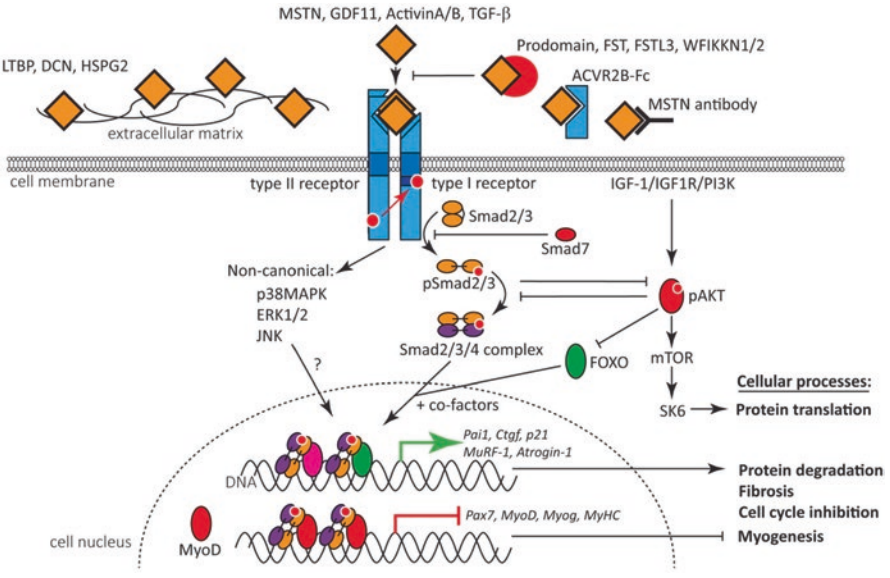

Fig. 8.1 Overview of the signaling pathway of myostatin and related TGF-β ligands, showing proteins/treatments that inhibit the activity of myostatin and related ligands, the effect of the canonical Smad2/3 pathway, and cross talk with Akt/mTOR pathway

mitogen-activated protein kinase (MAPK) and interact with other signaling pathways such as PI3K/Akt/mTOR (Fig. 8.1).

The *MSTN* gene consists of three exons that encode the signaling peptide, the prodomain, and the mature ligand domain (Fig. 8.2). In contrast to other related members of the TGF-β family, myostatin is mainly expressed in skeletal muscle, although expression is also detected in the circulation and in other tissues such as the heart [11, 12]. To understand more about the function of myostatin in skeletal muscle and the role of this pathway in the regulation of muscle mass and muscle atrophy, it is first important to discuss the downstream effect of myostatin in skeletal muscle in this section. In addition, we will describe knowns and unknowns of the relative contribution of downstream canonical and noncanonical pathways in myostatin signaling in more detail and discuss how myostatin activity is regulated.

8.2.1 Effect of Myostatin on Myoblast/Satellite Cell Function and Muscle Regeneration

After identification of myostatin as conserved regulator of muscle mass in mammals, multiple studies focused on the molecular and cellular mechanisms explaining the effect of myostatin on skeletal muscle. Importantly, myostatin knockout results in increased muscle mass due to both an increase in muscle fiber number (hyperplasia) and muscle fiber size (hypertrophy). Since postnatal skeletal muscle growth is exclusively mediated by muscle fiber hypertrophy, this suggests that the effect of myostatin knockout on skeletal muscle mass is at least partly mediated by enhanced function of muscle progenitor cells in the embryo resulting in increased muscle fiber formation and the double-muscled phenotype. Initial formation of skeletal muscles in the embryo is initiated by muscle progenitor cells, or myoblasts, which originate from the pharyngeal arches and the dermomyotome compartment of somites and which migrate to the different sites in the embryo to form skeletal muscle fibers by cell fusion [13]. In adult skeletal muscle, a population of muscle stem cells, or satellite cells, that originate from these embryonic myoblasts resides between the basal lamina and the sarcolemma of the muscle fibers. These satellite cells play an important role in the regulation of postnatal muscle growth and are required for skeletal muscle regeneration after muscle damage [14]. Myostatin expression is detected in the embryonic stage in the somites and developing limbs, suggesting a role for myostatin in the regulation of myogenesis [15, 1]. In addition, *Mstn* expression is induced upon myogenic differentiation in myoblasts, and the myogenic regulatory factor MyoD regulates myostatin promoter activity *in vitro* [16]. The role of myostatin in muscle formation in the embryo was confirmed in studies that determined the effect of myostatin in chicken embryos. Implantation of myostatin-coated beads in developing limbs of chicken embryos inhibits the expression of Pax3, MyoD, and Myog and inhibits proliferation of embryonic myoblasts [17]. In addition, another study showed that embryonic myostatin overexpression in

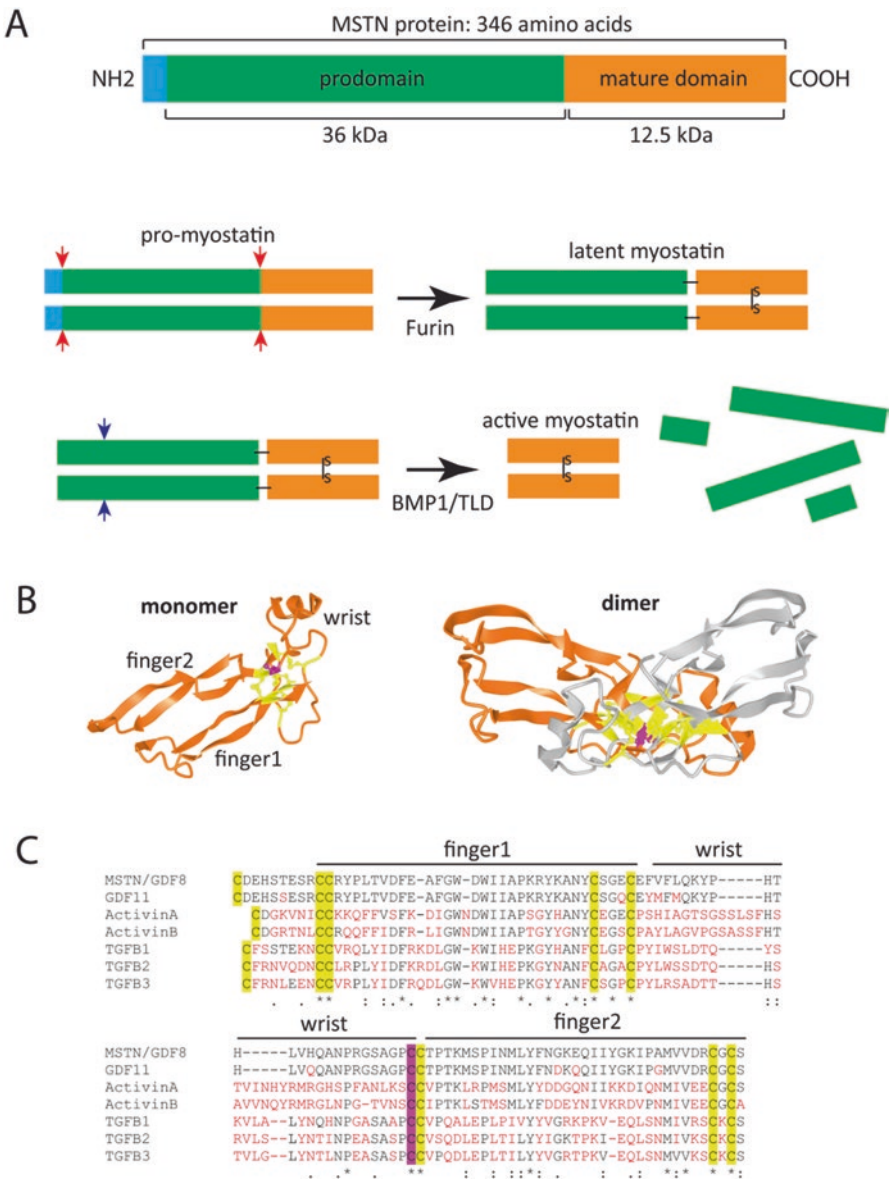

Fig. 8.2 Overview of myostatin protein and processing. (**a**) Overview of the myostatin protein and the different steps of myostatin processing. (**b**) Structure of the myostatin protein. On the left the myostatin monomer is shown with the different domains and on the right the active myostatin dimer (second monomer shown in gray). The conserved cystine knot with the interactions between the different cysteine residues is shown in yellow and in purple the cysteine responsible for dimer formation. Protein structure is derived from Protein Data Bank (PDB): 3HH2. (**c**) Alignment of the mature domains of myostatin and the different related TGF-β ligands that signal via Smad2/3. The conserved cysteine residues of the cystine knot are shown in yellow and in purple the cysteine responsible for dimer formation. The residues that differ from the myostatin protein are shown in red

chicken somites depleted the muscle progenitor cell population by inducing the expression of the cell cycle inhibitor *Cdkn1a* (*p21*) and stimulating premature differentiation, resulting in decreased muscle development in chicken embryos [15]. These studies support the hypothesis that myostatin inhibits the function of embryonic myoblasts and that genetic deletion of myostatin enhances embryonic muscle growth by expansion of the population of embryonic myoblasts and increased myogenesis.

In contrast, the effect of myostatin on satellite cells in mature skeletal muscle is controversial. Early studies suggesting that myostatin can inhibit myoblast proliferation and differentiation were mainly based on *in vitro* experiments in C_2C_{12} myoblasts. The C_2C_{12} myoblast cell line is a subclone of an immortalized cell line derived from myoblasts that were isolated from injured thigh muscle of 2-month-old wild type mice [18, 19]. In low serum medium, these myoblasts can fuse and differentiate to form multinucleated myotubes and are therefore frequently used as *in vitro* model for myogenesis and regeneration. High levels of myostatin protein or overexpression of myostatin inhibits proliferation of C_2C_{12} myoblasts [20–22] by increasing the expression of cyclin-dependent kinase inhibitor *Cdkn1a (p21)* and inducing the degradation of cyclin D1 [22]. Furthermore, myostatin inhibits myogenic differentiation of C_2C_{12} myoblasts by inhibiting the expression of myogenic regulatory factors *MyoD* and *Myog* [23, 24]. Apart from these experiments in C_2C_{12} myoblasts, other studies also suggest a role for myostatin in the regulation of satellite cells and muscle regeneration. Myostatin and its type II receptor Acvr2B are detected in satellite cells in adult mouse muscle sections, and myostatin knockout or inhibition increases proliferation rate of primary myoblasts and increases activation of satellite cells cultured in their muscle fiber niche [25–27]. In addition to the regulation of the cell cycle and myogenic differentiation, myostatin is also implicated in the regulation of satellite cell self-renewal by repressing the expression of satellite cell marker *Pax7* [28]. The functional implication of the effect of myostatin knockout on satellite cell function was furthermore shown by studies that compared the efficiency of muscle regeneration in *Mstn-/-* mice with wild type mice. Muscle regeneration is accelerated in *Mstn-/-* mice after muscle injury with cardiotoxin or laceration injury, as shown by increased levels of MyoD and Myog after damage and decreased fibrosis [29, 30], and this improved regenerative capacity was still observed in damaged muscles of senescent 24-month-old *Mstn-/-* mice [31].

However, results from other studies suggest that myostatin has either no or limited effect on satellite cells. *Mstn-/-* mice show muscle fiber hypertrophy without an increase in satellite cell number or number of myonuclei in muscle fibers, suggesting that in these mice, muscle fiber hypertrophy is not accompanied by increased satellite cell fusion [32]. Experiments with postnatal myostatin inhibition in mice also showed similar results, suggesting that specific myostatin-targeting therapies may not affect satellite cell function or number in skeletal muscle and therefore do not deplete the satellite cell population nor enhance satellite cell function [32]. Moreover, muscle hypertrophy was not inhibited by depleting the satellite cell population in *Mstn-/-* mice, demonstrating that hypertrophy occurred independently of satellite cell activity [33]. A recent study showed that inhibition of both myostatin

and activins with soluble ACVR2B-Fc in mice resulted in low but detectable levels of satellite cell activation [34]. Importantly, muscle fiber hypertrophy preceded satellite cell activation and myonuclear accretion after the treatment and thus occurred independently of satellite cell activity.

The reason for the seemingly conflicting effects of myostatin on satellite cells is currently unclear but likely depends on differences in the concentration and source of myostatin protein, culture conditions and type of cells (C_2C_{12}, primary myoblasts, or satellite cells in the muscle fiber niche), and the type, duration, and timing of *in vivo* treatment with inhibitors of these pathways. In addition, the effect of postnatal myostatin inhibition on muscle regeneration remains unclear, and further studies are warranted to determine the role of myostatin and related proteins in muscle regeneration. Notably, evidence from multiple studies suggests that myostatin also affects the function of other cells that play an important role in muscle regeneration, such as fibroblasts and macrophages, and moreover suggests that myostatin contributes directly to muscle fibrosis by stimulating fibroblast proliferation and survival [35, 36, 29, 30].

8.2.2 Effect of Myostatin on Skeletal Muscle Fibers

As mentioned before, genetic deletion of myostatin results in pronounced muscle hypertrophy caused by both an increase in muscle fiber hyperplasia and muscle fiber hypertrophy. To exclude the effects of myostatin inactivation on embryonic myogenesis, other studies determined the effect of conditional postnatal myostatin inactivation specifically in skeletal muscle. Importantly, inhibition of myostatin in adult mice resulted in muscle fiber hypertrophy and increase in muscle force production [37–39]. In contrast to myostatin loss of function, the direct catabolic effect of myostatin on skeletal muscle was demonstrated in studies that showed the effect of myostatin overexpression in mice, which resulted in pronounced muscle atrophy [40–42].

Several lines of evidence suggest that myostatin causes muscle atrophy by inducing catabolic pathways and repressing pathways involved in translation. Trim63 (MuRF-1) and Fbxo32 (MAFbx/Atrogin-1) are E3 ubiquitin ligases that are involved in proteasomal degradation of proteins in catabolic muscle-wasting conditions. Gene expression of *MuRF-1* and *Atrogin-1* is increased in different forms of muscle atrophy, resulting in specific degradation of muscle proteins in muscle fibers. Forkhead box O (FoxO) proteins are crucial mediators of muscle atrophy via transcriptional regulation of atrogenes such as *MuRF-1* and *Atrogin-1* and regulation of autophagy [43–45]. *In vitro*, high levels of myostatin (3–5μg/ml) increase Atrogin-1 and/or MuRF-1 protein and mRNA levels in differentiated C_2C_{12} myotubes, and this effect was found to be FoxO1 dependent [46–48]. *In vivo*, myostatin overexpression in skeletal muscle increases both *MuRF-1* and *Atrogin-1* expression in skeletal muscles in mice [48]. In addition, downstream mediator Smad3 synergistically induces the expression of *MuRF-1* together with Foxo1 [49]. This synergy between Smad3

and FoxO transcription factors in muscle cells is consistent with reports in other cell types showing that FoxO transcription factors are indispensable for the transcriptional control of a subset of target genes that are regulated by Smad2 and Smad3 [50, 51]. Furthermore, *in vitro* experiments showed that Foxo1 is also required for the transcription of myostatin together with Smad3 in C_2C_{12} myotubes and showed that myostatin increases *Foxo1* expression *in vitro* and *in vivo*, suggesting a positive feedback loop between these pathways [52, 48]. Together these studies suggest that the catabolic effects of FoxO and myostatin pathways on skeletal muscle are integrated and that these pathways have a synergistic effect on muscle atrophy.

The IGF-1/PI3K/Akt/mTOR pathway is a critical regulator of muscle protein translation and skeletal muscle growth [53, 54]. Importantly, multiple evidence points to cross-signaling between myostatin and the IGF-1/PI3K/Akt/mTOR pathway (Fig. 8.1). In myostatin knockout mice, the total and active phosphorylated Akt protein levels are higher in cardiac and skeletal muscle [55]. Furthermore, antibody-mediated postnatal inhibition of myostatin function in mice resulted in increased muscle protein synthesis and higher levels of phosphorylated active ribosomal protein S6 (p-rpS6) and p70 S6 kinase (pS6K), two downstream mTOR target proteins [56]. *In vitro* experiments furthermore demonstrated that myostatin inhibits IGF-1/PI3K/Akt/mTOR pathways in C_2C_{12} myoblasts and differentiated myotubes [55, 57]. Importantly, this regulation is reciprocal since IGF-1 also has an inhibitory effect on myostatin signaling in C_2C_{12} cells [58]. The mechanism of the crosstalk between these pathways is not entirely clear and may involve several different interactions. Akt is known to physically interact with Smad3, thereby preventing Smad3 phosphorylation, interaction with Smad4, and nuclear translocation [59, 60]. Furthermore, Akt prevents nuclear translocation of Foxo transcription factors [61], which may affect Smad2/3-dependent transcription and inhibit *Mstn* expression. Conversely, Smad3 indirectly induces expression of Akt/mTOR inhibitor *PTEN* by decreasing the expression of *microRNA-29* [62].

In addition to regulation of muscle fiber growth, myostatin signaling is also implicated in regulation of muscle fiber type and muscle fiber metabolism. Myostatin is predominantly expressed in fast-twitch muscles, and *Mstn* promoter activity is mainly detected in fast type IIB muscle fibers in mice [63, 64]. Interestingly, the myogenic transcription factor MyoD is also mainly expressed in fast-type muscles in mature skeletal muscle and implicated in regulation of myostatin transcription [65, 64, 16], suggesting that the fiber-type-specific expression of myostatin is regulated by MyoD. A direct role for myostatin in muscle fiber-type specification was found in *Mstn-/-* mice, which show a decrease in slow type I and type IIA and an increase in the percentage of fast glycolytic IIB fibers [66, 67]. Although *Mstn-/-* mice show pronounced increases in muscle mass, the specific force of *Mstn-/-* skeletal muscle (defined as the maximal tetanic force normalized by muscle weight) decreased compared to wild type mice [66]. Subsequent studies demonstrated that skeletal muscles of *Mstn-/-* mice show extreme fatigability associated with decreased oxidative capacity of muscle fibers and mitochondrial depletion [67, 68]. Postnatal myostatin inhibition by overexpression of the prodomain or injections with ACVR2B-Fc resulted in similar decrease of oxidative capacity and decrease in

fatigue resistance of skeletal muscle and in addition showed reduced muscle capillarization [68, 69]. Postnatal myostatin inhibition furthermore decreased expression of key enzymes and transcription factors involved in oxidative metabolism, such as *Pdk4*, *Cpt1b*, *Pgc1α*, *Pparβ*, and *Porin*, and resulted in a shift to anaerobic glycolysis in skeletal muscle [69]. However, in contrast to *Mstn-/-* mice, no fiber-type switch toward fast IIB muscle fibers was observed after treatment with myostatin inhibitors [70, 69]. Together these results suggest that postnatal myostatin inhibition negatively affects oxidative metabolism and endurance capacity of skeletal muscle. However, recent experiments showed that despite the negative effect on oxidative capacity and endurance, treatment of mice with ACVR2B-Fc did not compromise bioenergetic status during fatiguing exercise, and these mice showed increased muscle force generating capacity compared to control mice [71].

8.2.3 Regulation of Myostatin Activity

The active myostatin dimer signals via specific type I and type II receptors to activate downstream Smad2/3 pathways and other noncanonical pathways. *In vitro* affinity labeling assays showed that myostatin binds with high affinity to the type II receptor ACVR2B and to a lesser extent ACVR2A and forms a heteromeric receptor complex with either ALK4 (ACVR1B) or ALK5 (TGFBR1) [72, 73]. Systemic injections of a soluble compound composed of the receptor domain of ACVR2B and a soluble IgG Fc domain, ACVR2B-Fc, specifically result in muscle hypertrophy in mice which is reminiscent of the myostatin knockout phenotype [74]. We recently showed that the interaction of myostatin with type I receptors is cell type specific and regulated through interaction with the co-receptor Cripto [75]. Specifically, *in vitro* RNAi experiments showed that myostatin signaling was mediated via ALK4/ACVR1B in C_2C_{12} myoblasts and primary myoblasts and that expression of co-receptor Cripto was required for myostatin activity in these cells. In fibroblasts and mesenchymal stem cells, Cripto was absent, and myostatin signaling was mediated via ALK5/TGFBR1 [75]. The relevance of Cripto in skeletal muscle was demonstrated by overexpression of soluble Cripto, which resulted in muscle hypertrophy and accelerated muscle regeneration in mice [25]. In contrast Cripto knockout resulted in impaired regeneration [25]. In contrast to our study, the authors showed that Cripto counteracts myostatin signaling in satellite cells, although these results were based on overexpression of soluble Cripto instead of knockdown of endogenous Cripto [25]. Other co-receptors are also known to regulate TGF-β activity, such as betaglycan (TGFBR3), and knockdown of this co-receptor inhibits myostatin activity in mesenchymal stem cells but not in myoblasts [75]. This suggests that TGF-β co-receptors play an important role in cell type specificity of TGF-β ligands including myostatin. However, the role of different co-receptors in the regulation of myostatin activity *in vivo* remains to be determined.

Local myostatin activation depends on cleavage of pro-myostatin by furin proteases and the subsequent activation of the latent complex by cleavage of the

prodomain by BMP1/TLD-like proteases (Fig. 8.2). After initial cleavage of pro-myostatin by furin, the prodomain of myostatin binds the mature myostatin dimer via non-covalent interactions, thus forming a latent myostatin complex and preventing the binding of the mature dimer to the type II receptor. Subsequent proteolytic cleavage of the latent complex by BMP1/TLD-like proteases is crucial for releasing the mature dimer and activating downstream pathways (Fig. 8.2) [76]. Genetic knockout studies in mice showed that the protease tolloid-like 2 (TLL2) is likely at least partly responsible for proteolytic cleavage of the prodomain in skeletal muscle, since genetic knockout of this protein also results in significant muscle hypertrophy [77]. However, muscle hypertrophy in *Tll2-/-* mice was not as pronounced as observed in *Mstn-/-* mice, suggesting that other proteases also play a role in processing of myostatin prodomain [77]. Multiple studies showed that overexpression of the myostatin prodomain or a dominant-negative pro-myostatin protein that lacks the residues required for proteolytic cleavage (dnMstn) results in reduced myostatin activity *in vivo* and muscle hypertrophy in mice [78–80, 76]. In different preclinical animal models of muscle-wasting, treatment with these proteins shows promising results by counteracting muscle atrophy (Table 8.2).

Although myostatin is predominantly expressed in skeletal muscle [11, 1], it is also detected in the circulation [11]. However, studies showed that myostatin protein is inactive in serum because the mature active dimer is bound to inhibitory proteins including its own prodomain and a protein encoded by the follistatin-related gene (FLRG or FSTL3) [81]. In addition, activity of myostatin is also inhibited by interactions with other proteins, such as follistatin (FST) [82, 83] and GDF-associated serum protein-1 and protein-2 (GASP-1/GASP-2, also known as WFIKKN-2/WFIKKN-1, respectively) [84, 85]. The significance of these inhibitory proteins in regulation of muscle mass was shown by knockout experiments and overexpression experiments of the genes encoding these regulatory proteins. Genetic deletion of *Fst* [86] or *Wfikkn-1*/*Wfikkn-2* [87] in mice results in decreased muscle mass and impaired muscle regeneration upon injury. In contrast, overexpression of *FST*, *FLRG*, or *GASP-1/WFIKKN-2* in mice results in muscle hypertrophy and increased muscle strength [88, 38, 89, 72, 90]. In addition, muscle regeneration after injury is improved in transgenic mice overexpressing *FST*, which was associated with decreased fibrosis, increased angiogenesis, and decreased *Mstn* expression [91]. *FST*-based treatments so far have shown promising results in preclinical animal models of different muscle-wasting conditions (Table 8.2) and are currently being evaluated in different clinical trials (Table 8.3).

Local myostatin activity is furthermore regulated by the interaction of secreted myostatin with different extracellular matrix (ECM)-associated proteins. Decorin (DCN) is a proteoglycan that is highly expressed in skeletal muscle and is present in the extracellular matrix. DCN inhibits TGF-β and myostatin activity via interaction of the core protein domain with the ligands and moreover it was shown that overexpression of *DCN* antagonizes the inhibitory effect of myostatin on myoblast differentiation [92, 30]. In addition, overexpression of *DCN* in mice accelerates skeletal muscle regeneration and counteracts fibrosis upon injury [93]. Another proteoglycan, the ECM protein perlecan (HSPG2), also has been implicated in the

regulation of myostatin activity. Perlecan knockout mice display skeletal muscle hypertrophy and decreased levels of myostatin expression and myostatin protein [94]. *In vitro* experiments showed that the myostatin prodomain specifically interacts with glycosaminoglycan chains of perlecan [95]. As yet the nature and relevance of this interaction are unknown and it is unclear how perlecan can regulate myostatin expression. Myostatin can also bind latent TGF-β-binding proteins (LTBP), which are known to interact with ECM proteins and sequester TGF-β proteins to the ECM. Specifically, LTBP2 and LTBP3 bind pro-myostatin via non-covalent interactions that require both the prodomain and the mature domain [96]. LTBP3 can sequester the non-cleaved pro-myostatin to the ECM, and this form of unprocessed myostatin was found to be the main form present in the ECM of skeletal muscle [96, 97]. The relevance of this interaction was furthermore demonstrated by overexpression of *Ltbp3* in skeletal muscle *in vivo* in mice, which resulted in pronounced muscle hypertrophy [96]. In addition, interaction of myostatin with another LTBP, LTBP4, was shown in a recent study. Co-immunoprecipitation experiments demonstrated a direct interaction of myostatin with the amino-terminal part of LTBP4. In addition to myostatin, TGF-β and GDF11 also interact with LTBP4, suggesting this protein can bind and regulate activity of multiple TGF-β family members. The therapeutic potential of this protein was shown in transgenic mice overexpressing *Ltbp4*, which resulted in a muscular phenotype comparable to that of *Mstn-/-* mice [98]. In addition, overexpression improved muscle pathology in a mouse model for Duchenne muscular dystrophy via inhibition of TGF-β and myostatin, resulting in decreased fibrosis and improved histology [98]. Moreover, recent studies suggest that LTBP4 is an important modifier gene in muscle-wasting conditions. Polymorphisms in the *Ltbp4* gene are associated with increased TGF-β release/activity and aggravate pathology in mouse models of muscular dystrophy [99, 100]. Differences in the sequence of the human *LTBP4* gene result in proteins with shorter hinge regions compared to the mouse protein, which makes the human protein more susceptible to proteolytic degradation and results in higher TGF-β activity. Antibodies that stabilize the hinge region of LTBP4 and counteract proteolysis improve muscle pathology in *mdx* mice overexpressing the human LTBP4 protein, suggesting the therapeutic potential of such treatments for patients with muscular dystrophy.

In summary, multiple proteins are involved in the regulation of myostatin activity and therapies aimed at treatment of some of these proteins, such as the myostatin prodomain, ACVR2B-Fc, FST, FLRG, and LTBP4, show promise in stimulating muscle growth and muscle regeneration, and are therefore candidates to counteract muscle atrophy.

8.2.4 The Effect of Canonical Myostatin Pathway on Skeletal Muscle

Like the structurally related cytokines TGF-β1, TGF-β2, and TGF-β3, GDF11, and activins, myostatin activates downstream signaling via phosphorylation of Smad2 and Smad3 proteins [73, 101]. Multiple studies suggest that canonical signaling via Smad3 is likely responsible for the effect of myostatin on myogenesis and skeletal muscle mass. First, Smad3 is known to physically interact with MyoD, a known master regulator of myogenesis, and thereby interferes with the transcriptional activity of MyoD and inhibits myogenic differentiation of myoblasts induced by MyoD [102]. This interaction was found to be specific since overexpression of Smad3, but not Smad2, interfered with MyoD-induced myogenic differentiation and muscle-specific reporter gene activity [102]. Myostatin stimulated the interaction between Smad3 and MyoD in C_2C_{12} myoblasts, and the inhibitory effect of myostatin on MyoD transcription was counteracted by a dominant-negative Smad3 in these cells [23]. In addition to the effect on MyoD, Smad3 can also interfere with the interaction of another key myogenic factor, MEF2C, with coactivator GRIP-1 resulting in decreased transcriptional activity of MEF2C [103]. In addition to these downstream effects, myostatin also negatively autoregulates its own activity by Smad2/3-dependent upregulation of the gene encoding the inhibitory Smad protein Smad7, similar as described for TGF-β [104, 101]. Importantly, multiple studies show that Smad7 plays an important role in the regulation of muscle mass and muscle regeneration. *Smad7-/-* mice show muscle wasting and impaired muscle regeneration [105]. Conversely, Smad7 overexpression stimulates myoblast differentiation *in vitro* and increases muscle mass and protects against muscle atrophy *in vivo* [106, 107].

In addition to the effect on myogenesis, both Smad2 and Smad3 proteins play a role in the regulation of muscle mass and muscle fiber atrophy. It was shown that type I receptor-mediated muscle hypertrophy induced by overexpression of constitutively active ALK4 or ALK5 was Smad2/3 dependent and that RNAi-mediated inhibition of Smad2/3 promoted muscle hypertrophy in mice [108]. A recent study showed that unilateral sciatic nerve denervation and immobilization in mice result in muscle atrophy accompanied by upregulation of *MuRF-1* and *Atrogin-1* expression and increased levels of both total and phosphorylated Smad2 and Smad3 proteins [109]. Genetic deletion of combined but not individual Smad2 and Smad3 counteracted denervation-induced muscle atrophy in mice. Interestingly, increased expression of *MuRF-1* and *Atrogin-1* after denervation was counteracted specifically by Smad3 knockout, but not by Smad2 knockout, suggesting overlapping as well as different functions for Smad2 and Smad3 [109]. This is consistent with other studies reporting that Smad3 overexpression increased *Atrogin-1* transcription and muscle atrophy and increased protein synthesis and inhibition of Smad3 resulted in muscle hypertrophy [62].

In contrast to the catabolic and anti-myogenic functions of Smad3 we discussed, recent studies have suggested that Smad3 is required for proper function of satellite cells, muscle regeneration, and maintenance of skeletal muscle mass. Genetic deletion of Smad3 in mice surprisingly resulted in skeletal muscle atrophy and impaired muscle regeneration [110, 111]. In addition, *Smad3-/-* myoblasts showed decreased proliferation rates and impaired differentiation *in vitro* compared to wild type myoblasts [110]. Mechanistically, Smad3 knockout resulted in increased *Mstn* and *MuRF1* expression and protein levels in skeletal muscle and decreased *Igf-1* expression [110, 111]. No difference was observed in Atrogin-1 and Foxo1/pFoxo1 protein levels, suggesting that these atrogenes are not downstream of Smad3. Importantly, myostatin knockout reversed muscle atrophy in these mice, suggesting that myostatin is responsible for the observed effect in skeletal muscle of Smad3 knockout mice and mediates this effect via Smad2 or other noncanonical pathways. It will be important to determine whether the observed effects of Smad3 knockout are due to postnatal inhibition of Smad3 in skeletal muscle or due to effects on embryonic myogenesis. In addition, the potential overlapping and distinct functions of Smad2 and Smad3 in myostatin signaling remain as yet unresolved. Dissecting the specific functions of these R-Smads in more detail in skeletal muscle and determining their relative contribution to different forms of muscle atrophy will help us understand more about the different downstream effects of myostatin and related proteins.

8.2.5 Noncanonical Myostatin Pathways

Apart from canonical Smad2/3-mediated pathways, myostatin is also known to activate other intracellular pathways that may mediate important downstream functions of myostatin. *In vitro* studies showed that myostatin induces phosphorylation of mitogen-activated protein kinases (MAPK) JNK, p38MAPK, and ERK1/2 in myoblasts [112–114]. Although it is as yet not known what the relative contribution of these pathways is in the downstream functions of myostatin signaling *in vivo*, *in vitro* experiments suggest that ERK1/2 are required for the effect of myostatin on myogenic differentiation of myoblasts and satellite cell self-renewal. For instance, it was shown that small molecule inhibition of ERK1/2 counteracts the inhibitory effect of myostatin on myogenesis in C_2C_{12} myoblasts [114]. In addition, high levels of myostatin inhibited *Pax7* expression via ERK1/2 in primary myoblasts [28]. *In vitro* studies in C_2C_{12} myotubes and *in vivo* studies of knockout mice showed that ERK1/2 are required for the preservation of muscle mass [115, 116]. However, local increase in ERK1/2 phosphorylation in skeletal muscle is also associated with muscle atrophy, and myostatin/activin inhibition in mice prevents these changes, suggesting that these noncanonical pathways may contribute to muscle atrophy in some conditions [117–119]. Moreover, a direct role for ERK1/2 was shown in a recent study in which treatment of tumor-bearing mice with an ERK inhibitor prevented

cachexia-induced muscle wasting [120], suggesting that inhibiting this pathway may be potential therapy for muscle wasting conditions.

8.2.6 *Cross-Signaling of Myostatin Pathways with Other Signaling Pathways*

Recent studies indicate that myostatin signaling pathway interacts with several other pathways that are involved in muscle atrophy and regulation of muscle mass. Stat3 is a transcription factor that is activated by several cytokines, among others, TNF-α and IL-6, and that is known as an important cause of muscle atrophy in several muscle-wasting conditions [121–124]. Stat3 can stimulate expression of myostatin and atrogin-1 via CAAT/enhancer-binding protein δ (C/EBPδ) in catabolic muscle-wasting conditions such as cancer cachexia and chronic kidney disease (CKD) [124, 125]. In addition, TGF-β signaling is known to stimulate phosphorylation and activation of Stat3, and Stat3 is known to physically interact with Smad2 and Smad3 and depending on cell type can inhibit or potentiate Smad-dependent transcription [126–128]. Although it is not known whether Stat3 is required for the downstream effects of myostatin signaling and Smad2/3 function in skeletal muscle, it would be interesting to determine if this is the case and whether Stat3 inhibitors can be used to inhibit myostatin and/or TGF-β signaling. Notably, small molecule inhibitors of Stat3 have been identified as potential treatment to counteract muscle wasting during aging and in muscle degenerative diseases such as Duchenne muscular dystrophy [129, 130].

Recently, a link between Notch and myostatin signaling pathways has been established in myoblasts and skeletal muscle. Notch is an important signaling pathway that is involved in the regulation of satellite cell activation and myoblast proliferation and inhibits myogenic differentiation [131]. Notch signaling is mediated by intracellular cleavage of the Notch receptor, which results in the release of the Notch intracellular domain (NICD) and subsequent translocation of this protein to the cell nucleus where it regulates transcription of specific target genes. *In vitro* experiments in human myoblast cultures showed that myostatin stimulates the physical interaction of the NICD with Smad3 and induces expression of downstream Notch target genes *Hes1*, *Hes5*, and *Hey1* [132]. In addition, mice with genetic deletion of the gene encoding the Notch antagonist Numb show defective muscle regeneration, impaired satellite cell function, and increased *Mstn* expression [133]. This muscle phenotype in *Numb-/-* mice was counteracted by specifically inhibiting myostatin with RNAi [133]. TGF-β also induces the expression of Notch target genes in C_2C_{12} myoblasts and other cell types, and this effect is also dependent on the physical and transcriptional interaction between Smad3 and the NICD protein [134]. This suggests that the interaction between Notch- and Smad3-mediated pathways is a general feature and regulates a subset of target genes that are regulated by both these pathways. However, increased TGF-β activity and

Smad3 have also been reported to inhibit Notch signaling in muscles and myoblasts of old mice, suggesting the crosstalk between these pathways is highly context dependent (see chapter 8.4.3 below). Importantly, deregulation of Notch pathways has been reported in various muscle-wasting conditions and may contribute to muscle wasting in some conditions. For example, activation of Notch signaling pathways has been reported in mouse models for glucocorticoid-induced muscle atrophy and muscle dystrophy [135, 136]. In addition, decreased activity of Notch has been reported in satellite cells in aging skeletal muscle and restoring Notch signaling can restore the regenerative potential of skeletal muscle in old mice [231, 232]. It will therefore be interesting and important to determine how these pathways interact in different muscle wasting conditions.

Other studies also indicate crosstalk between Wnt and myostatin pathways. Transcriptional profiling of muscle tissue of *Mstn-/-* mice indicated that the expression of genes involved in the canonical β-catenin-mediated Wnt pathway was decreased, while the expression of genes involved in the noncanonical Wnt/calcium pathway was increased upon myostatin loss of function [137]. Gene expression of a noncanonical Wnt, *Wnt4*, was specifically increased in *Mstn-/-* mice, and *in vitro* experiments showed that myostatin inhibits expression of *Wnt4* [86]. Conversely, Wnt4 protein decreased the expression of *Mstn* in C_2C_{12} myoblasts and decreased myostatin activity in both C_2C_{12} myoblasts and primary myoblasts [138, 139]. Expression of *Wnt4* is induced during myogenic differentiation in C_2C_{12} myoblasts and primary myoblasts and during muscle regeneration *in vivo* in mice, and overexpression of Wnt4 stimulates myogenesis [138, 140].

Recent studies also highlighted an important function for bone morphogenetic proteins (BMPs) in the regulation of skeletal muscle mass and indicated cross talk of BMP signaling pathways with myostatin signaling pathways. As mentioned before, BMPs are members of the TGF-β family that mediate downstream signaling via interaction with distinct BMP type I receptors and type II activin/BMP receptors, resulting in phosphorylation of R-Smads Smad1/5/8 and activation or repression of specific downstream target genes. BMP signaling plays an important role in protection against muscle atrophy and mediates muscle hypertrophy. Overexpression of constitutively active BMP type I receptor ALK3 (caALK3) or Bmp7 in mouse skeletal muscle resulted in increased levels of phosphorylated Smad1/5/8 (pSmad1/5/8) and muscle hypertrophy and moreover counteracted denervation-induced muscle atrophy [141, 142]. In contrast, overexpression of the inhibitory Smad Smad6, the BMP antagonist Noggin, or intramuscular injection with small molecule BMP inhibitor LDN-193180 resulted in more pronounced skeletal muscle atrophy [141, 142]. BMP activity and expression of BMP family members *Gdf5* (*Bmp14*) and *Gdf6* (*Bmp13*) were induced in skeletal muscle during denervation [141, 142]. This implies that increased BMP activity is a protective response in catabolic conditions in skeletal muscle, which was confirmed by the finding that Gdf5 knockout mice show aggravated muscle atrophy after denervation. Analysis of the downstream effects and target genes involved in BMP-mediated regulation of muscle mass resulted in the identification of a new member of the ubiquitin ligase family of proteins, MUSA-1 (FBXO30) as a novel target for BMP signaling in

skeletal muscle [141]. In addition, inhibition of BMP pathways in skeletal muscle induced the expression of other atrogenes such as *MuRF1* and *Atrogin-1* and induced activity of HDAC4-myogenin pathway, which plays an important role in denervation-induced atrophy by stimulating the expression of atrogenes. Cross talk between BMP and myostatin pathways was demonstrated in myostatin knockout mice or follistatin overexpression in mice, which resulted in elevated pSmad1/5/8 levels, suggesting that the effect of myostatin inhibition was at least partly mediated by increased BMP activity [141, 142]. In addition, combined inhibition of activins and myostatin resulted in more pronounced hypertrophy and pSmad1/5/8 levels in skeletal muscle compared to overexpression of the individual prodomains [143]. There are several explanations possible for the observed crosstalk between myostatin/activin pathways and BMP pathways. First, inhibition of myostatin and/or activin may lead to increased BMP signaling via increased availability of type II activin receptors (ACVR2A/ACVR2B). Type II activin receptors are known receptors for some BMP ligands, such as BMP6 and BMP7, and *in vitro* experiments showed that myostatin competes with BMPs for interaction with these receptors [73]. Secondly, inhibition of myostatin and/or activin may lead to increased availability of the co-Smad Smad4 and therefore results in increased interaction with the BMP Smads Smad1/5/8.

In addition to the regulation of muscle hypertrophy and protection against muscle fiber atrophy, BMP signaling also plays an important role in the activation and expansion of the satellite cell population and prevention of premature differentiation [144, 145]. It is as yet however unknown whether myostatin signaling also crosstalks with BMP signaling during myogenesis and regeneration. Furthermore, considering the important role of BMPs in skeletal muscle and the potential involvement of myostatin and other TGF-β ligands in different muscle-wasting disorders, it is important to establish how these pathways interact and if deregulation of BMP signaling plays a role in different forms of muscle atrophy.

8.3 Function of Myostatin in Skeletal Muscle Atrophy

As mentioned before, artificially increased myostatin levels induce muscle atrophy *in vivo* in mice. However, such experiments do not provide direct evidence of the involvement of myostatin in different muscle-wasting conditions. Importantly, increased levels of myostatin expression and protein have been associated with some muscle-wasting conditions, suggesting a contribution of myostatin to muscle atrophy in some cases (Table 8.1). In addition, preclinical evidence suggests that targeting of myostatin and related pathways counteracts muscle atrophy in some conditions regardless whether myostatin is directly involved or not (Table 8.2). In the following section, we will discuss in more detail the effects of specific myostatin knockout and postnatal targeting in different preclinical animal models of muscle-wasting disorders.

Table 8.1 Association of myostatin levels with different muscle-wasting conditions

Condition	MSTN level (ref)	Species	Local/systemic
Muscle-wasting conditions			
Denervation atrophy	↑[258–261]	Mouse, rat, human	Local
Stroke	↑[262–264]	Mouse, human	Local
Unloading/disuse atrophy	↑[63, 146–148]	Mouse, rat, human-mouse	Local
	= [265]		Local
Glucocorticoid-induced atrophy	↑[184]	Rat	Local
Cachexia:	↑[11]	Human	Local, systemic
HIV-associated Cachexia			
Cancer cachexia	↑[266, 124]	Mouse, rat	Local
Chronic kidney disease (CKD)	↑[267, 181, 125]	Mouse, human	Local
Chronic obstructive pulmonary disease (COPD)	↑[268–271]	Rat, human	Local, systemic
Heart failure/congenital heart disease	↑[272–274, 12]	Mouse, sheep, human	Local (heart), systemic
Sarcopenia	↑[258, 275, 27, 276, 277]	Rat, human	Local, systemic
	= [278, 279, 209, 280]	Mouse, rat, human	Local, systemic
	↓[281–283]	Rat, human	Local, systemic
Neuromuscular diseases			
X-linked myotubular myopathy (XLMTM)	↓[218]	Mouse (*Mtm1-KO*)	Local
Sporadic inclusion body myositis (sIBM) Hereditary inclusion body myositis (HIBM)	↓[218, 284]	Human	Local
	↓[187]	Human	Systemic
Spinal muscular atrophy (SMA)	↓[218]	Human	Systemic
Duchenne muscular dystrophy (DMD)	↓[187, 218, 285]	Mouse (*mdx*), dog (GRMD), human	Local, systemic
Becker muscular dystrophy (BMD)	↓[187]	Human	Systemic
Limb-girdle muscular dystrophy (LGMD) 2A	↓[187]	Human	Systemic
Limb-girdle muscular dystrophy (LGMD) 2B	↓[187]	Human	Systemic
Limb-girdle muscular dystrophy (LGMD) 2D	↓[286]	Mouse (*Sgca-/-*)	Local
Limb-girdle muscular dystrophy (LGMD) 2F	↓[194, 286]	Mouse (*Sgcd-/-*)	Local

↑ upregulated compared to control, = no difference compared to control, ↓ downregulated compared to control

Table 8.2 Effect of myostatin targeting in different muscle-wasting models

Condition	Method of inhibition	Animal model	Result	References
Muscle-wasting conditions				
Denervation atrophy	Mstn-/-	Mouse	=	[109]
	Mstn prodomain	Rat	+	[153]
	ACVR2B-Fc	Mouse	=	[119]
	dnACVR2B	Mouse	+	[108]
	FST	Mouse	+ (treatment before)	[154]
			= (treatment after)	[154]
Spinal cord injury	ACVR2B-Fc	Mouse	=	[287]
Stroke	Mstn peptibody	Mouse	+	[242]
Unloading/disuse atrophy	Mstn-/-	Mouse	-	[149, 150]
	Mstn antibody	Mouse	+	[152, 151]
	ACVR2B-Fc	Mouse	+	[119]
Glucocorticoid-induced atrophy	Mstn-/-	Mouse	+	[185]
	Mstn antibody	Mouse	+	[152, 97]
	RNAi myostatin	Mouse	+	[184]
	Bimagrumab (BYM338)	Mouse	+	[186]
Cachexia	Mstn-/-	Mouse (cancer)	+	[175]
	Mstn antibody	Mouse (cancer)	+	[177, 178]
	Mstn peptibody	Mouse (CKD)	+	[181]
	ACVR2B-Fc	Mouse (cancer)	+	[173, 174, 179]
		Macaque (SIV)	+	[180]
	ACVR2 antibody	Mouse (cancer)	+	[176]
	Smad7	Mouse (cancer)	+	[107]
Heart failure/congenital heart disease	Mstn-/-	Mouse	+	[288]
	Mstn antibody	Mouse	+	[288]
Sarcopenia	Mstn+/-,Mstn-/-	Mouse	+	[168, 166, 167, 31]
	Mstn antibody	Mouse	+	[170, 169]
	Mstn prodomain	Mouse	+	[171, 172]
Neuromuscular diseases				
X-linked myotubular myopathy (XLMTM)	ACVR2B-Fc	Mouse (*Mtm1δ4*)	+	[195]
Nemaline myopathy (NM)	Mstn antibody (mRK-35)	Mouse (*TgACTA1D286G*)	+	[197]
	ACVR2B-Fc	Mouse (*TgACTA1D286G*)	+	[196]

(continued)

Table 8.2 (continued)

Condition	Method of inhibition	Animal model	Result	References
Spinal muscular atrophy (SMA)	Mstn-/-	Mouse (*SMAΔ7*; severe)	=	[158]
	dnMstn	Mouse (*C/C*; mild)	+	[162]
	ACVR2B-Fc	Mouse (*C/C*; mild)	+	[162]
	FST	Mouse (*SMAΔ7*; severe)	=	[159]
			=	
		Mouse (*SMAΔ7*; severe)	+	[160]
		Mouse (*SMAΔ7*; severe)	=	[159]
		Mouse (*SMAΔ7*; mild)	+	[161]
ALS	Mstn-/-	Mouse	+	[157]
	Mstn antibody	Mouse, rat	+ (early stage)	[155]
	ACVR2B-Fc	Mouse	+	[157]
	FST	Mouse	+	[156]
Duchenne muscular dystrophy (DMD)	Mstn-/-	Mouse (*mdx*)	+	[188]
		Mouse (*mdx*)	=	[32]
	Mstn+/- (whippet)	Dog (*GRMD*)	-	[289, 189]
	Mstn antibody	Mouse (*mdx*)	+ (young, adult)	[290, 191, 239]
		Mouse (*mdx*)	= (adult)	[191]
	Mstn prodomain	Mouse (*mdx*)	+	[291, 292, 79]
	dnMstn	Dog (*GRMD*)	+	[293]
	ACVR2B-Fc	Mouse (*mdx*)	+	[294, 292, 295]
		Mouse (*mdx*)	-	[69]
	FST	Mouse (*mdx*)	+	[38, 90, 257]
Limb-girdle muscular dystrophy (LGMD) 1C	Mstn prodomain	Mouse (*Cav3-/-*)	+	[296, 297]
	RNAi myostatin	Mouse (*Cav3-/-*)	+	[298, 299]
	ACVR2B-Fc	Mouse (*Cav3-/-*)	+	[296]
	Type I receptor inhibitor	Mouse (*Cav3-/-*)	+	[300]
Limb-girdle muscular dystrophy (LGMD) 2A	Mstn prodomain	Mouse (*Capn3-/-*)	+	[193]
Limb-girdle muscular dystrophy (LGMD) 2B	ACVR2B-Fc FST	Mouse (*Dysf-/-*)	+/-	[192]
		Mouse (*Dysf-/-*)	-	[192]
Limb-girdle muscular dystrophy (LGMD) 2C	Mstn antibody	Mouse(*Sgcg-/-*)	+	[301]

(continued)

Table 8.2 (continued)

Condition	Method of inhibition	Animal model	Result	References
Limb-girdle muscular dystrophy (LGMD) 2D	Mstn prodomain	Mouse (*Sgca-/-*)	=	[193]
Limb-girdle muscular dystrophy (LGMD) 2F	Mstn-/-	Mouse (*Sgcd-/-*)	+ (4wks)	[194]
	Mstn antibody	Mouse (*Sgcd-/-*)	+ (4wks)	[194]
			= (20wks)	
Merosin-deficient congenital muscular dystrophy (MDC1A)	Mstn-/-	Mouse (dyW/dyW)	-	[198]

+, muscle mass and/or function increased compared to control; =, no difference in muscle mass and/or function compared to control; -, pathology aggravated compared to control

8.3.1 Role of Myostatin in Disuse and Denervation-Induced Muscle Atrophy

Disuse, unloading, and denervation lead to catabolic conditions that result in muscle atrophy via increased expression of atrogenes such as *MuRF-1* and *Atrogin-1*. Myostatin mRNA and protein levels are induced after skeletal muscle unloading or disuse in mice, rats, and humans [63, 146–148]. Notably, myostatin mRNA and proteinlevels were induced during unloading-induced muscle atrophy in fast-twitch plantaris muscle but not in slow-twitch soleus muscle, suggesting that the change in myostatin levels is muscle fiber-type specific [63, 148]. The relevance of myostatin signaling in muscle atrophy in disuse and unloading conditions was shown in studies that determined the effects of myostatin knockout or postnatal inhibition in mouse models of these conditions (Table 8.2). Although myostatin knockout in mice results in muscle hypertrophy, the loss of muscle mass after 7 days of hind limb suspension was more pronounced in *Mstn-/-* mice compared to wild type mice, which was associated with impaired protein translation and more pronounced upregulation of *MuRF-1* and *Atrogin-1* [149, 150]. In contrast, inhibition of myostatin in mice using specific antibodies during limb cast immobilization or hind limb suspension counteracted the decline in muscle mass, increased muscle force, and decreased the expression of *MuRF-1* and *Atrogin-1* [151, 152]. Importantly, the positive effect of myostatin inhibition was observed during 14 days of immobilization but not after 21 days of immobilization, suggesting that postnatal inhibition of myostatin efficiently counteract disuse atrophy only during shorter periods of immobilization [151].

In contrast to immobilization-/unloading-induced muscle atrophy, the contribution of myostatin in denervation-induced atrophy is questionable. Although myostatin expression increases locally in skeletal muscles during denervation atrophy (Table 8.1), the effect of myostatin inhibition in different studies is conflicting. Gene delivery of the myostatin prodomain or a proteinase-resistant pro-myostatin mitigated botulinum toxin-induced denervation atrophy in rats [153]. In addition, *in*

vivo transfection of a dominant-negative myostatin type II receptor (dnACVR2B) that inhibits downstream signaling in mouse skeletal muscle partially protected muscles from denervation-induced muscle atrophy [108]. On the other hand, treatment with the soluble ACVR2B-Fc myostatin receptor domain efficiently counteracted muscle atrophy induced by immobilization but not after sciatic nerve denervation in mice [119]. AAV-mediated overexpression of follistatin counteracted denervation atrophy when mice were injected before surgical denervation but not when mice were injected after the procedure, suggesting that the timing of treatment is important [154]. In addition, *in vivo* experiments in mice showed that although Smad2/3 is required for denervation atrophy, myostatin is not required for Smad2/3-mediated atrophy, suggesting that another mechanism is involved [109]. Instead IGF-1 receptor deactivation contributed to the accumulation of Smad2/3 proteins independently of myostatin signaling [109].

Some forms of neuromuscular diseases also cause denervation-induced muscle atrophy, such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). ALS is a lethal neuromuscular disease caused by late-onset degeneration of motor neurons in the brain and spinal cord, which results in muscle atrophy. In mouse and rat models of ALS, myostatin inhibition alleviated muscle atrophy and decline in muscle force during the early stages of the disease, but treatment did not result in functional improvement during the end stage of the disease and failed to improve survival in these animal models of ALS [155–157]. Treatment of ALS mice with ACVR2B-Fc resulted in more pronounced improvements compared to the effects of myostatin knockout, suggesting that targeting other TGF-β family members in addition to myostatin is more effective [157].

In SMA animal models, the effect of myostatin inhibition appears to depend on the severity of disease. SMA is a lethal neuromuscular disease that is caused by degeneration of motor neurons in the spinal cord and can be divided in different subtypes depending on the severity and age of onset. Different animal models exist that model the mild or severe variants of the disease, such as the severely affected SMAΔ7 mice and the mildly affected C/C SMA mice. Myostatin knockout or ACVR2B-Fc treatment did not improve the pathology or survival of SMAΔ7 mice [158, 159]. The effect of FST treatment in SMAΔ7 mice is controversial, with one study showing no effect of the treatment and another study showing a positive effect on pathology, motor function, and survival [160, 159]. In mildly affected SMAΔ7 mice treated with a SMN2 splicing modifier, FST treatment effectively counteracted muscle atrophy [161]. Correspondingly, both a myostatin inhibitor and ACVR2B-Fc treatment alleviated muscle pathology in the mildly affected C/C SMA mice [162]. Interestingly, ACVR2B-Fc treatment was more efficient in counteracting muscle wasting compared to the myostatin inhibitor, suggesting that multi-targeting compounds are more effective therapeutics [162].

8.3.2 Role of Myostatin in Sarcopenia

Sarcopenia is generally defined as aging-related muscle loss resulting in pronounced muscle weakness in elderly and is considered as a multifactorial condition [163]. When we age, a combination of disuse, loss of motor units, changes in diet, and pathological changes due to the aging process all contribute to muscle fiber atrophy and loss of muscle fiber number [164]. In addition, multiple studies suggest that satellite cell function is impaired as we age, leading to impaired muscle regeneration and fibrosis and contributing to muscle atrophy in aging muscle [165]. Although the contribution of myostatin to sarcopenia is controversial since there is no clear consensus on changes in myostatin expression and protein in aging muscle and serum (Table 8.1), multiple studies determined the effect of myostatin inhibition on skeletal muscles during aging and showed that myostatin is a promising target to alleviate sarcopenia (Table 8.2). The therapeutic potential of myostatin inhibition to counteract sarcopenia was first shown in studies that determined the effect of myostatin knockout in aging mice. Myostatin knockout resulted in muscle fiber hypertrophy, increased muscle mass, increased satellite cell activation *in vitro*, and improvement of muscle regeneration in old mice compared to control mice, suggesting that myostatin loss of function protects against sarcopenia [166, 167]. In addition, myostatin heterozygous knockout in mice (*Mstn+/-*) also protected against loss of muscle mass and function during aging and resulted in significant increases in muscle mass and in both absolute and specific muscle force [168]. Hydroxyproline content and *Col1a2* expression decreased in old *Mstn+/-* mice compared to control mice, suggesting a decrease in fibrotic tissue [168]. Interestingly, in contrast to *Mstn-/-* mice, heterozygous loss of function of myostatin also resulted in significantly increased longevity in mice [168]. In addition to genetic deletion of *Mstn*, several approaches of myostatin inhibition in aging mice also resulted in significant improvements in muscle mass, structure, and function. Treatment of older mice with myostatin antibodies increased muscle mass and muscle force (absolute force/grip strength) and decreased apoptosis in skeletal muscle as demonstrated by decreased TUNEL staining and decreased *Casp3* expression [169, 170]. Treatment of older mice with soluble myostatin prodomain or overexpression of myostatin prodomain also increased muscle mass and absolute force and moreover resulted in decreased expression of *Foxo1* and *MuRF1* [171, 172]. Together, these preclinical studies suggest that myostatin inhibition may be a promising therapeutic strategy to counteract sarcopenia.

8.3.3 Role of Myostatin in Cachexia

Cachexia is a wasting syndrome that results in loss of weight, muscle fiber atrophy, fatigue, and frailty and is caused by chronic disease states, such as cancer, AIDS, chronic kidney disease (CKD), and chronic obstructive pulmonary disease (COPD).

The association of myostatin with muscle atrophy was first revealed in a study that compared serum protein and mRNA expression levels of myostatin in healthy and HIV-infected men [11]. Some HIV-infected men show pronounced skeletal muscle wasting due to the chronic nature of AIDS, and in this study, the authors showed that myostatin protein and mRNA expression levels in serum and skeletal muscle increased in these men compared to healthy men [11]. In addition, increased myostatin levels have been associated with other cachectic conditions such as cancer, CKD, and COPD (Table 8.1). Myostatin knockout or treatment with myostatin/ACVR2 antibody or ACVR2B-Fc counteracts muscle atrophy and improves muscle function in mouse models of cancer cachexia [173–179]. Myostatin knockout and ACVR2 antibody or ACVR2B-Fc treatment furthermore increased survival of tumor-bearing mice [175, 176, 179]. In addition, combined inhibition of activins and myostatin with specific prodomains counteracted cancer cachexia more efficiently compared to single treatments, showing that multi-targeting TGF-β ligands may be a more promising strategy to counteract cachexia-associated muscle wasting [143]. A different strategy of inhibiting TGF-β pathways, gene delivery of Smad7, also counteracted cancer cachexia-induced muscle wasting in mice [107]. In addition to cancer cachexia, myostatin inhibition also prevented muscle atrophy in other cachectic conditions in preclinical animal models, such as chronic kidney disease in mice and AIDS in SIV-infected rhesus macaques [180, 181]. Together these studies show great promise of targeting these pathways to counteract muscle wasting in cachexia.

8.3.4 Role of Myostatin in Glucocorticoid-Induced Muscle Atrophy

Patients with chronic inflammatory diseases or degenerative muscle diseases such as muscular dystrophy are frequently treated with glucocorticoids such as prednisone to suppress inflammation. However, long-term glucocorticoid treatment has several side effects including muscle atrophy [182]. In addition, glucocorticoid treatment is associated with satellite cell dysfunction and impaired muscle regeneration in skeletal muscle [183]. Glucocorticoids directly induce *Mstn* expression via glucocorticoid response elements present in the myostatin promoter, and glucocorticoid treatment is associated with increased *Mstn* expression in rats [184]. The effect of specific myostatin inhibition on glucocorticoid-induced muscle wasting was first shown in *Mstn-/-* mice, which are protected from dexamethasone-induced muscle atrophy [185]. In addition, treatment with myostatin or ACVR2 antibodies also prevents glucocorticoid-induced muscle wasting in mice, showing the therapeutic potential for such treatments in this context [186, 152, 97].

8.3.5 Role of Myostatin in Neuromuscular Diseases

Patients with inherited neuromuscular diseases that cause muscle degeneration may also benefit from myostatin-targeting therapies. Although the pathology of such disorders, such as muscular dystrophy, is typically caused by mutations in genes that are important for skeletal muscle function and targeting of myostatin will not restore these primary causes, inhibiting these pathways may still alleviate muscle atrophy and some of the other secondary pathological processes that contribute to the pathology of neuromuscular diseases, such as impaired muscle regeneration and fibrosis. In contrast to conditions that induce muscle fiber atrophy, recent studies show that degenerative muscle-wasting diseases such as muscular dystrophy are associated with decreased levels of myostatin protein and mRNA (Table 8.1). This suggests that myostatin most likely is not primarily responsible for some of the secondary pathological changes observed in these disorders, such as fibrosis and impaired regeneration. Moreover, myostatin levels were negatively associated with loss of ambulation in BMD/DMD, LGMD2A/2B, and HIBM patients, suggesting that progressive decline in muscle function results in further decrease in myostatin [187]. Nonetheless, a wealth of studies in preclinical animal models show that myostatin inhibition alleviates or counteracts muscle-wasting pathology in different neuromuscular diseases and may be a potential target regardless of the decreased myostatin levels detected in these muscle-wasting conditions (Table 8.2).

The first indication that myostatin targeting could be a promising therapy for patients with muscular dystrophy was provided by a study in 2002 that genetically crossed myostatin knockout mice with *mdx* mice, a mouse model for Duchenne muscular dystrophy (DMD) [188]. Importantly, this study showed that myostatin knockout in *mdx* background resulted in increased muscle mass as well as increased absolute muscle force, measured at different ages by grip strength in these mice compared to *mdx* control. In addition, the amount of fibrosis was decreased in these *mstn-/- mdx* mice [188]. However, these results are controversial since other studies failed to show a clear effect on muscle regeneration in *mstn-/- mdx* mice and even reported deleterious effects on oxidative metabolism and increased muscle fatigability after treatment with ACVR2B-Fc [32, 69]. Moreover, a recent study described the effect of cross-breeding whippets with a heterozygous myostatin mutation with golden retrievers that have DMD (GRMD dogs) and showed that the effects of genetic myostatin loss of function were deleterious and aggravated the dystrophic pathology in these so-called GRippet dogs [189]. However, a multitude of other studies showed positive effects of myostatin inhibition using antibodies, myostatin prodomain, ACVR2B-Fc, and FST in *mdx* mice (Table 8.2). It is furthermore important to realize that *Mdx* mice show a mild pathology that is not comparable to the progressive pathology in DMD patients. Therefore, the question is whether *mdx* mice are a suitable preclinical model to test such therapies for DMD. The diaphragm muscle is more severely affected in these mice and may therefore represent a more suitable muscle type to test myostatin-targeting therapies [190]. One study determined the effect of myostatin antibody treatment on the

pathology in diaphragm muscles of *mdx* mice and reported that the treatment counteracted some of the pathological changes in young *mdx* mice but not in older *mdx* mice, suggesting that the effect of the myostatin antibody treatment is age dependent and may only be effective in the early stage [191]. In addition to *mdx* mice, other potentially more suitable mouse models of DMD are available, such as the more severely affected *mdx utrn-/-* mice and *DBA/2J-mdx* mice. Further studies are needed to test the efficiency of myostatin-targeting treatments in these mice in order to determine the effect on the pathology of DMD in more detail.

In addition to DMD, the effect of targeting myostatin and related proteins has been tested in other muscular dystrophy mouse models, such as different mouse models of the different types of limb-girdle muscular dystrophy (Table 8.2). LGMD is also characterized by muscle degeneration, but the severity, age of onset, and disease progression vary among the different subtypes. Myostatin-targeting therapies show promising results in mouse models of LGMD1C, LGMD2A, and LGMD2C (Table 8.2). However, in a mouse model of LGMD2B (dysferlinopathy), the pathology of the disease worsened after treatment with FST or ACRV2B-Fc and accelerated muscle degeneration [192]. Furthermore, in a mouse model of LGMD2D, overexpression of myostatin prodomain did not result in any changes in the dystrophic pathology [193]. In a mouse model of LGMD2F, myostatin knockout and treatment with myostatin antibodies improved muscle pathology in 4-week-old mice but not in 20-week-old mice, suggesting that myostatin targeting is only efficient in the early stage of this disease [194].

In addition to muscular dystrophies, myostatin targeting has also shown promising results in mouse models of other myopathies, such as nemaline myopathy and X-linked myotubular myopathy (XLMTM). Notably, treatment with ACVR2B-Fc increased muscle mass and survival in these mouse models, and treatment with myostatin antibody improved pathology and muscle force in a NM mouse model [195–197]. However, myostatin knockout did not improve muscle pathology in a mouse model for merosin-deficient congenital muscular dystrophy (MDC1A) but instead increased postnatal lethality [198].

In summary, these results show that targeting myostatin and related proteins alleviates the pathology of some degenerative muscle-wasting diseases but may have no effect or even worsen the pathology of other myopathies. In addition, due to discrepancies between different studies and the use of animal models that do not accurately resemble the human pathology, the effect of such therapies in muscle-wasting diseases such as DMD is still uncertain, and further research in more relevant preclinical animal models is therefore warranted.

8.4 Function of Related TGF-β Pathways in Muscle Atrophy

The function of myostatin in the regulation in muscle mass and the role of this pathway in muscle atrophy have become more evident in the past years. However, in recent years multiple studies also identified a role for other related members of the

TGF-β family in the regulation of muscle mass. The involvement of other related members of the TGF-β family in skeletal muscle growth was first discovered in studies that investigated the effect of proteins that inhibit activity of multiple members of this family on skeletal muscle. These studies showed that overexpression of FST or treatment of mice with ACVR2B-Fc results in more pronounced increase of muscle mass compared to the effect of myostatin knockout alone [89, 72, 74]. In addition, overexpression of inhibitory proteins FLRG/FST in *Mstn-/-* mice results in additional increase of muscle mass in these mice, thereby almost quadrupling muscle mass compared to wild-type mice [89]. Indeed it is known that several additional members of the TGF-β family interact with FLRG, FST, and ACVR2B that have a similar effect on myogenesis as myostatin, such as GDF11, activins A and B, and TGF-β [199, 200]. Next, we will discuss the evidence showing the involvement of these related proteins in different muscle-wasting conditions.

8.4.1 The Role of GDF11 in Sarcopenia

GDF11 is the closest structurally related member of myostatin, showing ~90% homology to myostatin in the mature ligand domain (Fig. 8.2). In contrast to myostatin this protein is not expressed in skeletal muscle and genetic deletion of GDF11 does not result in muscle hypertrophy [201, 202]. Instead GDF11 plays an important role during embryonic development in the patterning and development of the axial skeleton [202]. A potential involvement of GDF11 in sarcopenia was first uncovered by a study showing that GDF11 levels are reduced in serum of aged mice and in elderly and that injection of GDF11 can alleviate aging-related muscle wasting in mice [203, 204]. It was already known that heterochronic parabiosis (the linkage of circulation of young mice to old mice) can alleviate sarcopenia, suggesting the presence of a rejuvenating factor in young blood. These studies therefore suggested that GDF11 might be the rejuvenating factor in young blood responsible for the observed effects of heterochronic parabiosis. However, these findings came as a surprise since GDF11 is highly homologous to myostatin. Recent studies demonstrated that GDF11 is a catabolic and anti-myogenic factor like myostatin and inhibits myogenesis and muscle regeneration and induces muscle wasting *in vivo* in mice [205–208]. In addition, differences found in GDF11 levels during aging were not reproducible due to methodological issues with the immuno-based assays used for detection, such as cross-reactivity of the Gdf11 antibody with the highly homologous myostatin protein and detection of non-specific background signals [205, 203]. Recent results from a different study using a highly specific liquid chromatography with tandem mass spectrometry (LC-MS/MS) assay showed that there were no significant changes in GDF11 levels in older men or women [209]. Although the results of different studies on the effect of GDF11 on aging skeletal muscle are not conclusive, potentially due to differences in experimental design, in our

opinion most evidence now suggests that it is unlikely that decreased levels of GDF11 contribute to the development of sarcopenia.

8.4.2 The Role of Activins in Muscle Atrophy and Muscle-Wasting Disorders

Activin A and activin B are closely related members of the TGF-β family that are encoded by the *INHBA* and *INHBB* genes. Activins mediate downstream signaling via interactions with activin type II receptor ACVR2A and ACVR2B and type I receptor ACVR1B (ALK4). Importantly, several studies showed that activin A and activin B are also important regulators of muscle mass and play a role in muscle atrophy. The association of activins with cancer cachexia was first shown in inhibin knockout mice, which show highly elevated levels of activin A and activin B and develop ovarian and testicular sex cord-stromal tumors accompanied by severe cachexia [210]. In addition, elevated levels of activin A are detected in cancer patients and are associated with the development of cachectic wasting symptoms including muscle atrophy [211, 212]. Importantly, systemic inhibition of myostatin and activins with soluble ACVR2B-Fc counteracted cancer cachexia and prolonged survival in mice [179]. A direct role of these proteins in muscle wasting was shown recently in a study which demonstrated that systemic overexpression of activin A and activin B with adeno-associated viral vectors resulted in pronounced muscle atrophy and muscle fibrosis in mice [213]. In addition this study showed that overexpression of activin A or activin B results in more pronounced muscle atrophy compared to myostatin and TGF-β [213]. Conversely, other studies showed that activin A knockout results in muscle hypertrophy in mice and that antibody-mediated activin A inhibition in combination with inhibition of myostatin results in synergistic increase in muscle mass in mice and monkeys comparable to the effect of ACVR2B-Fc [214, 86]. A recent study showed that specific inhibition of activins and myostatin with specific prodomains of these proteins prevented muscle atrophy in cancer cachexia in mice, showing the potential therapeutic value of targeting these pathways [143]. In addition to the effect on muscle mass, it is known that activins inhibit myogenesis in the embryo and inhibit myogenic differentiation of myoblasts *in vitro* [215, 216, 200]. Interestingly, pro-inflammatory cytokines TNF-α and IL-1α induce expression of activin A via TAK1/p38MAPK/NFkB-dependent pathways *in vitro*, and the anti-myogenic effect of these pathways was found to be mediated by activin A [217]. A recent study showed that systemic levels of activin A protein and *INHBA* mRNA expression in muscles of patients with different neuromuscular diseases did not differ [218]. Although these studies suggest that activin A does not directly contribute to the pathology of neuromuscular diseases, combined inhibition of myostatin and activins does improve muscle histology in mouse models of DMD and synergistically increase muscle mass [143]. Together these

results show that activins may be a potential therapeutic target to alleviate muscle wasting in muscle-wasting conditions such as cachexia and DMD.

8.4.3 The Role of TGF-β in Muscle Atrophy

TGF-β ligands mediate signaling via the type II TGF-β receptor TGFBR2 and type I receptor TGFBR1 (ALK5). Although three isoforms exist (TGF-β1-3), mainly TGF-β1 has been associated with the pathology of different muscle-wasting disorders. TGF-β1 plays an important role in wound healing and regulation of the immune system and is a key pro-fibrotic factor [219]. In addition, it is known that TGF-β inhibits myogenic differentiation of myoblasts *in vitro* [220, 221]. Given the role of TGF-β1 in fibrosis and regeneration, it is not surprising that the mRNA expression and protein levels of TGF-β1 are increased in patients with degenerative muscle-wasting diseases such as muscular dystrophies, where continuous breakdown and necrosis of muscle fibers result in chronic inflammation, fibrosis, and impaired muscle regeneration. The important contribution of TGF-β in the pathology of muscle-wasting diseases was shown in a study that investigated the effect of inhibiting the TGF-β pathway using either specific antibodies against all three isoforms or losartan in mouse models of DMD or Marfan syndrome [222]. Inhibition of these pathways resulted in improved muscle regeneration and decrease in fibrosis in mouse models of these diseases [222]. Since then multiple studies have shown the important role of TGF-β1 and TGF-β2 in degenerative muscle-wasting diseases such as DMD [223–228]. In addition to fibrosis and the inhibitory effect on muscle regeneration, other studies also showed that TGF-β can directly induce muscle fiber atrophy. Overexpression of TGF-β1 in skeletal muscles resulted in fibrosis and muscle fiber atrophy and increased expression of *MuRF1* [229]. In addition, *in vitro* experiments in C_2C_{12} myotubes showed that TGF-β induces atrophy and increases the expression of *MuRF1*. A recent study showed the relevance of TGF-β activity in cancer cachexia-induced muscle atrophy and introduced a new mechanism of how TGF-β can contribute to muscle weakness. Advanced cancer is associated with bone metastases and in mice this results in bone degradation and release of TGF-β [230]. Inhibition of TGF-β (all isoforms) counteracted muscle weakness, suggesting that TGF-β was directly responsible for the decline in muscle force in these mice [230]. Mechanistically, TGF-β increased expression of *Nox4*, which resulted in interaction of Nox4 with the RyR1 Ca2+ release channel and subsequent oxidization and leakage of RyR1 channel, contributing to reduced muscle contractility and muscle weakness.

In addition, increase in TGF-β activity is also associated with sarcopenia during aging. More specifically, in mice local and systemic increases of TGF-β are associated with elevated pSmad3 levels; increased expression of cyclin-dependent kinase inhibitors *p15*, *p16*, *p21*, and *p27*; and satellite cell dysfunction and impaired regeneration in aging skeletal muscle [231]. Hyperactivity of TGF-β pathway in old mouse satellite cells was also associated with a decrease in Notch activation,

suggesting crosstalk between these pathways [231]. Indeed, Smad3 and Notch physically interacted *in vitro*, and activation of Notch resulted in decreased recruitment of Smad3 to the promoter regions of *p15*, *p16*, *p21*, and *p27* [231]. Moreover, systemic TGF-β type I receptor inhibition, local RNAi-mediated Smad3 inhibition in the muscle, or Notch reactivation rescues the regeneration defect in aging mouse muscle, suggesting the therapeutic potential of modulating these pathways [231–233]. Experiments in human satellite cells showed that deregulation of these pathways is conserved during aging in humans [234].

8.5 Translation of Myostatin-Targeting Therapies to the Clinic

The prospect of inhibiting skeletal muscle atrophy using specific myostatin-targeting therapy has been tantalizing for many years since the discovery of myostatin in mice. First and foremost, this is because the muscle-specific expression and action of myostatin make it an appealing therapeutic target since potential harmful side effects in other tissues can be avoided. The second important reason is because evidence has accumulated that myostatin contributes to the pathology of some muscle-wasting conditions and targeting of myostatin and/or myostatin-related pathways can alleviate some forms of muscle wasting as evidenced from studies in different animal models as mentioned before. Different treatments based on different strategies of targeting myostatin have been translated to the clinic, and an overview of these treatments is provided in Table 8.3. It is important to distinguish between treatments that specifically target myostatin and treatments that in addition to myostatin also target other members of the TGF-β family, because the effects and efficiency of these distinct strategies can be quite different. Targeting of multiple targets may prove to be more efficient in muscle-wasting conditions but may also result in serious side effects in other tissues. In the following section, we will discuss the progress that has been made in recent years in the clinical translation of specific myostatin-targeting compounds and multi-targeting compounds.

8.5.1 Specific Myostatin Inhibitors in Clinical Trials

The first example of translation of a myostatin inhibitor to the clinic was a study published 10 years ago in 2008 that tested the monoclonal human myostatin antibody stamulumab (MYO-029), which was developed by Wyeth (now Pfizer). In a double-blind, placebo-controlled dose escalation study, three doses (1 mg/kg, 3 mg/kg, 10 mg/kg) were compared to placebo controls and injected once every 2 weeks during a 6-month treatment period in muscular dystrophy patients (BMD, FSHD, and LGMD). Although the safety profile of stamulumab was good, with few reported side

Table 8.3 Overview of different myostatin-targeting compounds in clinical trials

Name compound	Company/institute	Target	Mechanism of action	Conditions	Status clinical trials (refs)
Stamulumab (MYO-029)[a]	Wyeth Pharm.	MSTN	MSTN-specific antibody	BMD, FSHD, LGMD	Phase 1/2 completed (healthy)
					Phase 1/2 completed (BMD, FSHD, LGMD) [302, 236, 235]
Landogrozumab (LY2495655)	Lilly	MSTN	MSTN-specific antibody	Healthy, cancer cachexia, sarcopenia	Phase 2 completed (cancer cachexia)
					Phase 2 completed (sarcopenia) [240, 241]
Domagrozumab (PF-06252616)	Pfizer	MSTN	MSTN-specific antibody	Healthy, DMD, LGMD2I	Phase 1 completed (healthy) [238]
					Phase 1/2 active (LGMD2I)
					Phase 2 active/recruiting (DMD) [237]
Trevogrumab (REGN1033)	Regeneron	MSTN	MSTN-specific antibody	Healthy, sarcopenia	Phase 1 recruiting (healthy)
SRK-015	Scholar Rock	Latent MSTN	MSTN-specific antibody	SMA	Expected to start recruiting mid-2018
PINTA 745 (AMG 745)[a]	Atara Biotherapeutics/ Amgen	MSTN	MSTN peptibody, systemic	ADT (cancer), end-stage renal disease	Phase 1 completed (ADT) [303]
					Phase 2 completed (renal disease)
BMS-986089	Bristol-Myers Squibb	MSTN	MSTN adnectin	Healthy, DMD	Phase 1 completed/recruiting (healthy)
					Phase 1/2 active (DMD)
					Phase 2/3 recruiting (DMD)

Name compound	Company/institute	Target	Mechanism of action	Conditions	Status clinical trials (refs)
Bimagrumab (BYM338)	Novartis	ACVR2B	Type II receptor-specific antibody	sIBM, sarcopenia, cancer cachexia, disuse atrophy, COPD, diabetes	Phase 2 completed (cancer cachexia, COPD, sarcopenia, atrophy) [248, 249, 304]
					Phase 2b/3 completed (sIBM) [247]
					Phase 2 recruiting (sarcopenia, hip surgery, diabetes type 2)
ACE-031[a]	Acceleron	MSTN, ACTIVINS, BMPs	Soluble receptor domain ACVR2B	Healthy, DMD	Phase 1/2 completed (healthy) [244]
					Phase 1/2 discontinued (DMD) [245]
ACE-083	Acceleron	MSTN, ACTIVINS, BMPs	FST (locally active)	Healthy, FSHD, CMT	Phase 1 completed (healthy) [253]
					Phase 2 recruiting (FSHD)
					Phase 2 recruiting (CMT)
ACE-2494	Acceleron	MSTN, ACTIVINS	Soluble receptor domain ACVR2B	Healthy, Neuromuscular disease	Phase 1 recruiting (healthy)
rAAV1.CMV. huFollistatin344	Milo Biotechnology/ Nationwide Children's Hospital	MSTN, ACTIVINS	FST	BMD, sIBM	Phase 1 completed (BMD, sIBM) [250, 252, 251]

[a]further development of therapy discontinued

effects, the treatment with this antibody did not result in clear changes in muscle mass or function, which was supposedly mainly due to a small study size and lack of statistical power, after which further clinical studies were subsequently halted [235]. A recent study suggested that this antibody was less effective in monkeys compared to mice and that clearance of the antibody is higher in monkeys and humans, which may explain the limited efficiency of the treatment in the clinical study [236]. However, development of other potentially more efficient myostatin antibodies as treatment for muscle-wasting conditions is currently actively pursued by different companies (see Table 8.3).

Recently, the first results of a new antibody developed by Pfizer, domagrozumab (PF-06252616), showed that this antibody has a good safety profile in healthy volunteers, displayed a slow clearance rate, and showed that a concentration of 10 mg/kg induced whole-body lean mass and muscle volume (4.5% change from baseline) [237, 238]. In addition, the antibody was shown to efficiently increase whole-body lean mass (10–15% change from baseline) and muscle volume (24% change in baseline) in cynomolgus monkeys, and treatment with the mouse variant increased muscle mass and improved functional outcome measures in *mdx* mice [239]. At the time of writing of this review, the safety and pharmacodynamic profile as well as the functional effect of domagrozumab is being evaluated in a phase 2 clinical trial in DMD patients and a phase 1/2 trial in LGMD2I patients, both of which are randomized double-blind open-label multiple ascending dose escalation trials (active/not recruiting; clinicaltrials.gov identifier NCT02310763 and NCT02841267). In addition, a multicenter open-label extension study is planned in DMD patients and is currently recruiting participants (clinicaltrials.gov identifier NCT02907619).

A recent phase 2 clinical study tested the effect of a different humanized monoclonal myostatin antibody developed by Lilly, landogrozumab (LY2495655), on lean body mass and physical performance in older men and women aged >75 with low muscle mass and strength, who experienced recent falls [240]. The results showed that 20 weeks of treatment with this antibody (six s.c. injections of 315 mg in 20 weeks) significantly increased appendicular and total body lean mass (0.43 kg and 0.71 kg change, respectively) and improved some functional outcome measures, such as stair climbing and chair rise time, compared to placebo-treated individuals [240]. In a different phase 2 clinical trial study, the effect of different doses of landogrozumab (four s.c. injections of 35 mg, 105 mg, or 315 mg in 12 weeks) on muscle mass and function was evaluated in men and women aged >50 that received a hip replacement [241]. The results of this study were unfortunately less clear-cut with the primary endpoint, an increase in appendicular lean mass after 12 weeks, not met and no effect observed on exploratory outcome measures for muscle function. However, the results of this study did show increased appendicular lean mass after 8 weeks and 16 weeks with the two highest concentrations used [241]. Other clinical trials in cancer patients have been completed with this antibody (clinicaltrials.gov identifiers NCT01505530 and NCT01524224), but as yet the results from these studies have not been published, and it is unknown whether Lilly is planning other clinical trials in the future. Other companies that have developed myostatin-targeting antibodies are currently recruiting (trevogrumab (REGN1033);

developed by Regeneron) or planning to start recruitment of participants (SRK-015; developed by Scholar Rock). SRK-015 was shown to bind specifically to pro-myostatin and the latent domain of myostatin and inhibits proteolytic processing of myostatin, thereby inhibiting myostatin activity via a different mechanism compared to conventional myostatin antibodies that target the mature protein [97]. The company has announced on their website that they expect to start with a first trial in SMA patients in mid-2018.

In addition to antibodies, other specific myostatin-targeting methods have been developed that have been tested in clinical trials. A myostatin blocking peptide coupled to a IgG domain developed by Atara Biotherapeutics, PINTA 745, was reported to increase muscle mass and improve muscle function in stroke and CKD mouse models [242, 181]. A phase 1/2 clinical trial study of this compound in patients with end-stage renal disease was completed in 2016 (clinicaltrials.gov identifier NCT01958970), and the company announced that the primary endpoints were not met in this study and that the company would not continue further clinical development of this treatment (http://investors.atarabio.com/news-releases/news-release-details/atara-bio-announces-results-phase-2-proof-concept-pinta-745). A myostatin-targeting adnectin was developed by Bristol-Myers Squibb company and is currently being evaluated in DMD patients. Adnectins are genetically engineered variants of the 10th type III domain of human fibronectin. The myostatin adnectin is composed of a human Fc IgG1 domain fused to an adnectin domain that specifically targets myostatin [243]. A multicenter, randomized, double-blind, placebo-controlled phase 2/3 study with this compound is currently ongoing in DMD patients and is estimated to finish in 2020 (ClinicalTrials.gov Identifier: NCT03039686).

In summary, the first results of second-generation myostatin antibodies such as domagrozumab and landogrozumab in clinical trials show positive results in healthy volunteers and older individuals. The efficiency of these and other specific myostatin-targeting antibodies and compounds will become more evident in the coming years when the first results from new and ongoing clinical trials in patients with muscle-wasting disorders will be announced.

8.5.2 Multi-targeting Compounds in Clinical Trials

In addition to specific myostatin inhibitors, other clinical studies have concentrated on the effect of inhibitors that target other related TGF-β members in addition to myostatin (see Table 8.3). ACE-031 is the human variant of the ACVR2B receptor domain coupled to a soluble Fc domain (ACVR2B-Fc) developed by Acceleron. The ACVR2B receptor mediates signaling of myostatin, Gdf11, and activins and can also bind BMPs with lower affinity [200]. Initial clinical studies in healthy volunteers (postmenopausal women) showed that one s.c. injection of this compound was safe and resulted in significant increase of lean body mass and muscle hypertrophy (4% increase at the highest concentration; 3 mg/kg) [244]. Although a subsequent clinical trial showed a similar effect in DMD patients and moreover

suggested a trend toward improvement in functional outcome measures such as the 6-min walking test, further trials were halted due to non-muscle adverse effects, such as epistaxis (nose bleedings) and telangiectasias (dilated blood vessels) [245]. A systemically active variant of ACE-031 that targets myostatin and activins but shows reduced affinity for BMPs, ACE-2494, has been tested in mice and increases muscle mass as efficiently as ACE-031 and healthy volunteers are currently recruited for a phase I trial (NCT03478319).

Bimagrumab (BYM338) is a multi-target antibody developed by Novartis against the type II ACVR2A and ACVR2B receptors and blocks the interaction of these receptors with their ligands: myostatin, activins, and BMPs [246]. Studies in mice showed that treatment with this antibody results in more pronounced muscle hypertrophy compared to myostatin or activin targeting alone and counteracts glucocorticoid-induced atrophy in mice [186, 246]. In a first randomized controlled clinical trial in 2014, the safety and effect of bimagrumab were evaluated in 14 sporadic inclusion body myositis (sIBM) patients and demonstrated that a single injection of 30 mg/kg resulted in increased muscle mass and improvement in 6-min walking distance [247]. However, a subsequent phase 2b/3 clinical trial in sIBM patients unfortunately did not meet its primary endpoint, a change from baseline in 6-min walking distance (NCT01925209). Importantly, results of other clinical trials suggest that bimagrumab can alleviate muscle atrophy and may improve muscle function in other muscle-wasting conditions. In a recent phase 2 clinical study, the safety and effect of bimagrumab on muscle mass and mobility were tested in 40 individuals aged >65 with sarcopenia. Treatment of bimagrumab (30mg/kg) resulted in significant increases in muscle volume compared to placebo and furthermore showed improvement in gait speed and 6-min walking distance (NCT01601600) [248]. Similar results on muscle mass were shown in a phase 2 clinical trial in patients with casting-induced muscle atrophy, where treatment with a single dose of bimagrumab (30mg/kg) accelerated recovery of muscle volume (NCT01601600) [249]. In addition, in a different phase 2 trial COPD patients received two doses of either placebo or bimagrumab (30mg/kg) and bimagrumab was found to induce thigh muscle volume (5.0-7.8%)(NCT01669174) [304]. However, in this study no differences were found in functional outcome measures, such as 6-min walking distance. Notably, in different clinical trials the safety of bimagrumab treatment was also demonstrated with only mild adverse effects reported, such as muscle spasms, acne and diarrhea [248, 304]. Further phase 2 clinical studies are planned and are currently recruiting participants to evaluate the effect of this antibody on sarcopenia in a larger cohort of older people (NCT02333331) and test the effect on muscle atrophy in hip fracture surgery patients (NCT02152761).

Adeno-associated virus (AAV)-mediated follistatin (FST) gene therapy, rAAV1.CMV.huFollistatin344, also showed promising results in clinical trials in patients with muscle-wasting diseases. The isoform of FST used in these studies, FS344, is serum based and has lower affinity for activins compared to other FST isoforms [250]. A phase 1/2 clinical trial in a small cohort of Becker muscular dystrophy (BMD) patients (n=6) showed that a single bilateral intramuscular injection of two different doses of rAAV1.CMV.huFollistatin344 in the quadriceps (3×10^{11} vg/kg

or 6×10^{11} vg/kg) significantly increased 6-min walking distance in four out of six patients with no difference between doses [250, 251]. In addition, muscle biopsies showed signs of improved muscle histology at the highest dose as evidenced by decreased muscle fibrosis, reduced percentage of central nucleated muscle fibers, and muscle fiber hypertrophy [251]. Similarly, a phase 1/2 clinical trial in six sIBM patients also showed functional improvement in the 6-min walking distance after one bilateral intramuscular injection of 6×10^{11} vg/kg of rAAV1.CMV.huFollistatin344 [252].

A different follistatin-based compound developed by Acceleron, ACE-083, showed promising results in a phase 1 clinical trial in healthy volunteers. Local injection of different doses of ACE-083 (50–200 mg/kg) in TA or RF muscles resulted in a dose-dependent increase in muscle mass of up to 10% for the TA muscle and up to 15% for the RF muscle [253]. Clinical phase 2 trials with ACE-083 are planned in patients with Charcot-Marie-Tooth disease (CMT; NCT03124459) and facioscapulohumeral muscular dystrophy (FSHD; NCT02927080) and are currently recruiting participants.

Together these studies suggest that targeting multiple TGF-β ligands may efficiently induce muscle mass and improve muscle function in muscle-wasting conditions. Although serious adverse side effects have been reported for ACE-031, initial clinical trials with other compounds showed a good safety profile and therefore show promise as potential therapy to counteract muscle wasting.

8.6 Future Perspective

Preclinical studies in animal models of muscle-wasting disorders have demonstrated the potential of treatments that target myostatin and related signaling proteins in counteracting the decline in muscle mass, and some strategies show promising results in clinical trials as well. However, several important issues remain to be resolved before such treatments are to be considered as realistic treatment for different muscle-wasting conditions.

First, because of contradicting results from different preclinical studies, it is unclear whether targeting of myostatin and related pathways is actually a good strategy to counteract muscle atrophy and improve muscle function in some conditions such as denervation atrophy and muscular dystrophies. Recent reports of the detrimental effect of myostatin inhibition on the oxidative metabolism and endurance and the lack of effect of such treatments on muscle regeneration in DMD mouse models raise some concerns regarding the efficacy of such treatments in alleviating muscle wasting. Future studies in clinically more relevant animal models are therefore required, and results from clinical trials with myostatin inhibitors in DMD patients should result in more clarity on the effect of these treatments.

Second, it is important to distinguish between strategies that target myostatin specifically and treatments that target multiple members of the TGF-β family and to establish which strategy shows the highest efficiency in stimulating muscle growth

and muscle function without inducing serious adverse side effects in other tissues. Indeed, as we discussed, multiple preclinical experiments suggest that multi-targeting compounds are more efficient in counteracting muscle atrophy and muscle wasting and also show promising results in stimulating muscle regeneration in different animal models. Future studies are warranted to identify overlapping as well as different functions of different TGF-β ligands in muscle atrophy and should clarify which ligands or downstream pathways are valid targets for therapy.

Last, it is important to realize that targeting of myostatin and related pathways is not a definitive cure for neuromuscular diseases such as muscular dystrophy and should be considered as supportive therapy in such cases. Indeed, preclinical studies showed the potential of combination therapies aimed at restoring the genetic defect of muscular dystrophy and stimulating muscle growth with myostatin targeting [254–257]. In addition, multiple signaling pathways play a role in muscle wasting, but as yet it is largely unknown if and how these pathways interact. More detailed knowledge of cross talk between myostatin/activin/TGF-β signaling pathways with other important pathways that regulate muscle mass and/or regeneration such as BMPs, Wnts, and Notch could lead to identification of novel targets for muscle wasting.

Competing Financial Interests The authors declare no competing financial interests.

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Chapter 9
Hormones and Muscle Atrophy

Ana Isabel Martín, Teresa Priego, and Asunción López-Calderón

Abstract The endocrine system is an essential regulator of muscle metabolism in both health and disease. Hormones such as growth hormone (GH), insulin-like growth factor-I (IGF-I) and androgens are the main regulators of muscle metabolism in both health and disease; have profound influences on muscle, acting as anabolic factors; and are important regulators of muscle mass. On the contrary, glucocorticoids have direct catabolic effects and induce muscle protein loss. Muscle wasting is a systemic response to fasting and several diseases like cancer, sepsis, renal and cardiac failure and trauma. Muscle atrophy also occurs in specific muscles with denervation, immobilization or inactivity. All of these conditions are characterized by significant changes in the endocrine environment. The aim of this review was to describe the role of endocrine system on the development of muscle atrophy. Understanding hormonal regulation of the skeletal muscle in these conditions might facilitate the development of hormone-mediated therapies for muscle atrophy.

Keywords Hormones · GH · IGF-I · Glucocorticoids · Androgens · Testosterone · Thyroid hormones · Insulin · Leptin · Ghrelin

9.1 Introduction

The endocrine system plays an important role in regulating many functions such as development and growth, metabolism, energetic balance, reproduction, behaviour and adaptation to changes in the internal and external environments. Between these functions, the skeletal muscle is the target organ through which the endocrine system controls the different body functions.

Skeletal muscle mass is mainly regulated by exercise, nutrition and hormones. In the skeletal muscle, numerous hormones control anabolic-catabolic balance, glucose metabolism and muscle mass maintenance and reparation after injury. However, growth hormone (GH) and insulin-like growth factor I (IGF-I), testosterone, thyroid

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hormones (TH) and glucocorticoids (GCs) exert major effects on skeletal muscle growth and function.

Muscle atrophy can be due to several causes: chronic illnesses, aging, malnutrition, disuse and acute/chronic inflammatory conditions. The inflammatory process is characterized by an increase in pro-inflammatory cytokine production that triggers many endocrine responses. During the acute phase, proteolysis in the skeletal muscle provides substrates to fuel the necessary increases in immune activity. However, in chronic diseases where inflammation persists, continuous muscle protein breakdown leads to a profound depletion of the skeletal muscle.

Muscle wasting or cachexia in chronic diseases (such as cancer, sepsis, chronic kidney or heart failure, chronic obstructive pulmonary disease and rheumatoid arthritis) is associated with an increase in muscle proteolysis, whereas anorexia can be present or not. Cachexia is characterized by a decrease in the size of the muscle fibres, myonuclear number, protein content and muscle strength. In those conditions, fast glycolytic muscle fibres are more affected than oxidative-type fibres. On the contrary, muscle atrophy induced by aging or sarcopenia is associated with a decreased ability of muscle regeneration.

All conditions described above are characterized by decreased IGF-I levels, activation of the adrenal axis (characterized by an increased release of GCs), a decline in the gonadal axis (with a reduced secretion of gonadal steroids), an alteration in the thyroid axis and a dysregulation of the hormones involved in glucose and lipid metabolism (insulin and leptin). Even more, hormones involved in electrolyte metabolism (vitamin D and angiotensin II) seem to play a role in muscle wasting in some type of muscle atrophy. It is known that the dysregulation in the endocrine environment is the main mechanism involved in muscle atrophy, activating proteolysis and autophagy and, in some cases, inhibiting muscle regeneration (decreasing protein synthesis and myocyte proliferation) (Fig. 9.1).

One of the most frequent types of systemic muscle loss is sarcopenia, which is seen in older patients. This phenomenon differs from other types of atrophy, as the muscle loss develops gradually and occurs over several years. Even though muscle atrophy occurs without apparent disease, older patients have a combination of several factors, including decreased levels of GH and IGF-I, insulin resistance, prolonged periods of inactivity or bed rest, a decline in sex hormones, etc., which may directly contribute to the muscle wasting.

Muscle atrophy, also called disuse atrophy, occurs by prolonged reduction of physical activity. Muscle disuse includes joint immobilization, limb suspension, bed rest, denervation, microgravity and mechanical ventilation [1]. In these conditions, the skeletal muscle adapts to a prolonged reduction in physical activity by decreasing total muscle mass and myosin content [2] and changing fibre type from slow to fast.

It is important to note that each atrophic condition has its own specific characteristics, its particular hormonal environment and distinct mechanisms and pathways that lead to muscle wasting. Thus, in this chapter the main hormones involved in muscle atrophy and the main atrophic mechanisms in which the hormones are involved in each axis will be analysed.

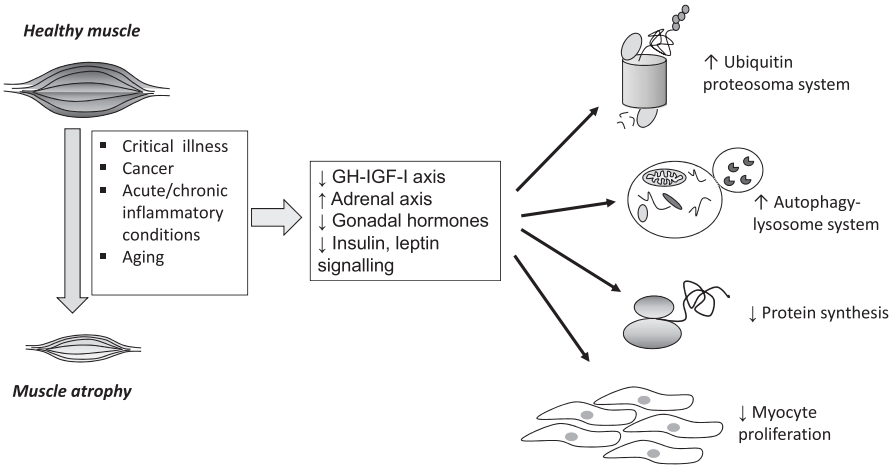

Fig. 9.1 Schematic diagram showing the main hormone alterations involved in several atrophic conditions with detrimental effects on the skeletal muscle

9.2 GH-IGF-I Axis

9.2.1 GH

GH or somatotropin is a peptide hormone synthetized by somatotrope cells in the adenohypophysis. This hormone regulates metabolism and has a crucial role in somatic growth and development. GH synthesis and secretion are stimulated by hypothalamic growth hormone-releasing hormone (GHRH) and by ghrelin mainly secreted by the stomach, whereas hypothalamic somatostatin and IGF-I, which are stimulated by GH, are the main inhibitors of GH. GH is secreted in a pulsatile mode, and pulse frequency is affected by many factors such as diet, deep sleep, exercise, stress and fasting.

GH stimulates IGF-I synthesis by the liver, and IGF-I is one of the main regulators of muscle mass. IGF-I has receptors in a wide range of cell types, and receptor activation depends on IGF-I concentration in plasma, as well as on local production of this growth factor. GH administration increases serum IGF-I levels, skeletal muscle weight and muscle fibre cross-sectional area. However, in mice lacking IGF-I-receptor function in the skeletal muscle, GH fails to reverse the impaired muscle function [3]. These data indicate that in vivo effects of GH on muscle mass and strength are primarily mediated by activation of the IGF-I receptor. Regardless of the indirect anabolic effect of GH due to IGF-I, GH binds to its receptor in myocytes, activates Janus kinase 2 (JAK2) signalling and may have IGF-I-independent effects in the skeletal muscle [4]. GH and IGF-I have opposite metabolic effects. GH is lipolytic; increases free fatty acids in serum, which in turn inhibit glucose

uptake to muscle and other organs; and may induce hyperglycaemia and insulin resistance. On the contrary, IGF-I has lipogenic and hypoglycaemic effects.

Due to the pulsatile secretion of GH, it is not easy to determine modifications in GH secretion in muscle atrophy induced by different conditions. Nevertheless, GH deficiency syndrome and hypogonadism, at a young age, are associated with lower muscle mass, muscle strength and physical performance. The decrease in muscle mass and function in GH deficiency is reversible by GH administration. GH increases muscle strength by increasing muscle mass without affecting contractile force or fibre composition, an effect that is IGF-dependent [5, 6].

In several diseases such as sepsis, surgical diseases, chronic heart failure and critical illness, the increase release of cytokines is associated with liver GH resistance, decreased circulating IGF-I levels and muscle atrophy, despite normal or even elevated circulating levels of GH [7, 8]. Similarly, cachectic colorectal cancer patients, but not gastric cancer patients, have acquired GH resistance: high GH but low IGF-I levels are corrected by radical surgery [9]. However, as these authors pointed out, GH resistance induced by cancer is not universal but depends on the cancer type.

Among the inflammatory mediators that induced GH resistance, pro-inflammatory cytokines (mainly TNF-α, IL-1β and IL-6) have been shown to inhibit GH signalling [10, 11]. There are two mechanisms by which cytokines induce GH resistance, TNF-α and IL-1β downregulate GH receptor (GHR), whereas IL-6 upregulate the members of the suppressors of cytokine signalling (SOCS) family [12]. In addition to cytokines, in several chronic illnesses and/or organ injury, other mediators can induce GH resistance. Growth differentiation factor 15 (GDF15), also called MIC-1, is a member of the transforming growth factor-β (TGF-β) family of cytokines. Levels of GDF15 are low under healthy conditions, but it is upregulated by organ injury in several chronic diseases such as chronic obstructive pulmonary disease, sepsis, cancer, heart failure and chronic kidney disease [13–15]. Circulating GDF15 in turn acts on the liver to inhibit growth hormone (GH) signalling and IGF-I synthesis [16], therefore inducing muscle wasting.

On the other side, rheumatoid arthritis inhibits the GH-IGF-I axis both in experimental animals and in humans and induces muscle wasting [17, 18]. GH treatment in patients with juvenile idiopathic arthritis increases growth, as well as bone and muscle cross-sectional area [19]. These data can be explained by the fact that contrary to sepsis or other inflammatory diseases, arthritis does not induce GH resistance, since GH treatment is able to increase circulating IGF-I as well as IGF-I expression in the liver and in skeletal muscle [20, 21].

One of the endocrine changes associated with aging is the somatopause, or the continuous decline in plasma concentration of GH and IGF-I to very low levels [22]. This decrease in GH secretion contributes to sarcopenia, since GH administration is able to ameliorate the decrease in muscle mass secondary to aging [4, 23]. However, the risks related to GH therapy, such as cancer development, lead to safety concerns [24].

9.2.2 IGF-I and IGFBP-3

As mentioned above, most of the GH actions on the skeletal muscle are IGF-I-dependent, since GH upregulates IGF-I synthesis in the liver, and therefore it increases plasma concentrations of this growth factor. IGF-I is the main stimulator of skeletal muscle mass, since this hormone increases protein synthesis and decreases proteolysis. In addition, IGF-I increases satellite cell proliferation [25], as well as myoblast proliferation and differentiation during normal growth or regeneration after skeletal muscle injury. Therefore, the effects of IGF-I in the skeletal muscle result in an increase in skeletal muscle mass and in improving the functional capacity of muscle.

In addition to circulating IGF-I, local IGF-I also plays an important role in the maintenance of muscle mass acting as a paracrine/autocrine growth factor. Muscle produces a local supply of IGF-I that is secreted from the fibres to the extracellular matrix [26]. It has been reported that local infusion of IGF-I increases muscle mass [27] and that muscle injury or resistance exercise training upregulates local IGF-I and induces muscle hypertrophy [28, 29]. Furthermore, muscle atrophy is higher after ablation of muscle IGF-I production than when liver IGF-I production is inhibited [30], suggesting that local IGF-I is a crucial factor for muscle growth. All these data indicate that local IGF-I effects are important for muscle hypertrophy. Skeletal muscle cells, as other mechanosensitive cells, respond to mechanical stimuli by producing a special IGF-I isoform called mechano-growth factor (MGF) or IGF-1Ec in humans and IGF-IEb in rodents. This IGF-I isoform can play a role in muscle regeneration, since in basal conditions MGF levels in muscle are very low, but they increase after muscle injury [29].

IGF-I acts predominantly via the IGF-I receptor (IGF-IR), a transmembrane receptor with tyrosine kinase activity, and through the PI3K/Akt/mTOR/FoxO pathways, it activates protein synthesis and inhibits proteolysis (Fig. 9.2). MGF, the IGF-I isoform, is unable to activate Akt. Activated Akt phosphorylates and, thereby, prevents nuclear translocation of the FoxO (forkhead box class O factors) family of transcription factors (FoxO-1 and FoxO-3) that decrease the activity of the two main proteolytic pathways: the ubiquitin-proteasome system and autophagy. In addition, Akt activation increases glucose and amino acid uptake and via its actions on mTOR increases protein synthesis. The other signalling pathway activated by both IGF-I and its isoform MGF is the Ras/Raf/ERK pathway that is able to increase cell proliferation in muscle cell cultures [31]. The hypertrophic action of IGF-I on the skeletal muscle is exerted on activated satellite cells. Under IGF-I stimulation, satellite cells divide and then differentiate in myoblast and fuse to muscle fibres or form new fibres [26]. It has been speculated that MGF is responsible for muscle progenitor proliferation through ERK activation, whereas mature IGF-I promotes differentiation and protein synthesis [32] and simultaneously decreases the proteolytic pathways.

IGF-I action is regulated by six IGF-I-binding proteins (IGFBPs), which can either stimulate or inhibit the effect of IGF-I. IGFBP-3 synthetized by the liver is the

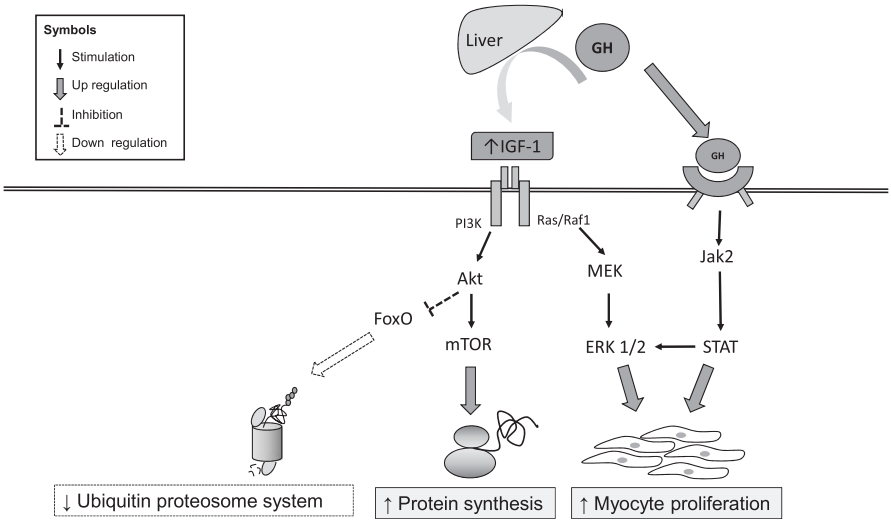

Fig. 9.2 Schematic representation of signalling pathways used by growth hormone (GH) and insulin-like growth factor-I (IGF-I) system to regulate the skeletal muscle. GH induces hepatic production of IGF-I. In muscle, GH activates JAK2/STAT pathway which transduce GH actions. The binding of IGF-I to its receptor in muscle can result in signal transduction via two pathways: PI3K/AKT and Ras/MEK. When activated, Akt stimulates protein synthesis through mTOR. Phosphorylation of FoxO by Akt inactivates this transcription factor, decreasing the activity of the proteolytic systems. The Ras/MEK pathway contains an elaborate kinase cascade that ultimately leads to stimulate myocyte proliferation
Akt protein kinase B, *ERK ½* extracellular signal-regulated kinases 1 y 2, *FoxO* forkhead box protein O, *mTOR* mammalian target of rapamycin, *MEK* dual specificity mitogen-activated protein kinase kinase, *PI3K* phosphatidylinositol-3 kinase, *STAT* signal transducer and activator of transcription

main IGF-I carrier in plasma. This protein is also expressed locally in tissues, where it binds to IGF-I and then impair the activation of its receptor IGF-IR. In addition to these effects, IGFBP-3 inhibits cell growth and promotes apoptosis by a non-IGF-dependent mechanism [33]. These and other data suggest that in the skeletal muscle, IGFBP-3 has opposite effect of IGF-I. Furthermore, IGFBP-3 can play an inhibitory role in the PI3K/Akt signalling pathway in different types of cells [34, 35]. In addition to their actions in muscle metabolism, GH and IGF-I also have different effects on the expression of the IGFBP-3. IGF-I significantly downregulates IGFBP-3 expression in the skeletal muscle, whereas GH is unable to modify the expression of this binding protein in the skeletal muscle [21].

Sepsis and acute inflammatory diseases induce GH resistance and decrease circulating IGF-I and its carrier protein IGFBP-3 by decreasing their synthesis in the liver [36–38]. However, in the skeletal muscle, IGF-I and IGFBP-3 expressions are affected differently, where muscle IGF-I is decreased by sepsis and IGFBP-3 is increased [39, 40]. IGFBP-3 is produced by myogenic cell cultures, and it suppresses proliferation in an IGF-dependent and IGF-independent manner [41]. Therefore, the

increased expression of IGFBP-3 in the skeletal muscle can contribute to inflammation-induced muscle wasting, together with the decrease in local IGF-I.

A decrease in circulating IGF-I and in muscle IGF-I has been reported in experimental cancer [42, 43]. Similarly, downregulation of muscle IGF-I expression has been observed in patients with gastric cancer [44]. These data, and others, suggest that downregulation of IGF-I is one of the causes of cachexia associated with certain, but not all, types of cancers. However, White et al. [43] detected in cancer cachexia a reduction in muscle IGF-I also during the first phases of cachexia progression, but not during the most severe period of wasting. It can be concluded that local IGF-I and IGF-I signalling in the skeletal muscle are inhibited during the initial phases of muscle atrophy, but not during the later stages of cachexia.

In rheumatoid arthritis, the decrease in circulating IGF-I has been reported as one of the causes of rheumatoid cachexia [45]. The decrease in plasma IGF-I in humans and in experimental animals with arthritis correlates with the disease severity, the decrease in body weight and muscle atrophy [17, 46]. In arthritic rats muscle IGF-I was not decreased [47], and systemic IGF-I administration was able to increase body and muscle weight [48]. These data indicate that the decrease in muscle mass seems to be secondary to the circulating IGF-I, rather to a decrease in muscular IGF-I. However, it is also possible that the increased IGFBP-3 expression observed in the skeletal muscles of arthritic animals [47] contributes to the inhibitory effect of arthritis on gastrocnemius mass by preventing IGF-I action, since IGF-I administration normalizes the increased IGFBP-3 levels in muscle [48].

Chronic heart failure is associated with exercise intolerance, decreased muscle strength and peripheral muscle wasting [49]. There is consensus that local IGF-I is downregulated in the skeletal muscle of patients with chronic heart failure [50, 51]. Furthermore, some authors found that exercise training programs reduced pro-inflammatory cytokines, increased local IGF-I production and attenuated muscle atrophy [50, 52]. However, the effect of this disease on circulating IGF-I is not very clear, since increased GH levels with normal or decrease IGF-I were reported [50, 53].

Although skeletal muscle disuse, immobilization or microgravity decrease muscle mass and strength, these atrophies are not associated with systemic changes in circulating hormones but rather with alteration in local anabolic factors such as IGF-I synthetized in muscle [54]. In this sense, local IGF-I injection is able to block disuse atrophy [55]. In contrast to muscle disuse, the decline observed during aging in circulating IGF-I plays a role in the development of sarcopenia. A decrease in IGF-I levels in plasma has been reported in sarcopenic women and men [56, 57]. As mentioned above, this decrease is secondary to alterations in GH secretion, but not to GH resistance, since GH treatment is able to ameliorate sarcopenia associated with aging. Similarly, low IGF-I levels during the chronic phase, but not during the acute one, of critical illness are less likely to be caused by GH resistance because they are not accompanied by elevated GH secretion and correlate positively with pulsatile GH secretion [58].

9.3 The Adrenal Axis: Glucocorticoids

Secretion of glucocorticoids (GCs) by the adrenal cortex belongs to the classical hypothalamus-pituitary-adrenal (HPA) axis. Corticotrophin-releasing hormone (CRH) is released from the paraventricular nucleus (PVN) of the hypothalamus and induces the release by the pituitary corticotrophs of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH stimulates cortisol (the main GC in humans) synthesis by the adrenal gland. This activation cascade is regulated by cortisol through negative feedback on hypothalamic CRH and on ACTH in the anterior pituitary [59–61]. Cortisol is secreted following a circadian rhythm, with the highest concentrations in the morning and the lowest levels at night. Cortisol acts by binding to the intracellular glucocorticoid receptor (GR), virtually expressed in all cells. The physiological actions of cortisol range from the suppression of inflammation regulating the immune system to the control of energy homeostasis (supplying enough glucose into the circulation for the brain); GCs ensure the survival of the organism in response to stress situations and in conditions of metabolic dysfunction, including fasting and starvation, insulin resistance, obesity-related diabetes and cachexia [62].

Multiple pathological conditions characterized by muscle wasting (sepsis, cachexia, starvation, chronic obstructive pulmonary disease, diabetes, acidosis, cancer, etc.) are associated with increased GC levels, suggesting that these hormones may contribute to muscle atrophy observed in different pathological states [63–66]. In addition, high doses and sustained treatment with GCs in a variety of inflammatory diseases represent an additional modus by which GC triggers muscular atrophy in humans and animals [67].

GC-induced muscle atrophy occurs predominantly in glycolytic muscles with fast-twitch (type II) muscle fibre more than in oxidative muscles composed by slow-twitch fibre (type I). In muscles with mixed fibre type, such as gastrocnemius muscle, type II fibres show greater atrophy than type I. This specificity by fast-twitch muscle atrophy comes from the vital role of slow-twitch muscle in maintenance of posture and respiration [68].

GCs induce muscle atrophy both decreasing the rate of protein synthesis and increasing the rate of protein degradation in the skeletal muscle. It is possible that GCs also alter angiogenesis producing a decrease in capillary number that could be related to skeletal muscle atrophy [69, 70]. In addition, GCs inhibit in the muscle the local production of IGF-I and the action of anabolic stimuli, such as insulin and IGF-I, and induce a decline of the amino acid-mediated signalling pathways involved in the control of muscle protein synthesis. The reduction in anabolic activity results from different mechanisms that converge to inhibit mTOR [71]. Several evidences indicate that GCs inhibit the PI3K/Akt pathway, which mediates the anabolic actions of insulin/IGF-I [72–74].

Several mechanisms are involved in GC-induced muscle protein degradation (Fig. 9.3). Firstly, GCs have been reported to stimulate atrogenes via the transcriptional factors FoxO [19] and the NF-κB (nuclear factor-kappa B) pathway. Secondly,

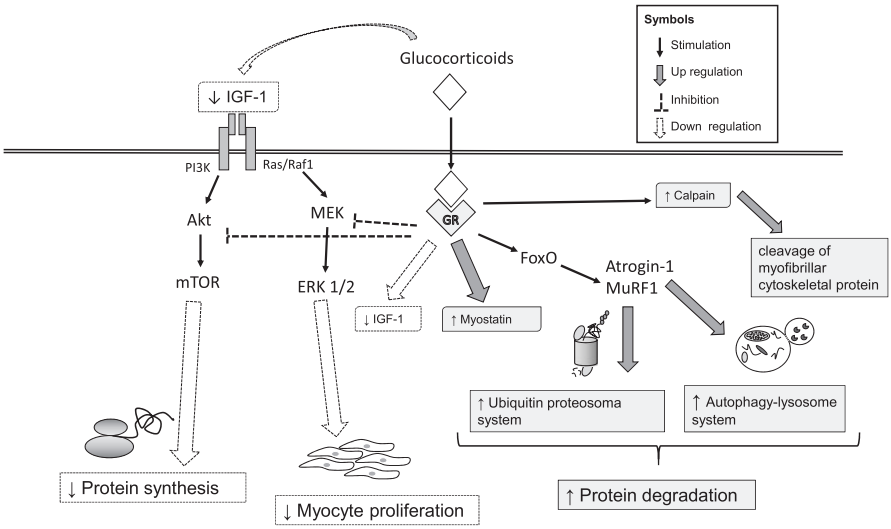

Fig. 9.3 Mechanism of action of glucocorticoids (GCs) on muscle. GCs interact with cytosolic glucocorticoid receptor (GR) and induce muscle atrophy mainly increasing protein breakdown and decreasing protein synthesis (by the inhibition of the local production of IGF-I and/or his action). Catabolic effects of GCs in muscle are mediated by specific transcription factors including FoxO family. Activation of these transcription factors upregulates atrogene expression (atrogin-1 and MuRF1). GCs also promote protein degradation via the induction of myostatin and the calpain proteolytic pathway

Akt protein kinase B, *ERK ½* extracellular signal-regulated kinases 1 y 2, *FoxO* forkhead box protein O, *IGF-I* insulin-like growth factor-I, *mTOR* mammalian target of rapamycin, *MEK* dual specificity mitogen-activated protein kinase kinase, *MuRF1* muscle RING-finger protein-1, *PI3K* phosphatidylinositol-3 kinase

GCs promote protein degradation via the induction of myostatin (a negative regulator of skeletal muscle development) [75–77]. As it has been mentioned, GC-induced muscle atrophy occurs predominantly in fast-twitch muscle fibres, which appear to have much higher myostatin gene expression [78]. Myostatin induces muscle wasting partly by activating the ubiquitin proteolytic system by downregulating the IGF-1/PI3K/AKT hypertrophic signalling pathway. This results in upregulation of atrogenic gene expression and inactivation of protein synthesis. In addition, myostatin inhibits the myogenic program by activating the SMAD complex and by MAPKs, thus resulting in a decrease of myoblast proliferation [79]. Recently, evidence has accumulated supporting that GCs act via a posttranscriptional mechanisms (such as microRNA miR-27a processing) to regulate myostatin expression [80]. Thirdly, GCs stimulate both the autophagic/lysosomal pathway [81] and the calpain pathway [82]. Autophagy-lysosome system is transcriptionally controlled through the expression of FoxOs [83]. FoxO3 is a critical factor for autophagy control in adult muscles [84]. MAPK pathway is also able to regulate the expression of autophagy-related genes independently of FoxO3 in cachectic muscle wasting [85]. The GC effect on calpain pathway could be mediated by calpeptin, a calpain inhibi-

tor, since it is able to block the dexamethasone-induced proteolysis [86]. In addition, Hayash et al. [82] reported that corticosterone administration increases calpain activity in muscle. A crosstalk between catabolic and anabolic processes in the skeletal muscle has been proposed [87]. In this sense, activation of the proteolytic systems by GCs stimulates the branched-chain amino acid degradation, which is believed to activate mTOR, and therefore indirectly inhibits mTOR-dependent protein synthesis.

Several experimental models have been used to investigate the effects of sepsis: peritonitis produced by caecal ligation and puncture, LPS administration on the skeletal muscle mass, etc. Sepsis, endotoxaemia and other acute/chronic inflammatory conditions are characterized by an increase in inflammatory cytokine production and a rapid and sustained elevation in GC levels. Although pro-inflammatory cytokines, in particular, TNF-α, IL-1β and IL-6, are sufficient to induce muscle atrophy [88], the increased GC levels evoked by inflammatory challenge are enough to induce atrophy [63]. Mice with specific deletion of the glucocorticoid receptor in muscle are more resistant to skeletal muscle atrophy induced by sepsis than control animals [89], which indicate that GCs are determinant in the inflammation-induced muscle atrophy. Furthermore, GCs by themselves have a direct effect in the skeletal muscle activating FoxO1 both in vivo and in vitro [90, 91]. In sepsis induced by caecal ligation and puncture, Wray et al. [92] reported that the glucocorticoid receptor antagonist RU-486 inhibits the upregulation of MuRF1 and atrogin-1/MAFbx in septic rats, supporting the important role of GCs for the development of muscle wasting. By contrast, Frost et al. [93] reported that sepsis-induced increase in muscle atrogin-1 and MuRF1 mRNA appears to be GC-independent, since pretreatment with RU-486 failed to ameliorate the sepsis-induced muscle atrophy. An explanation for this discrepancy is the use of smaller, immature rats in the first study versus adult rat in Frost et al. experiment. It is interesting to note that both studies report that fast-twitch muscle is more sensitive to the effects of sepsis than slow-twitch muscle.

In cancer, the role of GCs has been analysed in several studies. Braun et al. [89], using mice with specific deletion of the glucocorticoid receptor, demonstrate that GCs play a critical role in the pathogenesis of cancer muscle atrophy. Conversely, previous studies [94, 95] that utilized the glucocorticoid antagonist RU-486 in models of cancer did not demonstrate a significant protection of muscle mass, probably because RU-486 has only a 2 h half-life in rodents. In addition, it is also possible that muscle wasting in some tumour models depends on GCs, while others do not.

In chronic diseases, heart failure, chronic kidney disease (CKD) and chronic obstructive pulmonary disease (COPD), despite the diverse nature of these illnesses, they all seem to increase muscle proteolysis, primarily through the ubiquitin-proteasome system. The increased proteolysis and rapid muscle loss in these pathologies require GCs [96]. However, patients with CKD also have high levels of TNF-α, IL-6 and myostatin that seem to contribute to muscle loss. In these chronic diseases, the increase activity of the renin-angiotensin system also plays a critical role in skeletal muscle wasting. Alternately, in COPD increased myostatin expression has been reported in muscle of COPD patients with stable disease. Taking into

account that GCs increase myostatin expression [75], this could be one of the ways by which GCs trigger muscle atrophy.

Although it is unclear whether aging is associated with increased GC secretion [64], Waters et al. [97] reported that sarcopenic elderly persons have an increase in cortisol production compared with normal lean group. In women, Hassan-Smith et al. [98] have described that skeletal muscle 11β-hydroxysteroid dehydrogenase type 1 is upregulated with age and is associated with sarcopenia. This enzyme converts inactive GCs to their active form (cortisone to cortisol in humans). This increase of cortisol at the level of the skeletal muscle may contribute to the development of sarcopenia. In addition, GCs seem to be implicated in the delayed muscle mass recovery following a catabolic state in aged people. Muscle atrophy in old rats was due to depressed protein synthesis. In this sense, GCs induce a prolonged leucine resistance on muscle protein synthesis in old rats [99].

Type 1 diabetes mellitus (T1DM) arising from insulin deficiency is a catabolic state characterized by an increased protein degradation rate that produces an accelerated muscle atrophy [100]. Hyperglycaemia and hypoinsulinaemia play key roles in reduced muscle growth or increased proteolysis. GCs are one of the factors that contribute to muscle protein breakdown. Adrenalectomy blocks muscle loss in diabetic animals suggesting that GCs are necessary for stimulating muscle proteolysis. A combination of deficient insulin signalling and activation of the GCs in muscle decreases insulin receptor substrate (IRS), IRS-associated PI3K and p-Akt activities, leading to accelerated muscle wasting [73]. GCs and insulin pathways interact to modulate the anabolic and catabolic balance in the skeletal muscle. Endogenous GCs alone do not stimulate muscle protein breakdown; therefore a rise in GCs increases insulin to overcome proteolytic responses to GCs.

GCs do not appear to be required for disuse [101] or denervation-induced atrophy [102], bed rest or microgravity [103]. However, hypercortisolaemia may exacerbate bed rest-induced atrophy and functional loss in soleus type I fibres [104].

Paradoxically, in spite of muscle weakness and atrophy in response to GCs, chronic GC steroids are used to treat Duchenne muscular dystrophy with beneficial effects on muscle strength and function [105]. The positive effects of steroid treatment seem to depend on steroid dosing. Intermittent administration promotes muscle repair and increases muscle mass [106]. In addition, a low dose of GCs inhibits muscle inflammation, reduces fibre necrosis and increases myogenesis and low-dose inhibited muscle inflammation, reduced fibre necrosis and increased myogenesis [107].

9.4 Gonadal Steroids

Androgens and oestrogens are the main steroid hormones secreted by the testes and ovaries, respectively. They are essential for sexual and reproductive development and are regulated by the hypothalamic-pituitary-gonadal axis. The hypothalamus releases gonadotropin-releasing hormone (GnRH) that stimulates in the

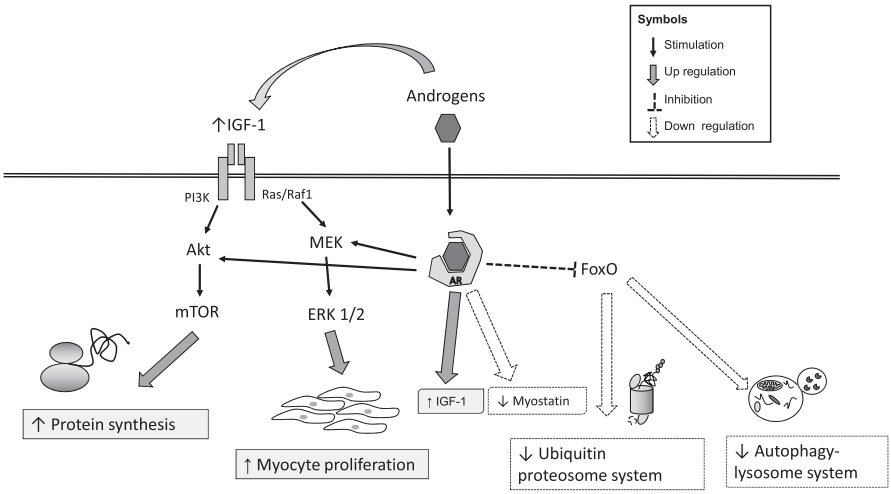

Fig. 9.4 Androgens (testosterone) activate PI3K/Akt signalling, either directly or through IGF-I stimulation. Activation of Akt leads to phosphorylation and activation of mTOR that increases protein synthesis. Androgen receptor activation (AR) leads to phosphorylation and inhibition of FoxO transcription factors, which are required for upregulation of the ubiquitin-proteasome system and autophagy lysosome, decreasing protein degradation. Testosterone also inhibits myostatin, which represses protein synthesis and increases muscle atrophy
Akt protein kinase B, *ERK ½* extracellular signal-regulated kinases 1 y 2, *FoxO* forkhead box protein O, *IGF-I* insulin-like growth factor-I, *mTOR* mammalian target of rapamycin, *MEK* dual specificity mitogen-activated protein kinase kinase, *PI3K* phosphatidylinositol-3 kinase

adenohypophysis the secretion of the two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both gonadotropins are essential for gonadal steroidogenesis and for gamete production. Steroid hormones released by the male and female gonads are the regulatory factors of the hypothalamic-pituitary-gonadal axis by negative feedback. Androgens and oestrogens, in a lesser extent, have a profound impact on muscle physiology and metabolism. They are involved in the process of growth, maintenance and repair of muscle mass [108]. Although both, oestrogens and androgens, have positive effects on muscle, there are important differences in the effects of either class of steroids on the skeletal muscle. In this sense, androgens have a predominant role in the regulation of muscle physiology in both sexes [108].

Sexual steroid action is mediated by intracellular receptors (AR and ER for androgens and oestrogens, respectively). These receptors are expressed in myocytes and satellite cells [108]. One of the mechanisms by which androgens activate myocyte growth is increasing the expression of muscle IGF-I [109]. In addition, androgens activate the expression of the IGF-I receptor, the downstream signalling (e.g. Akt) [110], and finally they activate the mTORC1 pathway [111] (Fig. 9.4). In this sense, it has been described a decline of the mTOR signalling pathway after castration in rodents and that the treatment with androgens restored the levels to those of sham-operated animals [112]. Androgens can also act independently of IGF-I path-

ways, stimulating directly myotube hypertrophy but not differentiation [110]. It has been reported a direct stimulation of Ras/MEK/ERK pathway by testosterone in muscle cells [113] and a suppression of the myostatin expression [114]. Testosterone has also a potent antiapoptotic effect in muscle, it maintains FoxO element inactivated, and it counteracts the upregulation of proapoptotic genes induced by H_2O_2 [115]. In this way, androgens can influence muscle mass decreasing protein breakdown and autophagy; the lack of these steroids increases those processes, and the replacement of the hormones reverts those effects [111]. Oestrogens act likewise activating the Akt/mTOR pathway [116, 117] and play an important role in muscle development by activating the p38/MAP pathway [118], but no clear actions of oestrogens were observed in the ERK signalling [118, 119] nor in the FoxO family factors [120], and apparently they have no effect on myostatin expression [120].

Knowing the important role of sexual hormones on muscle growth, low circulating levels of these steroids (physiological, pathological or medical treatment related) have a significant impact on muscle proliferation and maintenance. Men with hypogonadism have lower muscle size and strength [121]. Furthermore, individuals with low levels of androgens, as in androgen deprivation therapy for prostate cancer, showed an important decline in muscle strength and function [122].

In major illness, a decreased in testosterone levels, secondary to a decrease in gonadotropin secretion, has been reported [123]. The role of androgens in cancer cachexia is not well known because, as mentioned above, the causes, incidence and severity of cachexia can vary according to tumour type, site and mass. Nevertheless, hypogonadism is observed in the majority of patients with metastatic cancer and cachexia [124, 125]. Similarly, the decrease in testosterone levels, observed in COPD patients, can be one of the factors that contributes to the muscle atrophy and disability reported in those patients [126]. In muscle wasting induced by heart failure, a decrease in anabolic hormones such as testosterone and IGF-I has been reported [53].

The decline of oestrogens and androgens in aging contributes to the loss of muscle mass in sarcopenia [108]. In neuromuscular diseases, such as Duchenne muscular dystrophy, it has been described the positive effect of androgen receptor agonist treatment increasing the muscle mass [127]. In the same way to androgens, the replacement of oestrogens in ovariectomized rat models has positive effects on muscle contractile function and on proliferation of satellite cells [128–130]. In women, the age-associated muscle loss and accumulation of fat in muscle are dismissed by hormone replacement therapy [131], and a meta-analysis showed beneficial effects of oestrogens on muscle strength [132].

9.5 Thyroid Hormones

Thyroid hormone (TH) secretion is regulated by thyroid-stimulating hormone (TSH) or thyrotropin produced in a pulsatile fashion by the pituitary thyrotrope cells. TSH release is under the stimulatory control of the hypothalamic

thyrotropin-releasing hormone (TRH). The thyroid gland mainly produces tetraiodothyronine (T4) or thyroxine, but the biological activity of this thyroid hormone is exerted by triiodothyronine (T3). In target cells, T4 is modified by deiodinating enzymes (D1, D2 and D3). D1 and D2 convert the prohormone thyroxine (T4) into the active hormone T3 by outer-ring deiodination. In contrast, D3 converts T4 into the biologically inactive compound reverse T3 (rT3). Thyroid hormones control gene expression in various tissues by binding to nuclear thyroid hormone receptors that heterodimerize with the retinoid X receptor. Hypothalamic TRH and pituitary TSH secretions are controlled by thyroid hormones by a negative feedback [133]. The skeletal muscle is a principal target of TH, which is involved on contractile function, regeneration and skeletal muscle metabolism. T3 treatment increases maximal oxygen consumption, promotes appropriate muscle responsiveness to insulin and stimulates oxidative pathways by increasing mitochondrial biogenesis [134]. TH not only increase the number and diameter of muscle fibres, but they also participate in the determination of the normal pattern of fibre distributions in each muscle [135]. However, both an excess [136] and a deficiency of TH [137] cause muscle wasting and are detrimental for muscle regeneration [135].

Critical illness, sepsis and chronic inflammation are associated with changes in TH metabolism that can lead to altered muscle function. In the skeletal muscle, concentrations of TH depend on local levels of TH transporters, TH receptors and the activity of deiodinases. D2 and D3 have been identified in the skeletal muscle. Deiodinase, THR and TH transporter expressions are modulated in muscle during acute and chronic systemic inflammation [138].

The hypothalamic-pituitary-thyroid axis response is different in the acute phase of critical illness than in the prolonged one. The initial response of the thyroid axis is referred as “nonthyroidal illness syndrome” [139]. In this disease, the most typical alterations in plasma are low T3, low or normal T4 and elevated rT3 levels, together with normal TSH levels [138, 139]. This decrease in T3 and in T3/rT3 ratio could be the result of concomitant anorexia and fasting, rather than the illness per se. A combination of reduced serum T3 and T4 levels indicates poor prognosis in critically ill patients. Therefore, several investigators proposed T3 and/or T4 treatment to counteract this situation, but no beneficial and sometimes even harmful effects were observed (for review [140]). Since the skeletal muscle has the ability to store glucose, and houses nearly 75% of all protein in the body, muscle breakdown and atrophy in critically ill patients are proposed as physiological adaptations to save energy during acute illness. In these situations, the reduction of anabolic response in muscle mediated by the decreases in TH concentrations could favour energy preservation during illness. Through this response TH protect the organism against hypercatabolism, prevent muscle weakness and improve recovery.

In patients with prolonged critical illness, low plasma T4 concentrations and low T3 levels linked with low TSH secretion and hypothalamic TRH have been described. These data indicate central hypothyroidism with a lack of hypothalamic TRH-mediated stimulation of the thyrotropes with suppressed TSH-mediated activation of the thyroid gland [141]. It remains unclear the mechanism implicated in this response, but it has been proposed an increased in the expression of D2 in the hypo-

thalamus, which may increase the T3 supply to the TRH neurons, thus decreasing TRH secretion [142]. The skeletal muscle of patients suffering from prolonged critical illness adaptates to the low production of TH, increasing the local thyroid hormone receptor, thyroid hormone transporters and local activation of D2 [139, 141].

9.6 Other Hormones Related with Muscle Atrophy in Metabolic-Altered States

Secretion of hormones such as leptin, insulin and vitamin D is altered in several diseases related with metabolic dysfunction such as diabetes mellitus, obesity and aging [143, 144]. Therefore, they also play an important role in the muscle atrophy observed in these conditions.

Leptin is a hormone mostly secreted by the white adipose tissue, and it has a main role regulating energy balance [145]. Its level in blood depends on the fat stores and acts in the hypothalamus stimulating anorexigenic pathways and increasing energy expenditure [146]. Besides its central actions, this hormone has important peripheral actions, specifically on the muscle. Leptin actions are mediated by its receptor, the long form of leptin receptor (ObRb). This receptor contains intracellular motifs required for activation of the JAK/STAT signal transduction pathway, one of the main signalling cascades activated by leptin [147]. The ObRb receptor is expressed mainly in the brain, but it can be found in other peripheral tissues such as the liver, pancreas, adipose tissue and skeletal muscle [148]. In the muscle, leptin has an important role stimulating myoblast proliferation and differentiation [149, 150]. In addition, leptin inhibits muscle atrophy [151]. These actions of leptin are direct on the skeletal muscle, but also leptin can stimulate muscle growth indirectly by increasing both circulating and muscle-derived IGF-I [152, 153]. Both hypoleptinaemia and leptin insensibility are main factors related with the muscle wasting observed in malnutrition, anorexia, obesity and aging [143, 154, 155]. In this sense, treatment with leptin during aging has been proposed as a method to prevent sarcopenia [156].

Insulin, whose main role is the maintenance of glucose homeostasis, has also an important role in muscle growth. This hormone acts through an intracellular signalling pathway similar to that of IGF-I. Tyrosine phosphorylation of insulin receptor substrates (IRSs) leads to the activation of PI3K/AKT and ERK pathways. Both pathways activate muscle growth and protein turnover [157]. Hypoinsulinaemia (TIDM) and insulin insensitivity (obesity, TIIDM and aging) are also associated with muscle atrophy [100, 158]. A diabetic environment increases protein degradation in muscle [159], and it has been described diabetes mellitus as one of the major endocrine causes of sarcopenia [144]. In metabolic syndrome, characterized by abdominal obesity, hypertension, hyperglycaemia and hypertriglyceridemia, both leptin and insulin insensitivities are present, and muscle proliferation is impaired [143].

Vitamin D is a hormone with a main role in calcium homeostasis and bone metabolism. However, recently it has been related with skeletal muscle physiology [160]. Deficiency in vitamin D is associated with muscle weakness [161–163], and treatment with vitamin D seems to have a positive impact on muscle strength and mass [164, 165]. Aging and obesity are two conditions in which it has been described low levels of vitamin D, and treatment with this hormone has beneficial effects on the associated sarcopenia [166, 167]; thus, it can be assumed that vitamin D has an important role in muscle function and development.

Angiotensin II, a hormone involved in blood pressure control, may also play a role in skeletal muscle atrophy. Infusion of this hormone induces skeletal muscle atrophy by increasing proteolysis and decreasing both circulating and local IGF-1 [168]. In fact, angiotensin II has an inhibitory effect on the autocrine IGF-I system [169]. It has also been reported that angiotensin II increases hormones such as GC and myostatin and pro-inflammatory cytokines (TNF-α, IL-6) that contribute to the muscle atrophy. The renin-angiotensin system is activated in many catabolic conditions, and it has been suggested that angiotensin II is an active participant in the skeletal muscle wasting [170]. Congestive heart failure and chronic kidney disease are characterized by increased levels of angiotensin II and cachexia. In these illnesses, angiotensin-converting enzyme (ACE) inhibitor treatment improves the muscle loss [171]. Other situations in which angiotensin II is increased and may meditate skeletal muscle atrophy are obesity and aging in which the treatment with ACE inhibitors and angiotensin II receptor blockers showed beneficial effects on muscle [143, 172]. Therefore, the blockade of the renin-angiotensin system has been proposed as novel therapeutical tool for muscle atrophy [173].

9.7 Final Remarks

In summary, skeletal muscle atrophy is associated with a large assortment of conditions ranging from disuse or immobilization to chronic catabolic states that courses with cachexia. It is evident that muscle wasting is a complex and multifactorial condition and can be attributed to the complex interactions among several factors including alterations of the endocrine system. The correction of certain hormonal derangements may facilitate the development of improved hormone-mediated therapies for muscle-wasting conditions. Hormonal supplementation with growth hormone, leptin, testosterone or vitamin D could be possible therapeutic strategies, but their efficacy and safety need to be definitively established through larger-scale trials.

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Chapter 10
Ubiquitin-Proteasome Pathway and Muscle Atrophy

Rania Khalil

Abstract Many systemic diseases are featured by muscle atrophy. Cellular proteins are modified by covalent attachment to a small protein known as ubiquitin (Ub) through ubiquitination. This ubiquitination process serves as signal for protein turnover that leads to rapid muscle mass lack. This process is carried out through an enzymatic cascade, which includes three groups of enzymes termed ubiquitin E1 (activating enzyme), ubiquitin E2 (conjugating enzyme), and ubiquitin E3 (ligase). There are several ways of ubiquitin conjugation driving to ubiquitination of specific proteins through ubiquitin-proteasome system (UPS). A lot of UPS genes stated to be included in skeletal muscle atrophy. These genes do their effects by modifying different processes which affect muscle mass including myofibrillar protein degradation, myogenesis inhibition, and even modulation of autophagy as well as upstream regulatory pathways.

Keywords Muscle atrophy · Signal pathways · Ubiquitin · Ubiquitin ligases · Ubiquitin-proteasome system

10.1 Ubiquitin Ligases

Many systemic diseases are commonly featured by weakness through rapid atrophy of muscle that occurs in muscles upon disuse or nerve injury. These diseases include diabetes, cancer, sepsis, hyperthyroidism, and uremia [56]. A rapid lack of muscle mass and protein content occurred through general set of biochemical changes that described different types of muscle atrophy leading to an increase in the overall rate of breakdown of muscle proteins [54].

Many catabolic conditions have been distinguished by activation of protein degradation in muscle through a short protein containing 76 amino acids known as ubiquitin (Ub) which present in mainly all tissues of eukaryotes [6]. Cellular proteins are modified through covalent attachment to this Ub protein by cellular

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regulatory mechanisms called ubiquitination. The ability of the 26S proteasome complex to recognize ubiquitin chains attached to proteins can explain how cellular proteins can be targeted by ubiquitination for degradation. This protein system breakdown through proteasome complex is recognized as ubiquitin-proteasome system (UPS) [55].

There are four main endogenous proteolytic systems in which UPS is considered as one of them in vertebrates. UPS presents an important function in recycling of amino acids, controlling muscle protein turnover, or using it for energy production, as well as other roles in myogenesis. The other systems of proteolysis include cathepsins, calpains, and caspases [60].

The ubiquitination process serves as signal for protein turnover which is carried by an enzymatic cascade that involves three groups of enzymes termed ubiquitin E1 (activating enzyme), ubiquitin E2 (conjugating enzyme), and ubiquitin E3 (ligase enzyme). For some substrates, fourth enzyme, E4, lengthens short ubiquitin chains [61].

First, an ubiquitin E1-activating enzyme stimulates the carboxylic-terminal edge of ubiquitin by forming the highly reactive thiol-ester bond between it and a cysteine residue in the active site of the enzyme. The activated ubiquitin is secondly transferred onto the active-site cysteine residue of an ubiquitin E2 conjugating enzyme. Then E2 interacts with an ubiquitin E3 ligase that binds to the substrate [59]. Finally, E3 boosts the transfer of the ubiquitin onto the substrate. After recognition by the proteasome, the ubiquitin chains are elevated by deubiquitinating enzymes to permit ubiquitin recycling for reuse in other new conjugation responses [62].

In this process of ubiquitination, E2 acts as specific Ub-carrier protein responsible for attaching Ub to protein substrates. On the other hand, Ub-E3 protein ligase is considered as the key enzyme that catalyzes this boost of an activated form of Ub [29]. Ubiquitination role is not limited to act as the main contributor between the three protein degradation pathways by targeting substrates to the proteasome, the lysosomal system, and the autophagosome but also adjusts key cellular approaches including cell cycle progression, gene transcription, DNA repair, virus budding, receptor endocytosis, and apoptosis [64].

In spite that UPS is the main proteolytic pathway accountable for disposal of the damaged proteins, which accumulate in skeletal muscle; it is actually associated with enhancing of atrophy of skeletal muscle through its over-activation [18]. A lot of evidences showed that increased UPS expression may be occasional for skeletal muscle atrophy. The transient highly regulated manner for the ability of the UPS to target specific proteins for degradation arises from the large number of genes involved in regulating the state of protein ubiquitination [2].

The genes that are reported to be regulated in skeletal muscle wasting in the ubiquitin-proteasome system are nearly 35 E2s, nearly 750 E3s, and nearly 90 deubiquitinating enzymes known as atrogenes. These atrogenes spend their effects by modulating the various processes that determine muscle mass (myogenesis, protein synthesis, and degradation) as well as the upstream regulatory pathways [11]. These USP genes can be grouped into three functions: (1) myofibrillar protein degradation,

Table 10.1 Examples on some ubiquitin ligases and their related genes and conjugated enzymes

Ubiquitin genes	Ubiquitin-conjugated enzymes	Ubiquitin ligases
UbB	$E2_{14K}$/HR6B/UBC2	MAFbx/Atrogin-1
UbC	$E2_{20K}$ (203) UBC4/UBC5	MuRF1
UbA52		Cb1-b
UbS27A		E4
		E3α/UBR1
		E3α-II/UBR2UBR3

(2) myogenesis inhibition, and (3) autophagy modulation. Table 10.1 represents the list of genes in the UPS that are stated to be regulated in skeletal muscle atrophy [19].

10.1.1 UPS Genes Modulate Myofibrillar Protein Degradation

One of the first UPS genes oncoming to be fundamental for muscle atrophy is the ubiquitin-E3ligase muscle ring finger-1 (MuRF1). MuRF1 role occurs through myofibrillar proteins, which has been implicated in the myofibrils degradation. Actually, it links to and ubiquitinates myosin light chains 1 and 2, myosin heavy chain, and myosin-binding protein C and also troponin I. In spite that the effect of MuRF1 on troponin was monitored in non-muscle cell lines but could be pertinent also in skeletal muscle [50]. Experiments carried out on mutant form of MuRF1 support a certain role for it in targeting thick filaments for degradation. [13] stated that mice expressing a prevalent negative mutant form of MuRF1 showed degradation of thin filament with a simple loss of thick filaments in response to denervation, suggesting that MuRF1 is not involved in targeting of thin filaments [13].

On the other hand, thin filaments in other experiments have been found to be targeted by MuRF1 in vitro in cultured cells. Otherwise, exogenous corticosteroids lead to ubiquitinating actin. This conflict is explained by experiments carried out on purified monomeric actin that stated actin ubiquitinated by MuRF1 in vitro only, but the degradation of actin is independent of MuRF1 when present in myofibrils [14].

In addition to that, MURF1 gene is considered as the most important UPS component which precedes in vivo special function in skeletal muscle atrophy; several studies reported that MURF1 is downregulated in cardiac and skeletal myopathy [3, 4, 45, 58].

Another ligase which is known as muscle atrophy F-box/Atrogin-1 (MAFbx/atrogin-1) also ubiquitinates desmin. Also, intermediate filament protein, vimentin, is targeted by MAFbx/atrogin-1 in which vimentin is also associated with sarcomere Z-disk. These actions on Z-line proteins by ubiquitin ligases propose that ubiquitination plays presumed roles in mediating both the degradation of the filament proteins and the disassembly of myofibrils, leading to loss of muscle function through the loss of muscle mass and strength [9].

Subsequently, muscle atrophy treatment could be based on inhibition of UPS gene expression through inhibition of the two identified ligases MURF1 and MAFbx/atrogin-1. The treatment depends on the responsibility of these genes for the elevation of protein degeneration through the ubiquitin-proteasome system and is consistent in different models of muscle atrophy [7]. Recently, Khalil et al. [37] observed a significant decrease of MURF1 gene expression in muscle atrophied animals treated with taurine. Otherwise, a possible decrease of MURF2 and 3 activities in ischemic reperfusion injury was reported since 1977 by Crass and Lombardini [16].

10.1.2 UPS Genes Regulate the Myogenesis

The process whereby muscle satellite stem cells with positive Pax7 are stimulated to turn into proliferating myoblasts with positive MyoD is known as myogenesis. Pax7 expression is widely used as marker that approved to be ubiquitously expressed. On the other hand, myogenic activation could be detected by MyoD which is a basic helix-loop-helix transcription factor and is one of the four myogenic regulatory growth factors required for myogenesis [28]. The myoblasts that proliferated through myogenesis subsequently induce myogenin that fuse to form multinucleated myotubes. After birth, the myoblasts are going to fuse with the present myofibers, which is important for early life muscle growth. Under normal conditions, myofiber maintenance is not dependent on myogenesis; otherwise, upon subsequent aging, inducible depletion of muscle satellite stem cells in young adult does not affect muscle mass [47]. Also, mechanical loading-induced hypertrophy is not dependent on myogenesis. However, impaired myoblast fusion occurs in cancer cachexia that plays a significant role in the pathogenesis of the muscle atrophy, providing evidence that ongoing myoblast fusion is important in myofiber maintenance under catabolic situations [48].

Impairing myoblast fusion and differentiation is also one of the atrogin-1 ligase effects and, in addition to its effect on intermediate filaments, ubiquitinates and targets MyoD for degradation. The atrogenic effect of atrogin-1 appears to be mediated by this ubiquitination that is approved through mutant mice with a MyoD engineered to be resistant to atrogin-1 ubiquitination that is found to be significantly protected against muscle atrophy [52]. Also, atrogin-1 can inhibit fusion and expression of myofibrillar proteins through ubiquitinating myogenin. These effects are likely considered to myoblasts, while, in whole muscle both myogenin and MyoD are induced upon denervation. Actually, the promoters of MuRF1 and atrogin-1 are activated by this induced myogenin that is required for atrophy [22].

Another ubiquitin E3 ligase tripartite motif protein 32 (Trim32) participated also in myogenesis-dependent disuse atrophy. Its ubiquitination of the transcription fac-

tor NDRG2 is thought to cease the effect [44]. Trim32 role in egression from cell cycle and myogenesis, respectively, could be indicated from NDRG2 absence which leads to upregulation of cell cycle inhibitors and markers of differentiation. NDRG2 is confirmed to be phosphorylated by protein kinase B (Akt) and may intercede the myogenic-promoting activity of insulin and IGF-I [40].

The role of tripartite motif protein 72 (Trim72) is also studied in promoting myogenesis by its capability to modify fusion and myogenin expression. It aims to the focal adhesion kinase (FAK) for degradation, and FAK has been noticed to enhance the expression of the profusion genes caveolin-3 and 1D-integrin as well as myogenin [33].

Additionally, the expression of myogenin and myofibrillar proteins in muscle cells could be modulated by the USP19 deubiquitinating enzyme. This represents also the ability of USP19 to regulate muscle cell differentiation. Otherwise, USP19 has been shown to prevent cultured muscle cell fusion through inhibition of a transient induction of the unfolded protein response that is essential for the fusion of the myoblast [63]. Moreover, USP19 downregulates myogenin that has an important function in myogenesis which may suppose that inhibition of USP19 may be a therapeutic way for rising of muscle growth after injury [61].

Another ubiquitin E3 ligase, Nedd4–1 (neural precursor cell-expressed developmentally downregulated Nedd4–1), is characterized to mediate inactivity-induced muscle atrophy. The transcription factor Pax7 can be ubiquitinated by Nedd4–1, which via its differential effects on MyoD can act both as a promoter of myogenesis and as a repressor of myogenesis [46]. Therefore, it is believed that regulation of myogenesis can be controlled by the Pax7-to-MyoD ratio in which by ubiquitinating Pax7, Nedd4–1, transmits the balance in direction of MyoD and stimulates myogenesis [12].

Interestingly, tumor necrosis factor receptor-associated factor (TRAF) is an important binding protein of tumor necrosis factor (TNF) superfamily and the toll/IL-1 receptor (TIR) superfamily, which play an important role in innate immunity and acquired immunity. TRAF family has seven members (TRAF1–7), and TRAF6 has its special facture and biological function. Two domains which are N-terminal domain and C-terminal domain of TRAF6 could regulate signaling pathway function as ubiquitin E3 ligase through integration by multiple kinases [20].

The TRAF6 ubiquitin ligase stimulates ERK1/2 and JNK1/2 in satellite cells, leading to c-Jun activation and Pax7 induction, and the knockout of TRAF6 leads to impairment of muscle regeneration through increased Pax7 levels. This mechanism, along with the observation that TRAF6 is involved in the p38/mitogen-activated protein kinase (MAPK) and Akt pathways, can provide a mechanistic explanation for the impaired myogenesis seen in mice with silenced TRAF6 [24].

10.1.3 USP Genes Interact with Autophagy

Ubiquitin-proteasome system and autophagy are the two major mechanisms for protein degradation in eukaryotic cells. Autophagy is the mechanism by which cytoplasmic contents and organelles are delivered to lysosomes for degradation. LC3, an ubiquitin-like protein, plays an essential role in autophagy through its ability to be conjugated to phosphatidylethanolamine [57].

LC3 processing by the 20S proteasome requires both the N-terminal helices and the ubiquitin fold of LC3 in which addition of the N-terminal helices of ubiquitin to the N terminus of LC3 renders ubiquitin susceptible to 20S proteasomal activity [38]. Further, processing LC3 by the 20S proteasome in stepwise stages is considered. LC3 is cleaved firstly by its ubiquitin fold and thus holds up the conjugation function of it; thereafter and especially at high levels of the proteasome, LC3 is completely decayed. On the other hand, proteolysis of LC3 by the 20S proteasome can be prevented by an LC3-binding protein which known as p62, that intercede autophagic degradation of polyubiquitin assembles in cells [27].

However, complete/long-term inactivation of autophagy by knockout of the autophagy-related 7 (Atg7) gene leads to both atrophy and impaired muscle function, since this process plays a critical role in cell homeostasis through removal of dysfunctional mitochondria and protein aggregates. Therefore, both excessive autophagy, through excessive catabolism, and insufficient autophagy, through accumulation of proteins, generation of oxidative stress, and apoptosis, can lead to muscle atrophy [31].

TRAF6 ligase not only promotes myogenesis but also can modulate autophagy. TRAF6 ligase forms K63-linked ubiquitin chains on Beclin-1, a gene essential for the activation of autophagy. This ubiquitination does not target the protein for degradation but promotes the oligomerization of Beclin-1. TRAF6 may also interact with p62, a protein that plays a role in clearance of protein aggregates, and its inactivation in muscle leads to suppressed autophagy [30].

Moreover, knocking out the deubiquitinating enzyme USP19 results in downregulation of autophagy-promoting genes in muscle, indicating that USP19 may promote autophagy. A recent report indicates that USP19 can deubiquitinate and stabilizes Beclin-1, thereby promoting autophagy. These studies were carried out in non-muscle cell lines but, if relevant also in skeletal muscle, could be part of the mechanism by which USP19 promotes autophagy and muscle wasting [32].

Autophagy initiation is critically dependent on a serine/threonine kinase (ULK1), which acts as a substrate of the Cul3-KLHL20 ubiquitin ligase. During autophagy induction, ULK1 autophosphorylation facilitates its induction to KLHL20 for ubiquitination and proteolysis. This autophagy-stimulated, KLHL20-dependent ULK1 degradation holds the extent and duration of autophagy. Besides that, the breakdown of ATG13, ATG14, Beclin-1, and VPS34 are dominated by KLHL20 in extended starvation. Exhausted KLHL20 leads to disturbed autophagy and then muscle atrophy [41, 42].

10.2 Associated Signaling Pathways of Ubiquitin Ligases

A single E1 gene appears to exist in somatic cells and supplies activated ubiquitin to a larger family of E2s. Approximately 30 genes encode E2s in mammalian cells. Each E2 appears to interact with distinct E3s and different E3s recognize distinct substrates. Where multiple E2s can interact with an E3, the different E2s can mediate formation of different types of ubiquitin chain linkages. Thus, there are multiple pathways of ubiquitin conjugation leading to precise ubiquitination of specific proteins [5].

E3s can be organized into two major classes. One class (~90 human genes) contains a conserved C-terminal HECT domain (homologous to E6-AP carboxy-terminus – named after E6AP, the first E3 described in this class) and functions by first accepting ubiquitin from E2 onto a cysteine residue and then conjugating the ubiquitin to the substrate. The other E3 class (~800 human genes) contains a conserved RING finger motif 28–29 and functions by binding both substrate and the E221 and activating E2's conjugating activity. Ligases can exist as monomeric proteins or as multi-subunit complexes such as the family of cullin-RING ligases in which the substrate recognition and E2-binding functions are located on distinct subunits of the complex [7].

Forkhead box-containing, subfamily O3 (FoxO3) is the main transcription factor driving the expression of most of the atrogenes, such as those implied in the lysosomal and proteasomal pathways, which promote overall proteolysis. Two muscle-restricted ubiquitin ligases, atrogin-1 and muscle RING finger protein 1 (MuRF1), are dramatically upregulated by FoxO3 in all settings of muscle wasting. Molecules that block this activation of proteolysis or increase muscle protein synthesis might serve as pharmacological agents to combat wasting [8].

10.3 Signaling Pathways for Muscle Protein Loss

10.3.1 Toll–Like Receptor 4 (TLR4)

Toll-like receptors (TLRs) are an ancient conserved receptor family. The best-characterized member of this family is toll-like receptor 4 (TLR-4), the receptor for lipopolysaccharide (LPS), which is the best-known that can elicit cellular responses. Interaction between LPS and TLR-4 leads to the formation of an LPS signaling complex consisting of surface molecules, such as CD14 and MD2, as well as intracellular adaptor molecules, including myeloid differentiation primary response gene 88 (MyD88) and tumor necrosis factor (TNF)-α receptor association factor 6 (TRAF6), and activation of transcription factors such as nuclear factor κB (NFκB), which then induce activation of the inflammatory genes, such as TNF-α, interleukin (IL)-1, IL-6, and IL-8 [36].

Excessive inflammatory response has been recognized as a crucial mechanism for muscle atrophy in various models of the disease. Inflammatory cytokines levels in skeletal muscle of patients with cachexia and septicemia are higher than that in skeletal muscle of healthy individuals, and these cytokines contribute to maintain the pathological chronic inflammatory conditions [43]. Furthermore, upon immobilization in atrophied muscles, inflammatory cytokine gene expression is increased [49].

Although inflammatory cytokines are released from the immune and parenchyma cells (including muscle cells), they regulate pathways of intracellular signal transduction involved in muscle atrophy [15]. In vitro studies have shown that inflammatory cytokines enhance the expression of muscle-specific ubiquitin ligases such as MAFbx/atrogin 1 and MuRF1, which have been linked to the degradation of muscle proteins [10]. Therefore, immobilization causing local inflammation of skeletal muscles is associated with the development of muscle atrophy. However, the mechanisms underlying inflammation induced by immobilization causing muscle atrophy remain to be elucidated.

Activation of TLR4 signaling has been considered to be associated with inactivity-induced muscle atrophy. In fact, Schellekens et al. [53] reported that TLR4 knockout mice exhibit decreased mechanical ventilation-induced diaphragmatic muscle atrophy than that exhibited by wild-type mice. Interestingly, a recent study showed that even short-term bed rest can induce increased mRNA levels of inflammatory cytokines and protein levels of TLR4 in the skeletal muscles of healthy older adults [21]. Therefore, increased TLR4 expression, by inactivity such as immobilization, may be an important factor in muscle atrophy and excessive inflammatory response. The cast immobilization-induced muscle atrophy and inflammation is reduced in TLR4-defective C3H/HeJ mice [35].

Moreover, pro-inflammatory cytokines such as TNF-α promoted the loss of muscle protein in skeletal muscle. This is inconsistent with Frisard et al. [23] who found that TLR4 stimulation leads to activation in skeletal muscle; otherwise, upregulated TLR4 mRNA expression leads to further increased MyD88 mRNA expression in gastrocnemius muscle [65].

10.3.2 Nucleotide–Binding Oligomerization Domain Proteins (NODs)

Nucleotide-binding oligomerization domain protein (NOD) is among inflammatory signaling pathways as well as TLR4 that activate NF-κB to release pro-inflammatory cytokines. Loss of lean body mass can be caused by upregulation of the pro-inflammatory cytokines [34].

Among the NOD family, NOD1 and NOD2 are the best characterized members, which possess the ability to connect with the LPS and peptidoglycan and to trans-

duce a TLR-independent signal [25]. Multiple NODs are expressed in the skeletal muscle cells. They play major roles in the detection of microbial infection and the induction of innate antibacterial and inflammatory responses by recognition of pathogen-associated molecular patterns (PAMPs) [39]. Activation of TLRs or NODs by interaction with their specific PAMPs triggers downstream signaling pathways that results in activation of nuclear factor-κB (NF-κB). Activation of NF-κB further provokes the expression of pro-inflammatory genes, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6. These pro-inflammatory cytokines are key regulators that induce muscle atrophy directly [41, 42, 51].

10.3.3 Akt/Forkhead Box O (Akt/FOXO)

In addition, pro-inflammatory cytokines can lead to muscle atrophy partially via changing the Akt/forkhead box O (FOXO)/ubiquitin-proteasome proteolysis (UPP) pathway. Akt/FOXO signaling cascade is an important signaling mechanism in the pro-survival pathway. Seventy five percent of protein degradation during skeletal muscle atrophy is contributed by UPP, which can degrade most cell proteins. Therefore, controlling levels of specific proteins is considered as a critical function of UPP [17].

Phosphorylation of Akt inhibits proteolytic transcription factors. Phosphorylation is required for full activity of Akt, which stimulates protein synthesis and induces FOXO1 that initially stimulates protein degradation, and participates in MuRF1 and MAFbx transcription during muscle atrophy. Both MuRF1 and MAFbx are relied by UPP to degrade specific proteins within the cells [7].

10.3.4 Mammalian Target of Rapamycin (mTOR) Signaling Pathways

Moreover, many evidences have shown that mammalian target of rapamycin (mTOR) pathway also plays a very crucial role on protein synthesis. mTOR stimulation and eukaryotic initiation factor (eIF) 4E-binding protein-1 (4EBP1) phosphorylation are reported to increase protein synthesis. Control of protein synthesis could be by 4EBP1 that is one of the downstream targets in mTOR signaling pathway. Otherwise, activation of 4EBP1 could be via the Akt-dependent signaling pathway that prevent proteolysis and induce protein synthesis in muscle [26].

The excitatory amino acid transporters 3 (EAAT3), which was the glutamate transporter, exists in many tissues, including skeletal muscle. Almilaji et al. [1] reported that EAAT3 could be powerfully upregulated by mTOR and then later

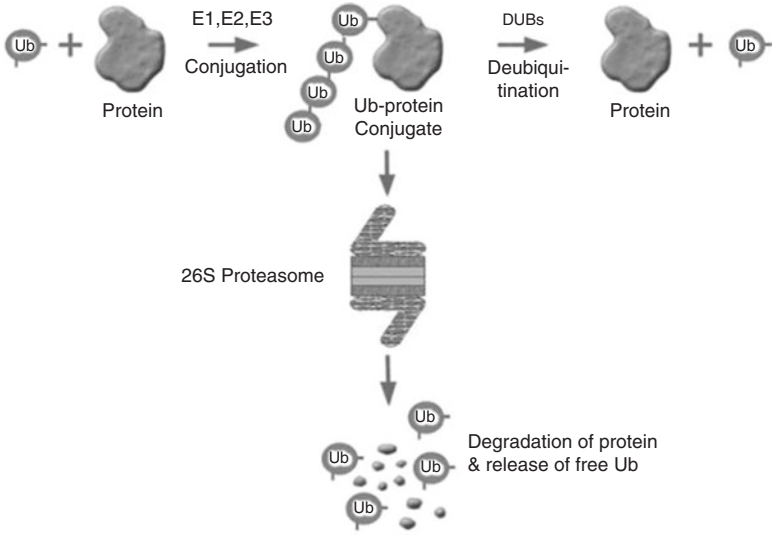

Fig. 10.1 The ubiquitin-proteasome system. *Ub* ubiquitin, *E1* ubiquitin activating enzyme, *E2* ubiquitin conjugating enzyme, *E3* ubiquitin protein ligase, *DUBs* deubiquitinating enzymes
Each Ub is conjugated to the other via Lys 48 and targets the protein substrate for recognition and degradation by the 26S proteasome. Conjugation is mediated by the sequential action of three enzymes – E1– which activates and transfers Ub to E2, which then works in concert with E3 to mediate ubiquitination. E3s recognize substrates. The conjugation can also be reversed by DUBs. Ref. [3]

could augment carrier protein concentration in the cell membrane. Therefore, mTOR induction leads to the increase of EAAT3 which could augment glutamate transposition increasing p-4EBP1/t-4EBP1 ratio (Fig. 10.1).

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Chapter 11
Noncoding RNAs in Muscle Atrophy

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Abstract Denervation, disuse, fasting, and various diseases could induce skeletal muscle atrophy, which results in the decline of life quality and increase of the mortality risk for patients. Noncoding RNAs (ncRNAs) are implicated important in regulating gene expression. Thus, ncRNAs, especially microRNAs and long noncoding RNAs (lncRNAs), have gained widespread attention as crucial players in numerous physiological and pathological processes, including skeletal muscle atrophy. In this review, we comprehensively described the potential of circulating microRNAs as biomarkers, summarized the profiling of microRNAs and lncRNAs in atrophying muscles, as well as discussed the effects and underlying mechanisms of microRNA machinery proteins, microRNAs, and lncRNAs in skeletal muscle atrophy. Considering the large quantity and variety of ncRNAs, the understanding of ncRNAs in muscle atrophy is still very limited. Future studies are needed to elucidate the possibility of ncRNAs as diagnosis biomarkers and therapeutic targets in muscle atrophy.

Keywords Noncoding RNAs · MicroRNAs · lncRNAs · Muscle atrophy · Muscular dystrophy

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11.1 Background

Muscle atrophy is characterized as the decrease in myofiber size, strength, protein content, and total muscle mass [1]. Muscle atrophy can be divided into primary muscular disease and secondary muscular disorders. Primary muscle atrophy is caused by direct diseases of the muscle such as Duchenne muscular dystrophy (DMD) [2] and myotonic dystrophy type 1 (DM1) diseases [3]. Secondary muscular disorders are usually the complications of other diseases, which include chronic kidney diseases (CKD) [4], sepsis [5], diabetes [6], cancers [7], renal and cardiac failure [8], burn injury [9, 10], and HIV/AIDS and neurodegenerative disorders [11]. Additionally, secondary muscular disorders can also occur in healthy individuals under the conditions such as spaceflight, starvation, aging, hindlimb unloading, bed rest, and immobilization [12]. It is well-known that muscle atrophy reduces the quality of life and increases the mortality risk for patients [13]. However, effective treatment methods for muscle atrophy are currently lacking. Thus, there is an urgent need to understand the molecular mechanisms that mediate muscle atrophy, which could greatly contribute to design therapies for alleviating muscle atrophy.

Accumulating evidence shows that noncoding RNAs (ncRNAs) play an important role in regulating distinct steps of muscle atrophy. ncRNAs comprise a large and heterogeneous family including microRNAs (miRs, miRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), PIWI-interacting RNAs (piRNAs), small nucleolar RNAs (snoRNAs), and tRNA derivatives. Among them, miRNAs and lncRNAs are the best-studied classes in different physiologic and pathological conditions, including muscle development and muscle diseases. miRNAs, short ncRNAs (~22 nucleotides), are endogenous and evolutionarily conserved, which mainly repress gene expression posttranscriptionally. One single miRNA has multiple target mRNAs, while individual mRNA can be modulated by numerous miRNAs [14, 15]. miRNAs collectively regulate the expression of 30% of human genes [16]. lncRNAs are a diverse class of noncoding RNAs which are more than 200 nucleotides in length. lncRNAs have been shown vital in regulating gene expression both transcriptionally and posttranscriptionally via various mechanisms. Given that aberrant gene expression underlies muscle atrophy, it is critically important to understand how gene expression is regulated by ncRNAs in response to diverse stresses or diseases which lead to muscle atrophy.

In this review, we will focus upon the ncRNAs (miRNAs and lncRNAs) involved in regulating muscle atrophy and the underlying molecular mechanisms.

11.2 MicroRNA Machinery Proteins in Muscle Atrophy

It is now evident that miRNAs play important roles in multiple physiological and pathological processes including muscle development, muscle regeneration, and muscle atrophy. After transcription by RNA polymerase II or III, miRNA precursors

are catalyzed by DROSHA/DGCR8 complex and exported from the nucleus to cytoplasm by Exportin-5 [17]. Then the enzyme Dicer processes the miRNAs into ~22 nt RNA duplex in cytoplasm, which are loaded onto RNA-induced silencing complex (RISC) and mediate translational repression/mRNA degradation [18, 19].

These proteins involved in miRNA biogenesis and production have been shown important in regulating muscle development and muscle atrophy. Loss of Dicer activity specifically in the myogenic compartment during embryogenesis reduced muscle-specific miRNAs, caused perinatal lethality, and resulted in decreased skeletal muscle mass and abnormal myofiber morphology [20]. Additionally, specific ablation of Dicer1 in postmitotic spinal motor neurons in mice from postnatal day 7 exhibited signs of denervation-related muscle atrophy, including myofiber type grouping, loss of muscle fibers with a large cross-sectional area, and the decreased total fiber diameter [21]. Another miRNA machinery protein Argonaute2 (Ago2) has also been shown important for regulating skeletal muscle atrophy [22]. Crystallin-B (CryAB), a small heat shock protein, interacts with the N and C termini of Ago2 [22]. When the endonuclease activity of Ago2 was significantly repressed through loss of CryAB in mice, the body weight and myofiber cross-sectional area were significantly reduced, while the fibrosis was increased in the skeletal muscle [22]. These results indicated that inhibition of Ago2 caused skeletal muscle atrophy.

In addition, some RNA-binding proteins were also found to negatively regulate miRNA biogenesis. For example, the nuclear factor 90 (NF90; also referred to as ILF3, NFAR1, or DRBP76)-nuclear factor 45 (NF45) complex suppresses miRNA processing through inhibition of pri-miRNA processing [23]. Adult NF90-NF45 double-transgenic mice exhibited skeletal muscle atrophy and centronuclear muscle fibers [24]. Compared with controls, microarray analysis demonstrated that NF90-NF45 overexpression reduced the expression of 23 miRNAs in skeletal muscles, including miR-133a, miR-133b, miR-1, and miR-378 which are reported to promote muscle development [24]. Among them, the processing of pri-miR-133a was found to be suppressed by NF90-NF45 complex [24]. And concomitantly, dynamin 2, a target of miR-133a, is elevated in the muscle of NF90-NF45 double-transgenic mice [24]. Therefore, the upstream regulation of miRNAs plays vital roles in muscle atrophy.

11.3 MicroRNAs Served as Potential Biomarkers in Muscle Atrophy

The reliable and sensitive blood biomarkers are useful, easily accessible, and convenient for the diagnosis, monitoring, and potential future therapy of diseases. miRNAs are found to be present in blood circulation and have been increasingly suggested as biomarkers for several diseases and clinical conditions [25]. As a consequence of fiber damage during atrophy, muscle-expressed miRNAs have been

found to be released into the blood, and their levels are usually correlated with the severity of muscle diseases. Thus, many scientific reports emphasize the possibility of muscle-specific miRNAs as circulating biomarkers for muscle atrophy induced by various stimuli.

Muscle atrophy and weakness are the primary characteristics of Duchenne muscular dystrophy and myotonic dystrophy type 1 patients. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis demonstrated that several muscle-specific miRNAs (miR-1, miR-133a, and miR-206) are increased in the serum of mouse and dog models of DMD [2]. Additional studies indicate that miR-1, miR-133a, and miR-206 are enriched in serum of DMD patients, and their levels were correlated with the severity of DMD disease, indicating that miR-1, miR-133a, and miR-206 are new biomarkers for the diagnosis of DMD and for evaluating the outcomes of therapeutic interventions in humans [26]. By multiplex qRT-PCR analysis of 381 miRNAs in 36 consecutive DM1 patients and 36 healthy controls, a signature of 9 deregulated miRNAs in plasma samples of DM1 patients was identified [3]. miR-133a, miR-193b, miR-191, miR-454, miR-574, miR-885-5p, and miR-886-3p were increased, while miR-27b was decreased in DM1 patients [3]. Among them, miR-133a was suggested to be used as candidate diagnostic biomarker for DM1 [3]. Another study demonstrated that miR-1, miR-133a, miR-133b, and miR-206 were increased in the serum from DM1 patients with progressive muscle atrophy compared to disease-stable DM1 patients [27]. And the levels of miR-1, miR-133a, miR-133b, and miR-206 were correlated with the progression of muscle atrophy in the DM1 patients, supporting their potential as useful and reliable biomarkers for DM1 patients [27].

Muscle atrophy is a common systemic complication of chronic obstructive pulmonary disease (COPD). The expression of muscle-specific miRNAs was determined in serum from 31 COPD patients with muscle atrophy and 14 healthy age-matched controls by qRT-PCR [28]. The expression of miR-1 was reduced in COPD patients compared with controls, but there was no significant difference in the expression of miR-499, miR-208, miR-181, miR-145, miR-206, and miR-133 [28].

Additionally, the serum levels of muscle-specific miRNAs (miR-1, miR-23a, miR-133, miR-206, miR-208b, and miR-499) were all significantly elevated after hindlimb unloading for 7 days in mice, which could induce severe muscle atrophy [29]. Moreover, the serum levels of miR-23a, miR-206, and miR-499 were increased, while miR-1, miR-206, and miR-208b were not changed in 15 healthy human participants after 45 days of head-down bed rest [29]. And the levels of miR-23a, miR-206, and miR-499 were positively correlated with the ratio of soleus volume loss induced by head-down bed rest [29], indicating that circulating miR-23a, miR-206, and miR-499 could be used as candidate biomarkers for the diagnosis of muscle atrophy induced by disuse.

One study selectively characterized the expression of miR-9, miR-206, and miR-132 in serum from spinal muscular atrophy (SMA) mice and patients [30]. Both miR-9 and miR-132 were elevated in the serum from SMA mice and patients [30]. Serum miR-206 was increased in SMA mice compared with controls, but its level

in SMA patients has no significant difference [30]. These results indicated the potential of miR-9 and miR-132 as candidate serum biomarkers for SMA.

Collectively, some miRNAs have been identified as possible circulating biomarkers for the diagnosis of DMD, SMA, and DM1 diseases, as well as muscle atrophy induced by hindlimb unloading, head-down bed rest, and COPD disease. However, the specific, sensitive, and reliable biomarkers are still lacking for muscle atrophy.

11.4 MicroRNAs in Muscle Atrophy

To understand the involvement of miRNAs in muscle atrophy, a large number of miRNA profiling have been performed in atrophying muscles under different conditions such as fasting, denervation, diabetes, disuse, and cancer cachexia. The miRNA signature of muscle atrophy has been found peculiar under each condition [31].

In primary muscle atrophy caused by direct diseases of the muscle, miRNA microarrays in muscle tissues identified 39 miRNAs such as miR-29a, miR-30c, miR-30b, miR-92, miR-29c, miR-423, miR-361, miR-299-3p, and miR-181d which were upregulated in Duchenne muscular dystrophy patients [32]. Sixty-two miRNAs such as miR-16, miR-279, miR-99a, miR-93, miR-455, miR-20b, miR-18a, miR-17-5p, miR-152, miR-106a, and miR-106b were upregulated in facioscapulohumeral muscular dystrophy patients [32]. The levels of miR-1 and miR-133a/b were significantly decreased, while miR-206 was significantly increased in muscles of 12 myotonic dystrophy type 1 patients as compared to 6 healthy controls [33].

Lipopolysaccharide, cancer cachexia, and chronic alcohol exposure are the pathological stimuli for muscle atrophy. Small RNA deep sequencing in pig skeletal muscles analyzed the miRNA expression profiles during lipopolysaccharide-induced wasting [34]. Four miRNAs (miR-146a-5p, miR-221-5p, miR-9860-5p, and miR-148b-3p) were significantly upregulated, while three miRNAs (miR-192, miR-215, and miR-429) were downregulated in the lipopolysaccharide-challenged samples [34]. Cancer cachexia-induced muscle atrophy is a direct cause in the functional decline of cancer patients [35]. By injecting Lewis lung carcinoma cells into C57BL/6 J mice to induce muscle atrophy, miRNA sequencing identified nine dysregulated miRNAs including miR-147-3p, miR-299a-3p, miR-1933-3p, miR-511-3p, miR-3473d, miR-233-3p, miR-431-5p, miR-665-3p, and miR-205-3p in the tibialis anterior muscles injected by Lewis lung carcinoma cells [36]. Utilizing a zebrafish model of muscle atrophy induced by chronic alcohol exposure, miRNA microarray identified that 14 miRNAs were upregulated, while 47 miRNAs were downregulated more than twofold in skeletal muscles [37]. Among them, miR-140-3p was downregulated, whereas miR-146a was upregulated. Interestingly, the potential targets of both miR-140-3p and miR-146a include several members of the Notch signaling pathway [37].

Recently, RNA sequencing was performed to assess the whole transcriptome in mouse models of denervation-induced muscle atrophy [38]. There were 671

differentially expressed miRNAs in gastrocnemius muscles at different time points (1 week, 2 weeks, 4 weeks, and 8 weeks) after nerve injury compared with controls [38]. At an early denervation stage, another miRNA microarray analysis in rats showed that miR-206, miR-195, miR-23a, and miR-30e were differentially expressed in the slow muscles, while other miRNA molecules (miR-214, miR-221, miR-222, miR-152, miR-320, and let-7e) were differentially expressed in the fast muscles compared to controls [39]. These studies indicated that miRNAs were dynamically altered in the progression of muscle atrophy and miRNAs in different types of skeletal muscles respond to the same stimuli in distinct ways.

Amyotrophic lateral sclerosis (ALS) is characterized by the signs of denervation-induced muscle atrophy. In human studies of ALS, miR-206 was elevated in muscles of four early-stage ALS patients [40] and characterized as a potential biomarker for ALS patients [41]. Using small RNA-seq, the expressions of small RNAs in muscle tissues of ALS patients and healthy age-matched controls were compared [42]. Nineteen miRNAs such as miR-100, miR-10a, miR-125a, miR-125b, miR-1260a, miR-128, miR-1291, miR-132, miR-133a, and miR-151a were upregulated, while 10 miRNAs such as miR-126, miR-1285, miR-1303, miR-150, miR-191, and miR-28 were downregulated in the ALS groups [42]. Interestingly, this study did not find changes in the expression of miR-206 in ALS patients [42], which might be due to the differences in study populations.

Spinal cord injury can induce severe skeletal muscle atrophy and the transformation toward fast-twitch, type II fibers. In human, miR-208b and miR-499-5p expressions were progressively declined in skeletal muscle during the first year after spinal cord injury [43]. Moreover, miR-208b and miR-499-5p were inversely correlated with the expression of myostatin, an inhibitor of muscle growth, in human skeletal muscle after spinal cord injury [43]. miR-208b reduced myostatin expression in intact mouse skeletal muscle after spinal cord injury, whereas miR-499-5p had no obvious effect [43].

Addition of dexamethasone (Dex) leads to a distinct atrophic phenotype in differentiated C2C12 myotubes, which is the in vitro model of Dex-induced muscle atrophy [44]. miR-1, miR-322, miR-351, and miR-503-3p were found to be upregulated in Dex-treated C2C12 cells compared to controls, while miR-708 and miR-147 were downregulated [44]. miR-18a expression is declined during C2C12 myoblast differentiation [45]. And in vitro overexpression of miR-18a induces myotube atrophy via the PI3K/AKT pathway through Igf1 [45]. miR-182 expression is dramatically decreased in C2C12 myotubes treated with Dex [46]. miR-182 was enriched in exosomes isolated from the media of C2C12 myotubes, and Dex treatment could increase its abundance in exosomes [46].

In addition to the miRNA profiling studies, functional studies using cellular and animal models have disclosed multiple important miRNAs in muscle atrophy. Spinal and bulbar muscular atrophy (SBMA) is an inherited neurodegenerative disorder caused by the expansion of a polyglutamine repeat in the androgen receptor (AR-polyQ) [47, 48]. SBMA is characterized by proximal muscular atrophy, weakness, contraction fasciculation, and bulbar involvement [49]. miRNA microarray analysis identified that miR-196a, miR-196b, miR-496, miR-323-3p,

and miR-29b-3p were upregulated more than twofold in the spinal cords of male SBMA mice expressing full-length human AR with 97 glutamine residues (AR-97Q) compared to the male mice expressing wild-type human AR [50]. Among them, miR-196a was found to enhance the decay of the AR mRNA by silencing CUGBP, Elav-like family member 2 (CELF2) [50]. Further studies demonstrated that adeno-associated virus (AAV) vector-mediated delivery of miR-196a exhibited the strong and continuous inhibition of CELF2 expression and ameliorated the SBMA phenotypes in a mouse model [50]. Importantly, miR-196a was upregulated and the CELF2 mRNA was downregulated in the thoracic spinal cord of patients with SBMA, and miR-196a treatment could downregulate both the AR and CELF2 mRNAs and proteins in the fibroblasts obtained from patients with SBMA [50]. Thus, overexpression of miR-196a can be considered as the potential strategy for treating SBMA. Another report found that miR-298 could ameliorate the phenotype of SBMA in mice [51]. In vitro studies demonstrated that miR-298 directly bound to the 3′-untranslated region (UTR) of the human AR transcripts and reduced AR mRNA and protein levels [51].

miR-1 is specifically expressed in muscles and plays important roles in myogenesis, muscle regeneration, as well as muscle atrophy. High doses of Dex or myostatin (Mstn) induce severe skeletal muscle atrophy [52]. miR-1 was found to be elevated in both C2C12 myotubes and mouse models of Dex-induced atrophy [52]. Both Dex and Mstn could induce miR-1 expression through glucocorticoid receptor (GR) [52]. And miR-1 elevation promotes skeletal muscle atrophy through targeting HSP70 and reducing its levels, which led to decreased phosphorylation of AKT, enhanced activation of FOXO3, and upregulation of MuRF1 and Atrogin-1 [52]. In addition, miR-1 was found to be unchanged in soleus muscle of rats with muscle atrophy induced by hindlimb suspension [53]. Similar to miR-1, miR-133 also has important roles in the myogenesis and muscle development [54, 55]. However, the functional study of miR-133 in muscle atrophy is much more less.

Denervation is a common cause of muscle atrophy, and miR-351, miR-21, and miR-206 have been identified as important regulators of denervation-induced muscle atrophy. Following sciatic nerve transection, miR-351 was gradually reduced with time, and overexpression of miR-351 significantly repressed the decrease of the wet weight ratio and cross-sectional area of the tibialis anterior muscle in rats [56]. Mechanically, miR-351 is able to downregulate TRAF6 expression by directly targeting its 3'-UTR [56] and negatively regulate the two downstream signaling molecules of TRAF6, MuRF1 and MAFBx, in tibialis anterior muscles after sciatic nerve transection [56]. By miRNA profiling in mouse denervated muscles, miR-21 and miR-206 were found to be strongly induced after denervation [31]. Induction of miR-206 and miR-21 in adult mouse muscle contributes to muscle atrophy induced by denervation, whereas repression of miR-206 and miR-21 partially protects against denervation-induced atrophy in vivo [31]. More importantly, luciferase assays confirmed that YY1 was the target gene of miR-21, and eIF4E3 and Pdcd10 were the target genes of both miR-21 and miR-206 in denervated muscles [31]. However, in rats, miR-206 was found to increase the number of differentiating (MyoD1+/Pax7+) satellite cells and counteract denervation-induced atrophy through TGF-β1/Smad3 signaling pathway [57]. Moreover, miR-206 is dramatically

increased in a mouse model of amyotrophic lateral sclerosis (ALS), which exhibited denervation and atrophy of targeted muscles [58]. miR-206-deficient mice form normal neuromuscular synapses during development, but loss of miR-206 accelerated ALS progression in mouse model and induced severe skeletal muscle atrophy through targeting histone deacetylase 4 (HDAC4) [58].

A loss of muscle mass during muscle atrophy results from an imbalance of protein synthesis and degradation with a reduction in synthesis. miR-424-5p expression was increased in patients with conditions associated with muscle wasting (COPD patients, patients undergoing aortic surgery, and patients with ICU-acquired weakness) [59]. In mice, overexpression of miR-322 (rodent miR-424 orthologue) promoted muscle atrophy and reduced ribosome RNA levels [59]. Ago2 pull-down assays showed that miR-424-5p bound to mRNAs encoding proteins required for ribosomal RNA transcription and protein synthesis, PolR1A and upstream binding transcription factors [59].

A common clinical feature in patients with severe burns is skeletal muscle atrophy. miR-628 was increased in tibialis anterior muscle after burn injury in rats [9, 10]. Overexpression of miR-628 in rat muscle activates the IRS1/Akt/FoxO3a signaling pathway and promotes cell apoptosis [9]. IRS1 was identified as direct target of miR-628 [9].

Most of miRNAs mentioned above have been shown vital for only one model of muscle atrophy. A systematic study using different models of muscle atrophy identified that miR-29b was elevated in multiple in vivo atrophy models (denervation, Dex, fasting, cancer cachexia, and aging), as well as the in vitro atrophy models (primary myoblasts treated with Dex and myotubes differentiated from C2C12 treated with Dex, TNF-α, or H_2O_2) [60]. miR-29b overexpression induces muscle atrophy, and its inhibition attenuates muscle atrophy induced by multiple stimuli both in vitro and in vivo [60]. IGF-1 and PI3K(p85α) were identified as the direct targets of miR-29b [60].

miR-23a has also been found to be important in multiple models of muscle atrophy. In patients with chronic kidney disease (CKD), a decline in muscle mass is associated with increased morbidity and mortality [4]. Exercise can ameliorate the phenotype of muscle atrophy induced by CKD [4]. miR-23a was decreased, while miR-27a was unchanged in CKD mice muscle, and resistance exercise elevated miR-23a and miR-27a expression in CKD mouse muscle [61]. Overexpression of miR-23a/miR-27a in CKD mice attenuated muscle loss, improved grip strength, reduced caspase activity, and increased markers of muscle regeneration [61]. In primary satellite cells, PTEN and caspase-7 were identified as targets of miR-23a and FoxO1 was identified as a target of miR-27a [61]. Ectopic expression of miR-23a was sufficient to prevent Dex-induced muscle atrophy both in vitro and in vivo [62]. Furthermore, miR-23a transgenic mice showed resistance against Dex-induced skeletal muscle atrophy [62]. miR-23a repressed the translation of both MAFbx/atrogin-1 and MuRF1 in a 3' UTR-dependent manner, which were involved in promoting atrophy-associated protein degradation [62]. miR-23a was also reduced both in the atrophying muscles of rats with acute streptozotocin-induced diabetes and the C2C12 myotubes treated with Dex [63]. In-depth study demonstrated that

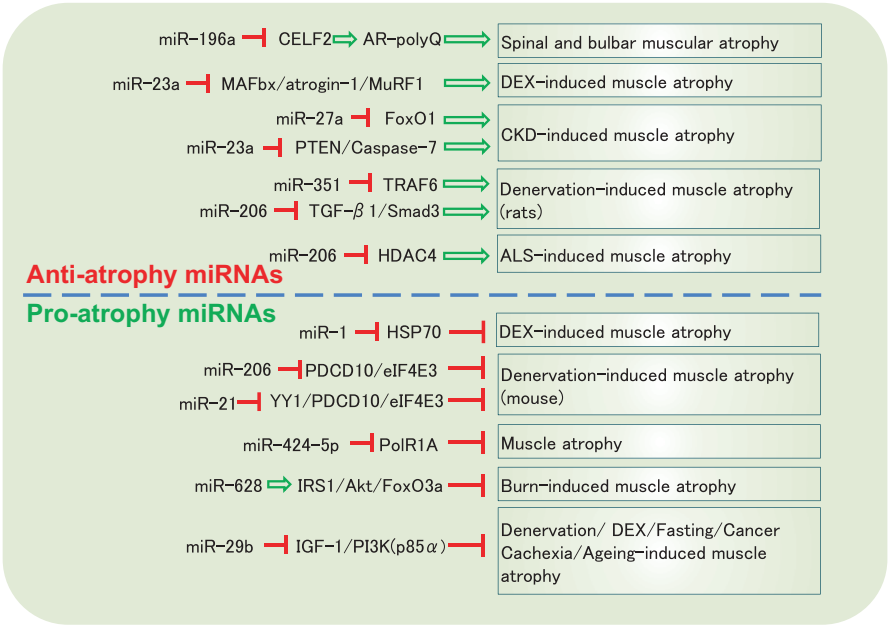

Fig. 11.1 MicroRNAs in muscle atrophy. *Dex* dexamethasone, *CKD* chronic kidney diseases, *ALS* amyotrophic lateral sclerosis

the decrease of miR-23a was due to the attenuation of calcineurin signaling and the promotion of exosome-mediated export of miR-23a caused by atrophy-inducing conditions [63].

Collectively, in vivo studies demonstrated that miR-196a, miR-298, miR-351, miR-23a, and miR-27a suppressed, while miR-1, miR-21, miR-424-5p, miR-628, and miR-29b promoted the progression of muscle atrophy (Fig. 11.1). Particularly, miR-206 suppressed ALS-induced muscle atrophy in mice and denervation-induced muscle atrophy in rats and promoted the denervation-induced muscle atrophy in mice (Fig. 11.1). Future studies based on these results will provide the potential therapeutic targets for muscle atrophy.

11.5 lncRNAs in Muscle Atrophy

lncRNAs are characterized as noncoding RNA sequences >200 nucleotides [64]. lncRNAs have been regarded as critical epigenetic regulators of gene expression in multiple physiological and pathological conditions [65]. The number of lncRNAs in the human genome is estimated to be no less than protein-coding genes [66]. A substantial number, but not all of the lncRNAs, are transcribed by RNA polymerase II, 5′-capped, spliced, and polyadenylated at the 3′ end, undergoing similar

posttranscriptional processing as mRNAs [67]. Compared with miRNAs, little is known about the biological roles of lncRNAs, and even less about their mechanism of action. In mammalian cells, the wide variety of subcellular localizations, expression levels, and stabilities of lncRNAs have been observed and a broad array of diverse mechanisms has been suggested. Based on the examples of well-studied lncRNAs, lncRNAs can either repress or activate gene expression through regulating gene transcription, mRNA stability, pre-mRNA splicing, protein translation, and protein stability [64]. Additionally, lncRNAs can serve as "sponge" RNAs for miRNAs through pairing to miRNAs and titrating them away from their mRNA targets [68]. Similarly, lncRNAs have been reported as a decoy that titrate the protein away from its potential targets, such as lncRNA *Gas5* and glucocorticoid receptor [69] and *sno-lncRNAs* and Fox2 [70]. To date, many studies mainly focused on the physiological function of lncRNAs in muscles, and the number of lncRNAs identified as regulators of muscle atrophy so far is still exiguous. Therefore, our understanding of lncRNAs in muscle atrophy, especially in stress-induced muscle atrophy, is much more limited.

Myogenesis is a complex process required for regeneration and growth of myofibers in adults and begins with the activation and differentiation of muscle stem cells. Multiple lncRNAs were reported to be associated with myogenesis and muscle regeneration. lncRNA SRA [71, 72], H19 [73], MUNC [74], lncMyoD [75], lnc-MD1 [76], lnc-mg [77], MAR1 [78], lnc-YY1 [79], Myolinc [80], and Dum [81] are confirmed as important positive regulators of myogenesis. In contrast, recent studies have shown that certain lncRNAs negatively regulate myogenesis, including SINE-containing lncRNAs [82], Yam-1 [83], Lnc-31 [84], Malat1 [85], and Sirt1 AS lncRNAs [86]. During muscle atrophy, impaired myogenesis is a common underlying mechanism [87]. Thus, the aberrant expression of these myogenesis-related lncRNAs might contribute to muscle atrophy. So far, among the lncRNAs mentioned above, only the roles of lncRNA MAR1 and lnc-mg have been investigated in cellular and animal models of muscle atrophy.

lncRNA MAR1 (muscle anabolic regulator 1) was significantly downregulated in the mouse gastrocnemius muscle during aging and unloading condition [78]. In C2C12 cells, MAR1 was found to promote the myogenic differentiation through serving as the sponges for miR-487b to regulate Wnt5a expression, which is an important factor during myogenesis [78]. Moreover, therapeutic enforced MAR1 expression in skeletal muscle of mice could counteract either age-related muscle atrophy or hindlimb suspension-induced muscle atrophy mice [78].

A myogenesis-associated lncRNA named as lnc-mg is specifically enriched in skeletal muscle and was shown to be induced in muscle stem cell differentiation [77]. According to the in vitro analysis of primary skeletal muscle cells and in vivo analysis of conditional knockout mice, lnc-mg promotes myogenesis by serving as a sponge for miR-125b to elevate the protein abundance of insulin-like growth factor 2 [77]. Conditional knockout of lnc-mg in mouse skeletal muscle results in muscle atrophy and the loss of muscular endurance during exercise [77]. However, muscle loss is not significantly improved after denervation in transgenic mice of lnc-mg [77]. Thus, the rescue effect of lnc-mg on stress-induced skeletal muscle atrophy needs to be carefully elucidated.

Spinal muscular atrophy is an inherited neuromuscular disorder, caused by recessive mutations of the survival motor neuron 1 (SMN1) gene and retention of variable copy numbers of the highly homologous SMN2 gene [88, 89]. lncRNA SMN-AS1 arises from the antisense strand of SMN and is highly enriched in neurons [90]. SMN-AS1 recruited PRC2 to the SMN promoter and transcriptionally repressed SMN expression [90]. Delivery of SMN-AS1 antisense oligonucleotides (ASOs) elevated the SMN expression in patient-derived fibroblast cells, cultured neurons, and a mouse model of severe SMA [90]. Combining SMN-AS1 ASOs with SMN2 splice-switching oligonucleotides additively increased SMN expression and ameliorated SMA in mouse model [90]. Similarly, another independent group also reported that selective disruption of SMN-AS1-mediated PRC2 recruitment could activate SMN and ameliorate SMA phenotypes in mice [91].

In addition to the myogenesis-related lncRNAs as potential candidates, lncRNA profiling has been performed to identify more important lncRNAs in the animal models of muscle atrophy. Severe thermal trauma covering more than 30% of the total body surface area triggers severe muscle atrophy. Microarray was used to determine the lncRNA expression levels in skeletal muscle tissues of three pairs of burned rats at the early flow phase, compared with sham rats [92]. An average of 117 lncRNAs were significantly differentially expressed (1.5-fold) [92]. Recently, the expression patterns of lncRNAs were also detected using RNA sequencing in the mouse gastrocnemius muscle after nerve injury at different time points and compared to that obtained in the control group [38]. There were 664 differentially expressed lncRNAs (75 upregulated and 87 downregulated at 1 week, 78 upregulated and 80 downregulated at 2 weeks, 89 upregulated and 77 downregulated at 4 weeks, and 76 upregulated and 102 downregulated at 8 weeks) in denervated muscle atrophy compared to control groups [38]. Two selected lncRNAs were validated using qRT-PCR and their changes were consistent with the RNA-seq data [38]. Another microarray analysis compares the differentially expressed lncRNAs in gastrocnemius muscle between adult (6-month-old) and aged mice (24-month-old) [78]. And 894 lncRNAs were identified to be downregulated, while 1051 lncRNAs were upregulated more than twofold in aged muscle tissues compared with controls [78].

Collectively, very few lncRNAs including lnc-mg, MAR1, and SMN-AS1 are uncovered to regulate muscle atrophy (Fig. 11.2). And the studies of myogenesis-related lncRNAs and profiling of lncRNAs in muscle atrophy have shown the deserving hints for further investigation of lncRNAs in muscle atrophy.

11.6 Conclusions and Perspectives

Skeletal muscle atrophy undergoes remarkable adaptations in response to numerous conditions, which significantly diminished quality of life. As we reviewed here, studies published in the past couple years emphasized identifying the potential

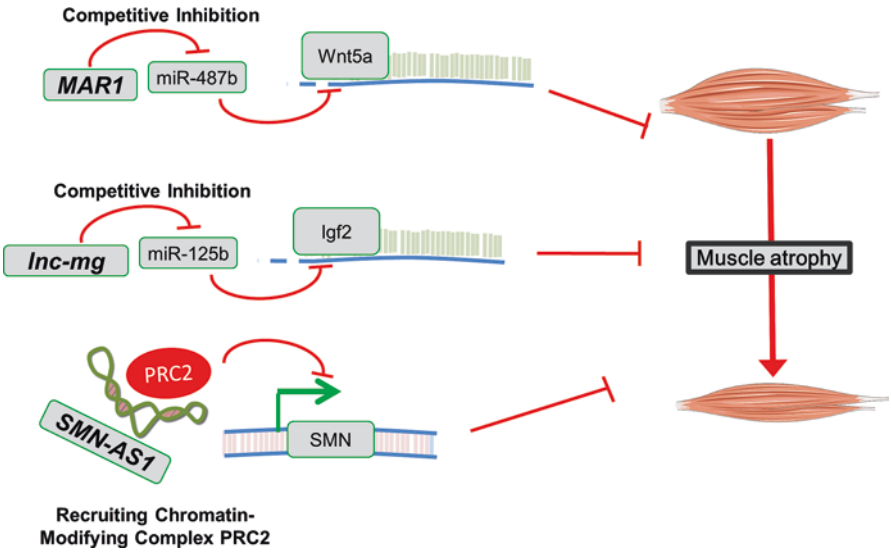

Fig. 11.2 lncRNAs in muscle atrophy

miRNAs as biomarkers, profiling the changes of miRNAs and lncRNAs, and uncovering the roles and mechanisms of miRNAs and lncRNAs in diverse muscle atrophy.

To date, numerous miRNAs have been found to be altered in the serum of patients with muscle atrophy compared with healthy controls. And several of them have been shown correlated with the different stages and severity of the diseases. However, the possible inconsistencies in the results and the specificity of this kind of biomarker remain the major critical challenges. One of the major reasons is the human subject variability, and therefore recruiting large cohorts of patients could greatly improve the future biomarker studies.

The quantity and variety of miRNAs and lncRNAs are very large, and many of them have been shown changed in atrophying muscles. However, at present, only a few miRNAs and exiguous lncRNAs were investigated in depth. Our current understanding about the mechanisms of miRNAs and especially the lncRNAs are still very limited. Besides, other ncRNAs such as circular RNAs are emerging as the vital regulators of various diseases. One recent RNA sequencing has identified 236 circular RNAs which were differentially expressed in the mouse gastrocnemius muscle after nerve injury at different time points [38] . Although this sequencing data provides a theoretical basis for studying circular RNAs in denervated muscle atrophy, the roles of circular RNAs in muscle atrophy are still unknown [38]. In the immediate future of ncRNA study, deciphering more important ncRNAs in muscle atrophy and uncovering their intrinsic mechanisms are highly needed, which will enhance our ability to gain a better understanding of muscle atrophy and provide novel diagnosis markers and therapeutic targets.

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Chapter 12
NF-kB and Inflammatory Cytokine Signalling: Role in Skeletal Muscle Atrophy

Anastasia Thoma and Adam P. Lightfoot

Abstract Atrophy is a classical hallmark of an array of disorders that affect skeletal muscle, ranging from inherited dystrophies, acquired inflammatory myopathies, ageing (sarcopenia) and critical illness (sepsis). The loss of muscle mass and function in these instances is associated with disability, poor quality of life and in some cases mortality. The mechanisms which underpin muscle atrophy are complex; however, significant research has demonstrated an important role for inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), mediated by the generation of reactive oxygen species (ROS) in muscle wasting. Moreover, activation of the transcription factor nuclear factor kappa B (NF-κB) is a key lynchpin in the overall processes that mediate muscle atrophy. The significance of NF-κB as a key regulator of muscle atrophy has been emphasised by several in vivo studies, which have demonstrated that NF-κB-targeted therapies can abrogate muscle atrophy. In this chapter, we will summarise current knowledge on the role of cytokines (TNF-α) and NF-κB in the loss of muscle mass and function and highlight perspectives towards future research and potential therapies to combat muscle atrophy.

Keywords TNF-α · Nuclear factor kappa B · Atrophy · Cytokines · Skeletal muscle

12.1 Introduction

Skeletal muscle is a robust and plastic organ; accounting for approximately 40% total body weight and 50% total protein and is responsible for ambulation, postural support, metabolic homeostasis and thermogenesis. Skeletal muscle is plastic in the sense of its capability of rapidly responding to load, in terms of training or disuse; and these features undoubtedly underpinned the success of our species in hunter-gather times [1]. However, in response to an array of pathological stimuli, it is

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dysregulation in mechanisms of plasticity which gives rise to atrophy of muscle. Skeletal muscle atrophy is defined as loss of muscle mass, derived from imbalance between rates of protein synthesis and degradation [2]. We observe muscle atrophy in an array of pathogenic states, ranging from inherited (DMD) and acquired (myositis) myopathies to sepsis (hyper-inflammation and disuse) and age-related loss of muscle mass (sarcopenia) [2]. In these instances, we observe a reduction in muscle fibre cross-sectional area and thus a reduction in force output – which manifests as muscle weakness and reduced capacity to exercise – collectively resulting in impaired quality of life. The cellular mechanisms, which are responsible for muscle atrophy, are indeed complex. However, significant research of the last ~20 years has indicated that nuclear factor kappa B (NF-κB) pathway activation and inflammatory cytokines such as TNF-α are key players in muscle atrophy. In this chapter, we discuss the basic biology of NF-κB signalling, the evidence demonstrating the role of NF-κB as a lynchpin in muscle atrophy – intertwined with the role of cytokines in atrophy – and how pharmacologically targeting NF-κB may be an avenue for therapy.

12.2 NF-kB and Muscle Atrophy

12.2.1 The NF-kB Signalling Pathway

NF-κB is a pleiotropic, redox-sensitive, nuclear transcription factor, which regulates the expression of a vast array of genes, associated with a diverse range of biological processes – ranging from innate and adaptive immune responses to cell growth, maturation and survival [3]. NF-κB plays a crucial role in allowing cells to adapt to a diverse array of environmental stimuli. In mammalian species NF-κB is comprised of the subunits p50, p52 p65 (RelA), c-Rel and RelB [4]. The individual protein subunits of NF-κB bind together to form heterodimers that are defined as the NF-κB complex. Dimerisation occurs at a region termed the rel-homology domain (RHD). The RHD is located on the N-terminus of each NF-κB unit and is approximately 300 amino acid bases in length [5]. There are 15 known dimers that have been identified to form NF-κB units. There is relative homology between the subunits, however key differences in p50 and p52 are apparent, whereby they lack a transactivational domain at their C-terminus; p50/52 homodimers do not activate transcription upon migration to the nucleus. One of the most characteristic dimers, which do activate transcription, is the p65/50 dimer [3].

NF-κB resides in the cytosol of cells in an inactive state, tightly bound to IκB, comprised of several subunits: IκBα, IκBβ, IκBγ and IκBε [3]. IκB forms covalent bonds with NF-κB that maintains it in a state of inactivity. Although inactive NF-κB is described as cytosolic, the NF-κB-IκB complex is constantly migrating in a cyclical fashion to and from the nucleus [6]. IκB prevents any significant binding of NF-κB to DNA, and the net export from the nucleus is greater than that of the

import – implying NF-κB to be cytosolic in origin [6]. NF-κB activation occurs by severing of covalent bonds with IκB via the action of the IκB kinase (IKK). IKK is a kinase, which phosphorylates IκB and initiates IκB degradation via the ubiquitin-proteasome pathway – leaving NF-κB free and active, which then translocates to the nucleus and binds to requisite promoter sequences at the κB domains [4].

NF-κB activation can occur in response to a variety of stimuli from viral and bacterial components to pro-inflammatory cytokines – however, one of the most well-characterised activators is TNF-α [6]. The canonical activation of NF-κB due to degradation of the inhibitor of kappa B alpha/beta (IKBα/β) by IκB kinase (IKK) is TNF-dependent [3]. The activation of IKKβ by TNF-α occurs due to translocation of IKKβ to the membrane by the chaperones CDC37 and HSP90; the activation of IKKβ is RIP-dependent. IKKβ phosphorylates the IKBα and IKBβ subunits which bind to and stabilise NF-κB in an inactivate state in the cytoplasm. TNF-α is produced by a variety of cell types, such as monocytes, macrophages, NK cells, endothelial cells, smooth muscle cells [7] in skeletal muscle [8] and adipocytes [9].

12.2.2 NF-κB in Muscle Disease

There is an overwhelming body of evidence delineating the important role for NF-κB in muscle wasting – in part, derived from a pivotal study in 2000. Authors demonstrated a key role for NF-κB in the loss of MyoD in cachexia – mediated via TNF-α/IFN-γ gamma signalling [10]. Research in more recent years has expanded our understanding in this context, with in vitro, in vivo, and now strong clinical evidence – reporting NF-κB as a key lynchpin in muscle atrophy.

Sarcopenia is the age-related loss of muscle mass – which typically occurs from the fifth decade of life onwards – with upwards of 50% loss of muscle mass observed in the eighth decade [11]. Loss of muscle mass and function in ageing is associated with frailty and impaired quality of life – and is an overall significant socioeconomic burden. During ageing we observed a loss of overall muscle fibre number and a reduction in cross-sectional area of those remaining fibres. Studies examining the role of NF-κB in the context of ageing have demonstrated elevated NF-κB content was fourfold higher in the medial vastus lateralis of elderly men (70 ± 1 years) when compared with young men (28 ± 1 years) [12]. In murine studies, anterior tibialis muscle of aged mice showed an aberrant persistent activation of NF-κB DNA binding activity [13]. Collectively, these studies illustrate a constitutive activation of NF-κB in aged muscle; however, the precise mechanism of action in the context of sarcopenia is poorly understood.

The idiopathic inflammatory myopathies, collectively termed myositis, are a group of heterogeneous acquired autoimmune disease, which primarily target skeletal muscle. Myositis can be subcategorised into polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM) characterised by profound muscle wasting, weakness and disability. Elevated circulating and muscle levels of

cytokines, such as TNF-α and IFN-γ alongside deposition of CD4/CD8 T-cells in muscle, are all hallmarks of disease [14]. The NF-κB pathway has been investigated in the context of myositis, with both PM and DM biopsies showing NF-κB activation [15]. Moreover, immunohistochemical investigations of biopsies from IBM patients showed increased deposition of p50 and p65 subunits in diseased muscle fibres [16]. An intriguing hallmark of myositis is the overexpression of major histocompatibility complex (MHC) I on the muscle fibre surface [17]. Mechanistic in vitro and in vivo studies have demonstrated that MHC I overexpression can drive NF-κB activation in muscle [18].

In terms of inherited myopathies, the X-linked recessive disorder Duchenne muscular dystrophy (DMD) has received significant attention in the context of NF-κB. DMD is a chronic degenerative neuromuscular disease, characterised by muscle lacking functional dystrophin protein [19]. Consequently, profound damage to the muscle fibre membrane occurs, which is a key driver of the degeneration of muscle in DMD. The muscle of DMD patients undergoes cyclical bouts of damage (degeneration) and regeneration – with invasion of immune cells, a secondary feature of the disease. Analysis of biopsy tissue from patients with DMD showed enhanced NF-κB DNA binding activity, determined by electrophoretic mobility shift assay (EMSA) [15]. Furthermore, studies in the *mdx* model of DMD have further highlighted NF-κB pathway activation in muscle using EMSA [20]. There is a prevailing theory that dysregulation of NF-κB signalling in DMD contributes the muscle inflammation and degradation. Thus, there is interest in pursuing novel NF-κB-targeted therapies to combat this process. Collectively, there is significant evidence to demonstrate a potential role for NF-κB in mediating the pathogenesis in a range of acquired and inherited myopathies.

12.2.3 Mechanisms of NF-κB-Mediated Muscle Atrophy

Here we highlight mechanisms researchers have identified, which muscle atrophy and wasting are mediated through, in the context of NF-κB pathway activation (Fig. 12.1). As a pleiotropic transcription factor, NF-κB regulates a plethora of genes, of which a proportion encode an array of cytokines and chemokines. Given the aforementioned myopathies in this chapter harbour significant inflammatory cell components (either as a primary in IIM or secondary pathogenic feature in DMD) to their pathogenesis, it is not surprising to see NF-κB as a lynchpin to some of those effects. Moreover, the notion that skeletal muscle is now considered an endocrine organ, capable of releasing an array of proteins and peptides – such as certain cytokines and chemokines – offers an interesting perspective. Studies have shown that treatment of C2C12 myotubes with TNF-α induces the upregulation of inflammatory cytokine gene expression and release [21, 22]. Moreover, cytokine and chemokine release is regulated by NF-κB activation, mediated by free radical

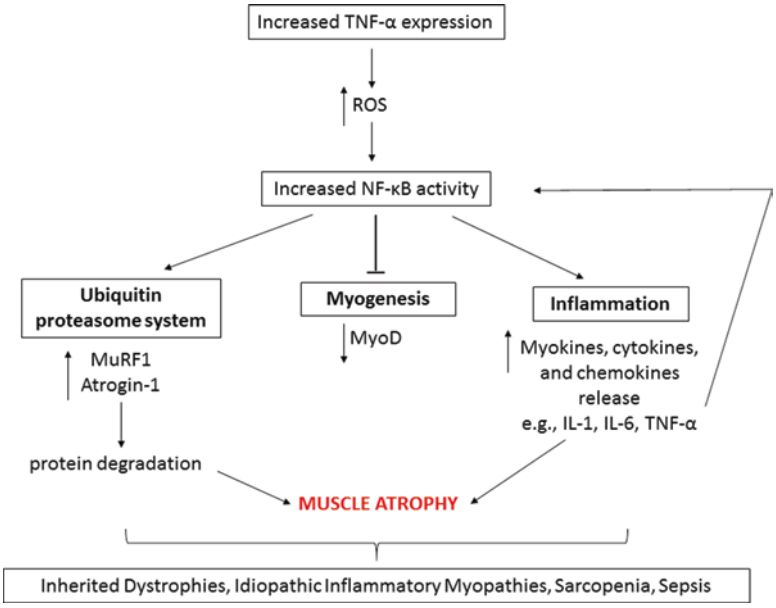

Fig. 12.1 NF-κB pathway

generation by the mitochondria [22]. The release of catabolic cytokines such as IL-6 may have paracrine signalling effects on neighbouring fibres and may self-perpetuate atrophy. The perspective of muscle-derived cytokines (myokines) rather than solely derived from immune cells is an additional facet to disease pathogenesis in myopathologies.

In muscle atrophy, we typically observe an imbalance in protein synthetic and degradative pathways. Specifically, we see activation of the ubiquitin-proteasome network – which regulates protein degradation. Poly-ubiquitination of proteins by the E3 ubiquitin ligases muscle RING finger protein 1 (MuRF1) and atrogin-1 targets proteins for degradation via the proteasome [23, 24]. There is now elegant evidence which describes how NF-κB signalling and the ubiquitin-proteasome pathway are intertwined in the context of atrophy. Overexpression of IKKβ in a murine model was elevated MuRF1 expression – which was ablated in MuRF1-knockout cross strain [25]. Moreover, a study using a muscle-specific knockout of IKKβ in a mouse model, prevented NF-κB activation, and subsequent muscle wasting in response to denervation [26]. Thus, there is elegant evidence which shows the interplay between protein degradation pathways and NF-κB activation in terms of muscle atrophy. Mechanistically, this evidence provides a strong justification in the pursuit of NF-κB-modifying therapies and agents in an effort to combat muscle-wasting disorders.

12.2.4 Therapeutic Targeting of NF-κB Activation

There is now a bourgeoning array of both synthetic and natural compounds, which have been characterised to target different aspects of NF-κB signalling. Given the strong association with NF-κB and muscle atrophy, it is perhaps logical to pursue interventions in this context. Some focus has been on targeting in the activators of NF-κB activation, with the focus on TNF-α. There is an array of biologics, comprising either monoclonal antibodies to TNF-α (e.g. infliximab) or decoy TNF receptors (e.g. etanercept) – which have been put to great use in the rheumatic diseases [27]. Both of these drugs have been tested and shown some beneficial effects in the *mdx* model of DMD – reducing myonecrosis and suppressing overall inflammation [28, 29]. In contrast, in patients with myositis, who often have elevated expression of TNF-α in muscle, the effectiveness of anti-TNF therapies is not convincing [30]. If indeed NF-κB signalling has a role to play in muscle atrophy in myositis, then perhaps more NF-κB-centric therapies may be worthy of pursuit. In terms of more NF-κB-centric/selective therapies, the NEMO-binding domain (NBD) peptide offers that opportunity. The NBD peptide disrupts the correct assembly of the IKK complex – which prevents canonical NF-κB pathway activation. Utilisation of NBD peptide in the *mdx* model of DMD significantly reduced macrophage invasion into muscle and reduced overall membrane damage/lysis [20] . The salicylates have also been shown to have the capacity to inhibit NF-κB activation [31]. Administration of sodium salicylate in aged mice results in downregulation in inflammatory gene expression and improved repair of muscle [32]. In terms of natural compounds to target NF-κB signalling, curcumin (the primary curcumoid component of turmeric) harbours anti-NF-κB properties [33]. Treatment of *mdx* mice with curcumin resulted in improved muscle strength, increased sarcolemmal integrity and a downregulation of inflammatory markers [34].

12.3 Cytokines in Muscle Atrophy

12.3.1 Tumour Necrosis Factor-Alpha

TNF-α is a 157-amino-acid-long peptide encoded on the short arm of chromosome 6 in humans [35] and exists in both soluble and membrane-bound forms. TNF-α is initially produced as 26 kDa membrane-spanning protein, anchored in place due to a 79-amino acid precursor sequence. Subsequent proteolytic cleavage frees TNF-α from the membrane into a 17 kDa soluble form [36]. TNF-α exists in circulation as a homotrimer, approximately 52 kDa in size [37], which binds to approximately 25 different receptors [7]; however, the most prevalent and well characterised are TNF receptors 1 and 2 (TNFR-1/2) [38]. TNFR-1 is fairly ubiquitously expressed across a range of cell types, whereas TNFR-2 seems to be more confined to cells of a haematopoietic origin [39]. Moreover, the vast majority of biological functions of

TNF-α occur via TNFR-1 [40]. The signalling cascade initiated via TNF-α binding of TNFR-1 is very well characterised (Fig. 12.1). The TNF-α homotrimer binds the TNFR-1 forming the TNF-TNFR-1 complex, where the intracellular domain is recognised and recruits TNF-receptor-associated death domain (TRADD) to the complex. Additional adaptor proteins are recruited to the complex, namely, receptor-interacting protein (RIP) and TNF-R-associated factor 2 (TRAF-2). The function of TRAF-2 is to recruit the protein cellular inhibitor of apoptosis 1 (cIAP-1) which also activates the mitogen-associated protein kinase pathway (MAPK) [40]. However, RIP is a key component of TNF-α signalling by the activation of nuclear factor kappa B (NF-κB).

12.3.2 TNF-α and Skeletal Muscle Wasting

The biological importance of TNF-α was demonstrated in several key studies throughout the 1970s and 1980s. TNF-α was originally discovered over 30 years ago as a serum soluble molecule, released by macrophages, which suppressed tumour growth significantly in mice [41]. TNF-α was characterised to be the hormone termed cachectin, which induced profound cachexia in mice [42]. Treatment of rats with recombinant TNF-α was found to induce a state of septic shock [43]. Administration of anti-TNF-α antibodies during endotoxin-induced insult provided protection against septic shock-induced cachexia and reduced overall morbidity [44]. These important studies provided a key insight into the deleterious role of TNF-α during instances of profound bacterial infection and that TNF-α is likely to be a key mediator of cachexia (muscle atrophy). Sepsis patients characteristically present with profound elevations in circulating levels of TNF-α [45]. Elevated circulating TNF-α is a key driver in the significant loss of total protein ~16%, which occurs over a 3-week period in patients with severe sepsis [46]. Moreover, experimental rodent models of sepsis have shown that reduced protein synthesis is associated with disrupted ribosomal s6 kinase phosphorylation in a TNF-α-dependent manner [47].

The exposure of muscle to TNF-α results in a loss of total muscle protein, a process that is reported to be regulated by NF-κB; additionally the loss of muscle protein demonstrated in this study was correlated with elevated ubiquitin conjugation and augmented by endogenous production of ROS [48]. Overexpression of the IκBα protein (which holds NF-κB in its inactive state) in muscle results in resistance to TNF-α-induced protein loss [49]. Studies examining the inhibition of NF-κB activation in vivo demonstrated improved skeletal muscle regeneration following trauma [50]. Thus, there is a clear association between TNF-α, NF-κB activation and muscle atrophy.

Although the loss of muscle protein as a consequence of TNF-α exposure is profound, it has been reported that the loss of muscle protein is superseded by a significant fall in specific force generation by muscle [51]. Studies into muscle contractility

in the diaphragm consistently report a fall in specific force generation in response to elevated levels of TNF-α [52]. Moreover this occurrence has been reported in the absence of muscle wasting [53]. Further studies have demonstrated loss of muscle function in the absence of atrophy, via TNF-α-induced activation of caspase-3, which may be due to the loss of the actin and myosin contractile filaments [54]. Studies have reported that TNF-α-induced loss of muscle protein occurs via the ubiquitin-proteasome pathway [55]. The ubiquitin-proteasome controls cellular proteolytic degradation of ubiquitinated proteins [56]. TNF-α administration induces elevation in ubiquitin expression and upregulation of markers associated with proteolytic degradation [55]. Upregulation of ubiquitin-conjugating activity in skeletal muscle has been reported to occur in a TNF-α/NF-κB-dependent manner [57].

The loss of muscle mass and significant reduction in muscle force as a result of TNF-α exposure have been widely described to be associated with elevated production of ROS [48]. Using a rodent model of TNF-α-induced cachexia, muscle loss was found to be ablated following pre-treatment with nitro-L-arginine, a known nitric oxide synthase (NOS) inhibitor [58]. The upregulation of NF-κB by TNF-α in skeletal muscle is reported to be controlled, in part, by the glutathione pathway; suppression of glutathione reductase activity reduced TNF-α-induced NF-κB activation [59]. More recently, treatment of muscle fibres with the antioxidant trolox (a vitamin E derivative) resulted in attenuation in the TNF-α-induced fall in specific force generation by muscle [51]. Moreover, the specific effect of ROS on muscle wasting has been investigated widely. Treatment of C2C12 myotubes with hydrogen peroxide (H_2O_2) resulted in the upregulation of the expression of ubiquitin ligases responsible for controlling protein degradation via the proteasome [60]. ROS-mediated muscle proteolysis has also been associated with Ca^{2+} calpain activity. Elevated formation of reactive aldehyde complexes by ROS causes accumulation of Ca^{2+} in the cytosol, due to disruption of Ca^{2+} transport across the plasma membrane [61], thus, inducing calpain-mediated cleavage of key proteins such as titin and nebulin, which are components of the contractile architecture [62]. Although the effect of ROS on skeletal muscle is profound, it is still unclear whether elevated ROS forms part of a downstream signalling cascade that mediates muscle atrophy.

12.3.3 Role of Other Cytokines in Muscle Atrophy

Although TNF-α is arguably one of the most well-studied cytokines in the context of muscle atrophy, there are other cytokines/chemokines which have an important role to play. Interleukin-6 (IL-6) is a classical pro-inflammatory cytokine, which harbours ancillary function in terms of influencing metabolism [63, 64]. A seminal study in the mid-1990s, whereby treatment of transgenic IL-6 overexpressor mice with an IL-6 receptor antibody, ameliorated muscle atrophy in this model [65]. Similarly, more recent evidence in the Apc (Min/+) murine model exhibit IL-6-dependent muscle atrophy – mediated through activation of atrogin-1 [66]. In a further rodent study, IL-6 was reported to induce atrophy via downregulation of

ribosomal S6 kinase phosphorylation – favouring a more catabolic state [67]. Moreover, in vitro studies in murine C2C12 cells have demonstrated IL-6 to inhibit myogenic differentiation [68]. There is also clinically relevant evidence for an important role for IL-6 in muscle atrophy. Patients with polymyositis and dermatomyositis present with elevated circulating levels of Il-6, which correlate with disease severity [69]. Moreover, use of an anti-IL-6R monoclonal antibody ameliorated disease progression in a murine C-reactive protein-induced model of myositis [70]. In addition a small cohort of treatment refractory polymyositis patients treated with the commercial anti-IL-6R tocilizumab has showed beneficial clinical outcomes – evidenced by reduced circulating creatine kinase levels and suppressed myo-oedema [71]. Interestingly, there has been an observation of acquired inflammatory myopathy developing in a patient treated with tocilizumab – however, this is an exceptionally rare occurrence [30]. Overall, there is strong mechanistic evidence for the role of IL-6 in muscle atrophy – with significant interest from global pharma in pursuing trials of anti-IL-6 therapies in a range of myopathies.

12.4 Future Perspectives

Our understanding of the basic biology, which mediates the impact of NF-κB and inflammatory cytokines on muscle, has developed exponentially over the last decade. The potential to target NF-κB signalling to target muscle wasting in a range of myopathologies is an attractive proposition. Currently, however the vast majority of success has been in animal models – with limited evidence in humans. Thus, there is still a crucial need to better understand the precise impact and potential long-term effects of NF-κB-modulating therapies.

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Chapter 13
Redox Homeostasis in Age-Related Muscle Atrophy

Giorgos K. Sakellariou and Brian McDonagh

Abstract Muscle atrophy and weakness, characterized by loss of lean muscle mass and function, has a significant effect on the independence and quality of life of older people. The cellular mechanisms that drive the age-related decline in neuromuscular integrity and function are multifactorial. Quiescent and contracting skeletal muscle can endogenously generate reactive oxygen and nitrogen species (RONS) from various cellular sites. Excessive RONS can potentially cause oxidative damage and disruption of cellular signaling pathways contributing to the initiation and progression of age-related muscle atrophy. Altered redox homeostasis and modulation of intracellular signal transduction processes have been proposed as an underlying mechanism of sarcopenia. This chapter summarizes the current evidence that has associated disrupted redox homeostasis and muscle atrophy as a result of skeletal muscle inactivity and aging.

Keywords Sarcopenia · Redox signaling · Antioxidants · Nerve · Superoxide

13.1 Background

Loss of skeletal muscle mass and function is among the most consistent and striking change associated with the advance of age [1]. Age-related muscle atrophy (sarcopenia) is described as a progressive loss of lean muscle mass and muscle function, which has a significant effect on the quality of life of older people and overall morbidity. A reduction in overall muscle function with age is linked to an increased mortality risk [2], which leads to instability, a subsequent increased risk of falls and consequently an increased demand for medical and social care. Deficits in skeletal muscle begin at a relatively young age and continue until the end of life [3]; human studies have reported that by the age of 70, there is a 25–30% reduction in the fiber

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cross-sectional area of skeletal muscle and a subsequent reduction in muscle strength by 30–40% [4].

Reduced muscle mass and contractile force inherent with aging have been extensively studied in both murine models and humans and are associated with various neurological impairments including loss of motor units [5, 6], structural alterations and degeneration of neuromuscular junctions (NMJ) [7–10], a decline in motor nerve function (partial denervation) [9, 11–13], impaired nerve redox signaling [14], and changes in fiber type related to continual cycles of denervation and reinnervation [15]. While physical activity can inhibit the decline of muscle functional deficits [16], even physically active older adults exhibit age-related deficits in muscle mass and function [17]. Age-related muscle atrophy and weakness is a lifelong process with a multifactorial and complex etiology that involves both extrinsic and intrinsic factors [15]. However, elucidation of the primary molecular and biochemical mechanisms underlying the age-related decline in neuromuscular integrity and function has yet to be determined.

13.2 Reactive Oxygen and Nitrogen Species (RONS) Produced by Skeletal Muscle

The cellular damage induced by O_2 toxicity was first reported more than 50 years ago and related to the increased generation of reactive species [18, 19], as a result of derivatives of O_2 (Fig. 13.1). Studies in the 1980s reported that reactive species are endogenously generated in skeletal muscle [20–22]. It has since been determined that both resting and contracting myofibers can generate reactive oxygen and nitrogen species (RONS). Reactive oxygen species (ROS) refer to O_2-derived molecules that are reactive species including O_2-centered radicals but also non-radical species which are reactive derivatives of O_2 [23]. Similarly, the term reactive nitrogen species (RNS) refers to both nitrogen radicals along with other reactive molecules where the reactive center is nitrogen [24–26]. RONS generation by skeletal muscle has been detected and quantified by a variety of methods including fluorescence-based

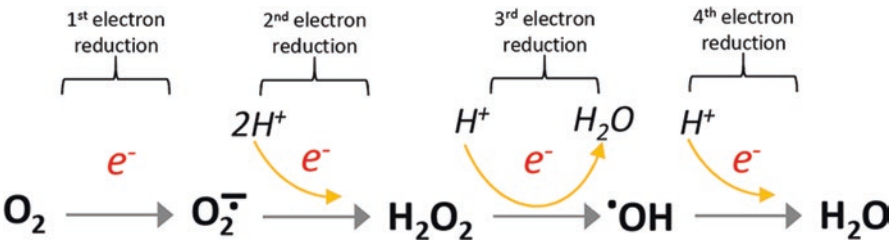

Fig. 13.1 Reactive oxygen derivatives produced by the sequential reduction of $O_2$to H_2O. Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$). (Redrawn from Sakellariou et al. [88])

microscopic assays [27, 28], spectrophotometry [29, 30], chemiluminescence [31, 32], HPLC techniques [33, 34], electron spin resonance spectroscopy (also known as electron paramagnetic resonance, EPR) [35, 36], and transfection methods including in vivo [37, 38] and in vitro [39]. Using a combination of the above techniques, it has been determined that the primary radical species generated by skeletal muscle include superoxide and nitric oxide (NO) [26, 40, 41].

13.2.1 Superoxide

Superoxide is derived either from the incomplete reduction of O_2 during metabolism in the electron transport chain (ETC) or as a specific product of dedicated enzymatic systems [42]. The subcellular location of superoxide generation in skeletal muscle is dependent on whether the muscle is quiescent or contracting, as different pathways are involved. Figures 13.2 and 13.3 depict the different sites within skeletal muscle and proposed reactions for RONS generation. Superoxide generation is associated with electron leakage and incomplete O_2 metabolism by mitochondrial ETC including complex I and complex III [43, 44] but also more recently complex II [45–47]. However, dedicated enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes including NOX2, NOX4, DUOX1, and DUOX2 [28, 29, 32, 48], xanthine oxidase (XO) [49, 50], and the lipoxygenases (LOXs) [51] which are linked to arachidonic acid (AA) release by the phospholipase A_2 enzymes (PLA_2) [52, 53] are also sources of superoxide; for a detailed review, see Ref. [54].

13.2.2 Nitric Oxide

Nitric oxide (NO) is endogenously generated within cells by the nitric oxide synthases (NOS), through the conversion of L-arginine to citrulline utilizing NADPH as a cofactor [55]. NO is a primary radical, and its concentration has been demonstrated to be regulated by NOS isoenzymes: the neuronal NOS (type I or nNOS), the inducible NOS (type II or iNOS), and the endothelial NOS isoenzyme (type III or eNOS) [54, 56]. nNOS was originally discovered in neuronal tissue but has also been shown to be expressed in the plasma membrane of skeletal muscle fibers where it interacts with the dystrophin-glycoprotein complex via a linkage to α1-syntrophin [57]. The eNOS isoenzyme was originally described in the endothelium where it is associated with caveolin-1; in skeletal muscle it is localized in the mitochondria and has been reported to be activated by heat shock protein 90 (HSP90) [58]. The expression of iNOS in skeletal muscle is increased in response to inflammatory conditions or following a septic challenge [59, 60]. NO has shown to interact with a number of different cytoskeletal proteins mainly through reactive cysteine residues and the formation of S-nitrosated residues [61]. The nNOS isoform is particularly

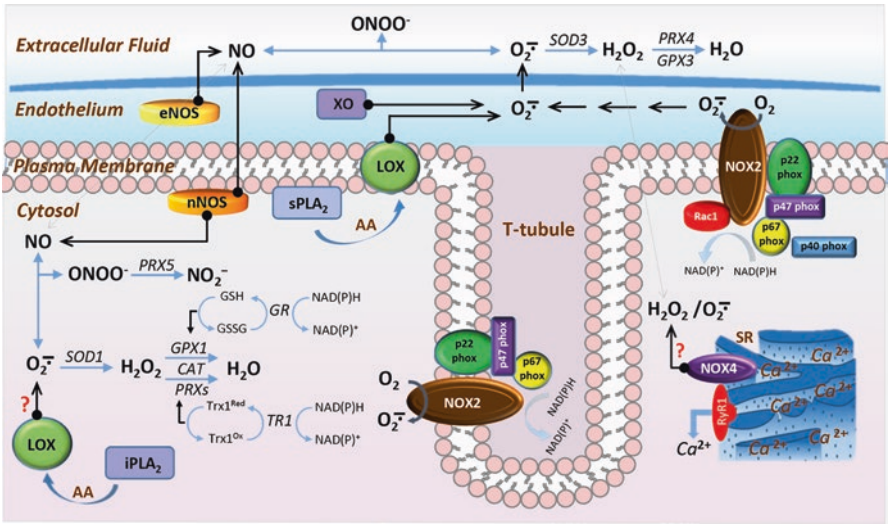

Fig. 13.2 Schematic representation of the non-mitochondrial sites for nitric oxide and superoxide production in skeletal muscle. Superoxide ($O_2^{\bullet-}$) is produced by multicomponent NAD(P)H oxidase 2 (*NOX2*), xanthine oxidase (*XO*), and the lipoxygenases (*LOX*) which activity is regulated by the phospholipase A_2 enzymes (PLA_2). Arachidonic acid (*AA*) release by the membrane bound calcium-dependent PLA_2 ($sPLA_2$) facilitates extracellular $O_2^{\bullet-}$ release by the membrane bound LOX. It is uncertain whether the cytosolic LOX enzymes contribute to intracellular $O_2^{\bullet-}$ changes which substrate availability might be regulated by the cytosolic calcium-independent PLA_2 ($iPLA_2$). NAD(P)H oxidase 4 (*NOX4*) also contributes to ROS changes, though the primary ROS product, $O_2^{\bullet-}$ or hydrogen peroxide (H_2O_2) of NOX4 is uncertain. Cytosolic and extracellular $O_2^{\bullet-}$ is dismuted into H_2O_2 by superoxide dismutase (*SOD*), SOD1 and SOD3, respectively, or reacts rapidly with membrane permeant nitric oxide (*NO*) produced by the endothelial and neuronal nitric oxide synthase (*eNOs* and *nNOS*) to form peroxynitrite ($ONOO^-$). H_2O_2 formed within the extracellular space is reduced into H_2O by the action of glutathione peroxidase 3 (*GPX3*) or peroxiredoxin IV (*PRX4*), while cytosolic H_2O_2 is reduced into H_2O by glutathione peroxidase 1 (*GPX1*), catalase (*CAT*), or peroxiredoxins (*PRXs*). Reduced glutathione (*GSH*) provides the electrons to GPX to catalyze the reduction of H_2O_2; GSH is oxidized to glutathione disulfide (*GSSG*). Reduction of GSSG is catalyzed by glutathione reductase (*GR*), where NAD(P)H is used as the reducing agent. Cytosolic *PRXs* utilize thioredoxin 1 ($Trx1^{Red}$) for their reducing action. Oxidized form of Trx1 ($Trx1^{Ox}$) is reduced by thioredoxin reductase 1 (*TR1*), by utilizing electrons from NAD(P)H. $ONOO^-$ can be reduced predominantly into nitrite ($NO2^-$) by peroxiredoxin V (*PRX5*). Sarcoplasmic reticulum (*SR*). (Redrawn from Sakellariou et al. [88])

expressed in glycolytic or fast muscle fibers [62] and has been suggested to be the primary source of NO release from myocytes [63]. The close proximity of nNOS to the dystrophin-glycoprotein complex has a pivotal role in skeletal muscle physiology as highlighted from studies utilizing the mdx mice [64] but also in humans suffering from muscle dystrophy [57, 65]. It has been suggested that NO has a direct functional signaling role via the formation of S-nitrosylated sites with effects on protein activity or indirectly by interactions with heme or nonheme Fe and Cu [66].

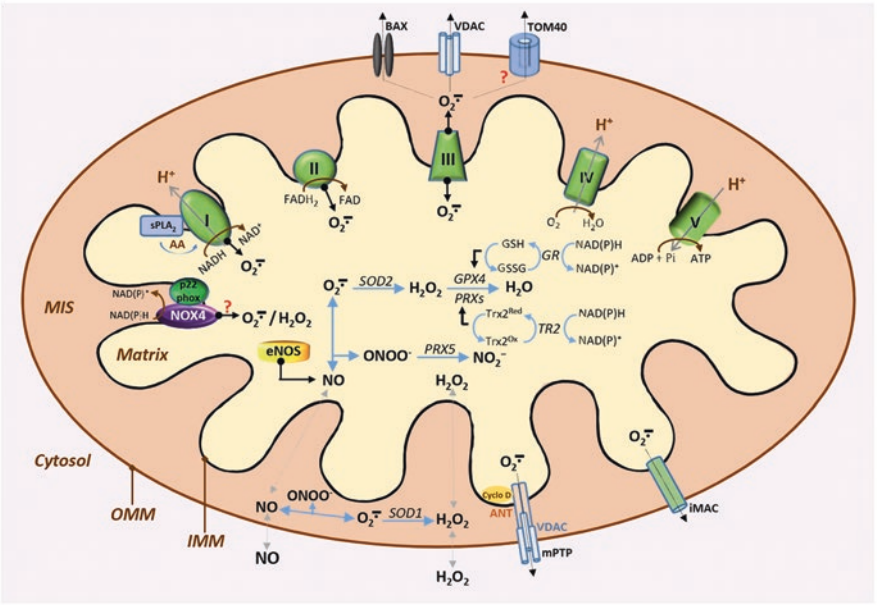

Fig. 13.3 Schematic representation of the mitochondrial sites for nitric oxide and superoxide production and the channels that mediate the release of superoxide to the cytosolic compartment in skeletal muscle. Superoxide ($O_2^{\bullet-}$) is produced by complex I, complex II, and complex III of the mitochondrial electron transport chain (ETC) of the inner mitochondrial membrane (*IMM*) and released into the matrix and the mitochondrial intermembrane space (*MIS*). NAD(P)H oxidase 4 (*NOX4*) also contributes to ROS changes, though the primary ROS product, $O_2^{\bullet-}$ or hydrogen peroxide (H_2O_2) of NOX4 is uncertain. Arachidonic acid (*AA*) release by the calcium-dependent phospholipase A_2 enzymes ($sPLA_2$) interacts with complex I and enhances superoxide generation by this complex. $O_2^{\bullet-}$ released into the matrix, and the MIS is dismuted into H_2O_2 by superoxide dismutase (*SOD*), SOD2 and SOD1, respectively, or reacts rapidly with nitric oxide (*NO*) produced by the endothelial nitric oxide synthase (*eNOS*) to form peroxynitrite ($ONOO^-$). H_2O_2 is reduced into H_2O by the action of glutathione peroxidase 4 (*GPX4*) or peroxiredoxins (*PRXs)*. Reduced glutathione (*GSH*) provides the electrons to GPX4 to catalyze the reduction of H_2O_2; GSH is oxidized to glutathione disulfide (*GSSG*). Reduction of GSSG is catalyzed by glutathione reductase (*GR*), where NAD(P)H is used as the reducing agent. Mitochondrial *PRXs* utilize thioredoxin 2 ($Trx2^{Red}$) for their reducing action. Oxidized form of Trx2 ($Trx2^{Ox}$) is reduced by thioredoxin reductase 2 (*TR2*), by utilizing electrons from NAD(P)H. $ONOO^-$ can be reduced predominantly into nitrite ($NO2^-$) by peroxiredoxin V (*PRX5*). $O_2^{\bullet-}$ is essentially membrane impermeant, while H_2O_2 is readily diffusible. Matrix $O_2^{\bullet-}$ can diffuse to the cytosol through the inner membrane anion channel (*iMAC*) that spans the IMM and the outer mitochondrial membrane (*OMM*) or via the mitochondrial permeability transition pore (*mPTP*) comprised of the voltage-dependent anion channels (*VDAC*) on the OMM, the adenine nucleotide translocator (*ANT*) located on the IMM, and cyclophilin D (*Cyclo D*) located in the matrix. Channels of the OMM including VDAC, BAX, and possibly the translocase of outer membrane 40 (*TOM40*) can also mediate the release of $O_2^{\bullet-}$ from the MIS to the cytosol. (Redrawn from Sakellariou et al. [88])

13.2.3 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a relatively stable molecule in comparison with the other reactive species with a longer half-life; hence H_2O_2 been suggested as the most likely candidate for redox signaling pathways [67]. H_2O_2 can interact with redox-sensitive components or pathways typically via oxidation of sensitive Cys residues and has been demonstrated to regulate the activity of a variety of transcription factors in skeletal muscle [68]. In aqueous solutions, superoxide can be protonated to produce hydroperoxyl radical or reduced undergoing a dismutation reaction to produce H_2O_2 [69]. In addition, a number of enzyme systems have also been reported to generate H_2O_2 including NOX4 [70, 71], urate, and amino acid oxidases [72]. Moreover, recent evidence supports endoplasmic reticulum (ER) H_2O_2 generation in vivo [73] via thiol-disulfide exchange mechanisms [74]. The catalytic activity of a wide range of metabolic enzymes can be modulated by H_2O_2, typically by oxidation of catalytic Cys residues or residues essential for disulfide bonds [75]. In addition there are a number of different enzymes that use H_2O_2 as a substrate including the peroxiredoxins, glutathione peroxidases, and catalase; isoforms of these enzymes are located in specific cellular locations which would suggest that it plays an important physiological signaling role.

13.2.4 Hydroxyl Radical

The hydroxyl radical is a highly reactive molecule due to its strong oxidizing potential and can rapidly react with biomolecules located close to its site of generation. In skeletal muscle fibers and other biological systems, hydroxyl radicals are typically generated as a result of the Fenton reaction that involves the reductive decomposition of H_2O_2 with reduced transition metal ions, copper (Cu) or iron (Fe) [76]. Oxidation of FeS cluster enzymes can result in an increase of "free iron" within the cell, allowing for the formation of hydroxyl radicals and altered redox homeostasis [77]. Similar to the Fenton reaction, the Haber-Weiss reaction can also generate hydroxyl radicals by Fenton chemistry, Fe or Cu is maintained in a reduced form by superoxide, which can result in the formation of hydroxyls from H_2O_2 [78]. There is some in vivo evidence to suggest that during skeletal muscle contractile activity, there is enhanced hydroxyl radical generation [79]. An increased intracellular concentration of highly reactive hydroxyl radicals can affect calcium dynamics and maximum force of skeletal myofibers [76]. There are a number of neuromuscular disorders such as including glucocorticoid-induced myopathy [80] and immobilization-induced skeletal muscle atrophy [81] that have reported an increase in hydroxyl radical formation.

13.2.5 Peroxynitrite

Peroxynitrite is another endogenously generated reactive species that can act as an intracellular oxidant; it is primarily generated by the reaction between NO and superoxide, often as a result of the close proximity of NOX and NOS enzymes [82]. Further evidence to support endogenous generation of peroxynitrite in skeletal muscle is shown in studies using transgenic animals where the levels of NO and/or superoxide were elevated [34]. Similar to the some of the other reactive species, peroxynitrite can oxidize sensitive Cys residues involved in disulfides or catalytic sites [83]. The protonated form, peroxynitrous acid, is also highly reactive and can oxidize Cys residues resulting in protein oxidation, phospholipid and DNA damage [82, 84]. It has also been reported that peroxynitrite is involved in tyrosine nitration [85] as well as the formation of S-nitrosylated Cys residues [86]; mass spectrometry approaches have identified an increasing number of proteins being nitrosylated and nitrated in skeletal muscle. In conditions where there are high concentrations of peroxynitrite, it can result in reversible and irreversible oxidation of cellular compartments of myofibers [34, 87], affecting overall enzymatic activity through structural modifications, including altered cytoskeletal dynamics and an impair cell signal transduction [82].

13.3 Primary Antioxidant Enzymes Expressed in Skeletal Muscle

Skeletal muscle expresses a sophisticated system to control the production of oxidants and protect the myofibers from oxidative damage. The system that functions to prevent oxidative damage consists of enzymatic and nonenzymatic antioxidants that work in a coordinated fashion to regulate redox disturbances in the muscle cell. An extended coverage of these goes beyond the scope of this chapter (for detailed review, see Ref. [88]. However, we summarize the most important enzymatic systems expressed in skeletal muscle including superoxide dismutases, catalase, glutathione peroxidases, peroxiredoxins, and glutaredoxins.

Superoxide dismutase (SOD) was discovered in 1969 and represents a family of metalloenzymes that catalyze the one electron dismutation of superoxide into O_2 and H_2O_2 [26]. There are three SOD isoenzymes depending on the metal ion bound to the active site. Skeletal muscle expresses copper-zinc SOD (*SOD1* or CuZnSOD), which is a highly stable enzyme present within the cytosol and the mitochondrial intermembrane space (MIS), and manganese-SOD (*SOD2* or MnSOD) which is found in the mitochondrial matrix [89]. There is however an additional isoform of SOD, the extracellular SOD isoenzyme (*SOD3* or EcSOD) [90] which is present in the interstitial spaces of tissues and extracellular fluids of many cell types and tissues and its primary function is to reduce superoxide formed outside the cell membrane [90].

Catalase (CAT), a homotetramer with a molecular mass of 240kDa catalyzes the reduction of H_2O_2 into H_2O and O_2. CAT is mainly found in the cytosolic compartment of the muscle fibers and requires heme (Fe^{3+}) bound at the enzyme's active site for its catalytic function [91]. CAT enzymatic activity increases with increased H_2O_2, and reports have shown that protein expression and activity is higher in highly oxidative myofibers [92]. CAT does not require reducing equivalents to function as a H_2O_2 reducer; thus CAT is considered an energy-efficient antioxidant [93].

Glutathione peroxidase (GPX), a homotetramer with each 22kDa subunit containing a selenium atom in the form of a selenocysteine, also catalyzes the reduction of H_2O_2 to H_2O or organic hydroperoxides (ROOH) to alcohol, using reduced glutathione (GSH) or in some cases thioredoxin (TRX) or glutaredoxin (GRX) as an electron donor [94]. In addition, reports also suggest that GPX is also implicated in the reduction of hydroxyl radical by elimination of H_2O_2 [95]. Mammalian cells express five isoforms of GPX (GPX1-GPX5), which differ in cellular localization and substrate specificity [96] with GPX1 as the cytosolic form [97] and GPX4 as the most widely expressed. GPX4 is a membrane-associated enzyme, partly localized to the MIS. GPX3 also known as plasma or extracellular GPX is present in the extracellular space [98, 99], whereas GPX2 is mainly expressed in the gastrointestinal system [100]. GPX5 is expressed in the epididymis in the mammalian male reproductive tract and is the least studied isoenzyme [100, 101]. The expression of the GPX genes is controlled by different mechanisms including O_2 tension, metabolic rate, toxins, and xenobiotics [23] as well as growth and development [102]. Similarly, to CAT, oxidative muscle fibers express higher amounts of GPX compared with glycolytic myofibers [100]. Though there is an overlap between the function of GPX and CAT, GPX has a higher affinity for H_2O_2 at low concentrations. However, under conditions where H_2O_2 is significantly increased, CAT becomes more significant in protecting biological systems, and its catalytic function prevails since it cannot be saturated under any H_2O_2 concentration since there is no apparent Vmax [103].

Peroxiredoxins (PRXs) initially described as thiol-specific antioxidants [104] were discovered in the late 1980s [105, 106] and are a family of cysteine-dependent thioredoxin peroxidases [107]. PRXs are capable of reducing both ROOH and H_2O_2 [108] with the use of electrons provided by thioredoxins [108]. Skeletal muscles express six isoforms of PRXs, which are present in the cytosolic compartment (PRX I, II, VI), the mitochondrion (PRX III), the extracellular space, and endoplasmic reticulum (PRX IV) [42]. PRXV is expressed in the cytosol, mitochondria, nuclei, and perixosomes [108] and is considered a peroxynitrite reductase [109]. PRX proteins have recently received much attention as they have shown to play a key role in transmitting redox signals into a dynamic biological response and to have subtle changes in both abundance and oxidative state with age [35, 110, 111].

Glutaredoxins (GRXs) are small ubiquitous disulfide oxidoreductases which share many of the functions of TRXs but are reduced by GSH rather than a specific reductase [122]. GRXs are small redox enzymes that exist in either a reduced or oxidized form and are involved in the protection and repair of protein and nonprotein thiols during compromised redox homeostasis [112]. GRXs are divided into

monothiol (Cys-X-X-Ser) and dithiol (Cys-X-X-Cys) GRXs [113]. Dithiol GPXs participate in the regulation of H_2O_2 via PRX pathways [114], proliferation and differentiation [115], transcription regulation via modulating the activity of nuclear factor κB (NFκB) [116], and apoptosis [117]. Monothiol GRXs are implicated in iron sulfur (FeS) cluster biosynthesis and Fe homeostasis [118]. GRX1 prevents oxidative damage and apoptosis and is found in the cytosol, and the MIS. GPX1 has also shown to translocate into the nucleus and exported from the cell [113]. GRX2 is localized in the mitochondria [119] and GRX3 in the nuclear and cytosolic compartment. Monothiol GRX5 has a mitochondrial translocation signal and shares the active-site motif of GRX3 [120]. Reports have also revealed that the GRX system can also catalyze reversible protein glutathionylation [121] and regulate the redox state of thiol groups [122] during aberrant redox control.

In addition to the main antioxidant enzyme defense network, skeletal muscle also expresses glucose-6-phosphate dehydrogenase (G6PD) and isocitrate dehydrogenase (IDH) which do not directly scavenge RONS but play a pivotal role in redox regulation by providing reducing power in the form of NADPH to the antioxidant enzymatic systems [123]. In addition, skeletal muscle also contains nonenzymatic antioxidants, which regulate reactive species and protect muscle cells from oxidative injury. These are H_2O soluble and fat soluble and are classified into two categories: (i) the endogenously produced and (ii) dietary antioxidants which cannot be synthesized or induced and must be taken from the diet. The main nonenzymatic antioxidants found in myofibers include GSH, uric acid, bilirubin, and coenzyme Q_{10} endogenously produced antioxidants but also dietary antioxidants including vitamin C, vitamin E, and carotenoids. An extended coverage of the nonenzymatic defense systems in skeletal muscle goes beyond the scope of this review; for a detailed review, see Refs. [124, 125].

13.4 Age-Related Muscle Atrophy Is Linked to Increased Oxidative Damage

The dual role of RONS to act as signaling molecules at low concentrations but also damage critical cellular compartment when produced at high concentrations is fundamental in skeletal muscle physiology/pathology. Reports in humans [126–128] and rodents [87, 129, 130] have provided evidence that age-related muscle atrophy is linked to an altered oxidative status of redox-responsive proteins [131], elevated concentration of oxidized macromolecules including an increase in DNA damage [126, 132], increased levels of lipid peroxidation [133, 134], and accumulation of oxidized proteins [127, 128]. Increased DNA damage has been shown to alter genetic stability which may induce the expression of genes that regulate cell proliferation and/or block the expression of certain genes, thus permitting damage with increasing age [135]. RONS-induced DNA sequence changes or mutations have been suggested to affect the cellular state of differentiation [23, 136] and

accumulation of mitochondrial DNA damage [132] which may prevent the rejuvenation of the mitochondrial population and lead to bioenergetic decline and cellular death [137]. In addition, aged skeletal muscle exhibits an accumulation of catalytically inactive or less active forms of enzymes and the observed age-related changes in catalytic activity have been suggested to occur due to oxidative modifications induced by RONS [138, 139].

Recent reports have provided evidence that increased oxidative damage inherent with aging is linked to age-associated changes in RONS, with myofibers from old rodents exhibiting increased intracellular RONS levels compared to young/adult rodents [140, 141]. Oxidants can modulate various intracellular signal transduction pathways, and age-related disruption of these processes due to compromised redox homeostasis has been suggested as contributing factor to muscle atrophy inherent with aging. The role of redox homeostasis in age-related muscle atrophy and weakness has been studied in various model organisms (reviewed in [88]) which have undergone genetic manipulations (transgenic and knockout models) and have provided insight into the function of RONS regulatory systems in neuromuscular aging.

13.5 Deletion of Cu-Zn Superoxide Dismutase in *SOD1*$^{-/-}$ Mice Leads to Accelerated Neuromuscular Aging and Functional Deficits

The association between redox regulation and age-related atrophy has been studied in several mammalian models which have undergone genetic manipulations (reviewed in [88]), to enable the study of disrupted redox signaling on the aging process. Deletion of CuZnSOD in mice (*SOD1*$^{-/-}$ mice) leads to a reduction in lifespan and an accelerated aging phenotype associated with myofiber atrophy (Fig. 13.4), neurological impairments (Fig. 13.5), and functional deficits [142]. Elevated oxidative damage has also been observed in skeletal muscles from

Fig. 13.4 Gross morphology of skinned hindlimb and forelimb muscles of *SOD1*$^{-/-}$ and *WT* mice at 12 months of age. Arrows indicate the phenotypic hindlimb muscle changes observed in *SOD1*$^{-/-}$ compared to *WT* mice. (Redrawn from Sakellariou et al. [14])

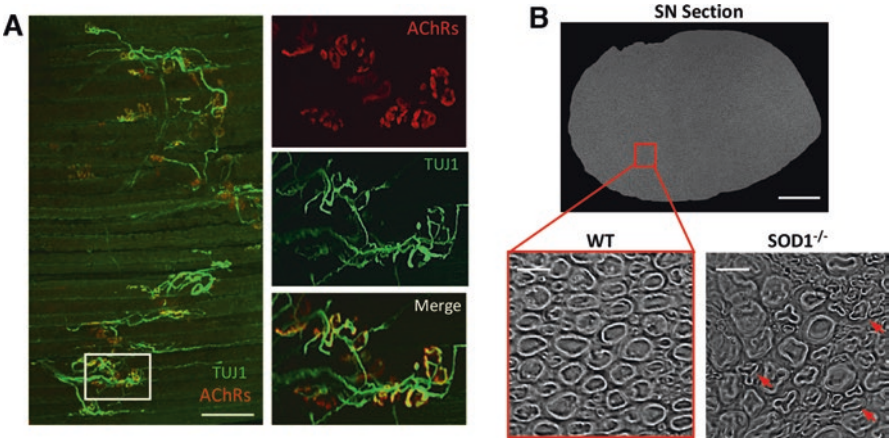

Fig. 13.5 Neuromuscular junction structure and peripheral nerve integrity in *SOD1*$^{-/-}$mice. (**a**) Intravital immunofluorescence imaging of neuromuscular junctions (NMJ) of an *AT* muscle from a *SOD1*$^{-/-}$ mouse. Presynaptic motor neurons immunolabeled with neuronal class III β-tubulin monoclonal antibody (TUJ1), a neuronal marker (green), and postsynaptic motor endplate acetylcholine receptors (AChRs) stained with Alexa Fluor 594-conjugated α-bungarotoxin (red). Right panels show enlarged area marked by white box in the left panel. 10x original magnification (left panel). Scale bar, 150μm. (**b**) Transverse section of a sciatic nerve (*SN*) from a *WT* (*SOD1*$^{+/+}$) mouse (top panel). 20x original magnification. Scale bar, 100μm; Bottom left panel shows enlarged area marked by red box in the top panel to show the morphology and myelin thickness of motor axons of the peripheral nerve. 60x original magnification. Scale bar, 10μm; Transverse section of a *SN* from a *SOD1*$^{-/-}$ mouse (bottom right panel). Note reduced myelin thickness of motor axons from peripheral nerve of the *SOD1*$^{-/-}$ model, indicated by arrowheads. 60x original magnification. Scale bar, 10μm. (Redrawn from Sakellariou et al. [14])

SOD1$^{-/-}$ mice [34, 143–149], and many features of the muscles of *SOD1*$^{-/-}$ mice including loss of fibers, reduction in contractile force, a constitutive activation of redox-sensitive transcription factors [146], degeneration of neuromuscular junctions (NMJ), and of loss of innervation resemble those observed in old wild-type mice [144, 145] and in older humans [13, 144]. Hence, it has been suggested that the *SOD1*$^{-/-}$ model may potentially provide a useful model to study the role of chronic oxidative stress in loss of skeletal muscle and to uncover potential targets for intervention for preventing age-related muscle wasting.

The prominent sarcopenic phenotype observed in the *SOD1*$^{-/-}$ model is associated with a number of neurological impairments (Fig. 13.5), including striking alterations in NMJ and peripheral nerve integrity/function (Fig. 13.5), motor axon degeneration, postsynaptic endplate fragmentation, terminal sprouting and axon thinning and irregular swelling, reduced occupancy of the motor endplates by axons, loss of innervation and motor function [143], impaired neurotransmitter release [150], and reduction in isometric force [145]. Collectively, these findings may suggest that the muscle atrophy phenotype shown in the *SOD1*$^{-/-}$ model might be initiated by disrupted redox signaling in motor neurons.

Disrupted redox signaling in motor neurons as a potential mechanism of sarcopenia in *SOD1*$^{-/-}$ mice has recently been assessed in genetically engineered mouse models including models with targeted deletion of CuZnSOD specifically in skeletal muscle alone [149] or motor neurons [148] but also in a "nerve rescue" *SOD1*$^{-/-}$ mouse model with neuron-specific expression of CuZnSOD [147], using a transgenic SOD1$^{-/-}$ mouse model in which SOD1 was expressed under control of the synapsin 1 promoter. The data from these studies provided evidence that CuZnSOD deficits in either the muscle or motor neuron alone are not sufficient to initiate a full sarcopenic phenotype and that deficits in both tissues are required to recapitulate the loss of muscle and function observed in the SOD1$^{-/-}$ model. Moreover, the data further showed that neuron-specific insertion of SOD1 corrected the skeletal muscle aging phenotype observed in SOD1$^{-/-}$ mice indicating that deficits in redox homeostasis in motor nerves appear to be the underlying factor that initiates mitochondrial dysfunction and oxidative damage which triggers a retrograde response leading to further NMJ degeneration and dysfunction. These reports have provided insight into the understanding of (i) the defective redox signaling events that underlie age-related atrophy and (ii) the redox-mediated cross talk between motor neurons and skeletal muscle.

13.6 Neuromuscular Aging Is Associated with Redox Proteomic Changes

In order to unravel the mechanisms responsible for the structural and functional changes associated with neuromuscular aging, many laboratories have begun to investigate both the proteome and site-specific redox modifications within skeletal muscle, to identify those proteins that change in abundance but also to identify those proteins that are particularly sensitive to redox changes.

Site-specific RONS-induced redox modifications of key regulatory enzymes can alter a wide variety of metabolic pathways related to cellular response to energy and stress. Modulation of the activity of downstream protein targets by redox modifications can also influence a variety of key regulators of distinct posttranslational modifications (PTMs) such as phosphorylation, ubiquitination, and acetylation, including components that control metabolic rate such as AMP-activated protein kinase (AMPK), protein kinase C (PKC), sirtuin 1, and mammalian target of rapamycin (mTOR) [131]. In skeletal muscle a number of redox-sensitive proteins are involved in excitation-contraction coupling; these modifications can specifically affect calcium homeostasis including calcium release, binding, and sequestration through site-specific redox modifications of specific cysteine (Cys), e.g., sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and ryanodine receptor 1 (Ryr1) [151, 152]. The nature or type of RONS-induced redox modification is dependent on a number of factors including the residues modified (typically Cys), the species and concentration of RONS generated, and the properties of the amino acids

surrounding the modified residue which can influence the sensitivity to modifications. One of the goals of redox proteomic approaches is to identify the RONS modification, the amino acid residue that has been modified, and the relative quantification of the modified amino acid, including both reversible and irreversible modifications which have shown to influence contractile force [48, 111, 153]. The major reversible RONS-induced modifications of Cys residues include sulfenylation (-SOH), glutathionylation (-SSG), nitrosylation (-SNO), and inter-/intradisulfide bond formation (-S-S-) [131]. The largely irreversible modifications include sulfonic ($-SO_3H$) or sulfinic (SO_2H) acid formation [154].

Neuromuscular aging exhibits an altered redox proteome with subsequent biochemical and physiological effects on the cytoskeleton, mitochondria, calcium signaling and sequestration [155–157]. Redox proteomic approaches have demonstrated that skeletal muscle aging is correlated with altered catalytic activity of a number of regulatory enzymes and an overall reduction in the identification of redox-sensitive proteins particularly involved in the generation of precursor metabolites and energy metabolism [111, 131]. These results suggest that age-related redox changes have a significant role in the loss of skeletal muscle mass and function inherent with aging. Reversible redox modifications on specific proteins are essential for correct adaptive response to contractile activity with activation of specific pathways, and skeletal muscle has shown to develop a dysregulated redox response with aging [111, 131]. However, irreversible oxidative modifications as a result of excessive RONS can lead to insoluble protein aggregates and protein degradation, which have been reported to increase in neurodegenerative diseases and aging [158]. Recent reports have demonstrated that reversible and irreversible redox modifications of myofilament proteins can modify both structure and function [159]; several regulatory and cytoskeletal myofilament proteins including troponin C [160], actin, α-actinin [111, 159], and myosin heavy chains [161–163] are susceptible to RONS-induced oxidative modifications, thus affecting Ca^{2+} dynamics and Ca^{2+} sensitivity [164] and as a result cross-bridge cycling [160] which ultimately affects contractile function.

13.7 Causative Links Between Disrupted Redox Signaling and Muscle Atrophy

There are a number of studies that have demonstrated a link between increased intracellular RONS concentrations and an altered redox environment in skeletal muscle atrophy, as a result of either muscle disuse [165] or disease [166]. The causative links between redox homeostasis and skeletal muscle atrophy include signaling pathways that regulate both protein synthesis and protein breakdown [167–169]. Regular exercise can help maintain skeletal muscle mass, yet contracting skeletal muscle generates RONS predominantly from NOX and NOS systems [28], which in turn are thought to acutely activate a variety of redox-regulated transcription factors (Nrf-2, NF-κB) required for adaptation to exercise [170]. In exercise studies it has

been reported that ingesting high doses of vitamin C and E can blunt the beneficial and adaptive responses induced by exercise in skeletal muscle presumably by disrupting the RONS signaling cascade [71]. However, in skeletal muscle from older individuals, there is a higher basal level of RONS, and as a result, chronic activation of many redox-regulated transcription factors may blunt many of the beneficial adaptive responses following an acute RONS-dependent increase during exercise [172].

The IGF1-Akt pathway is one of the key global regulators of protein synthesis; a number of studies have demonstrated that activation of IGF1 receptor can promote muscle hypertrophy, while inactivation is related to an impairment of muscle growth. [173]. The role of oxidative damage in relation to IGF1 signaling is unclear with reports suggesting that it may result in the promotion and inhibition of Akt signaling [174]. Studies using C2C12 myotubes have shown that oxidative damage due to chronic exposure to low levels of H_2O_2 attenuates Akt phosphorylation which would be predicted to result in an overall decrease in protein synthesis, increased proteolysis, and as a result increased muscle atrophy [174]. In support of this finding, a recent report demonstrated that administration of the mitochondrial targeted antioxidant peptide SS-31 resulted in an increase in the phosphorylated form of Akt and mTORC1 indicating that aberrant redox homeostasis can attenuate muscle protein synthesis by inhibiting the Akt/mTORC1 signaling pathway [175].

Growing evidence suggests that disrupted redox signaling due to enhanced RONS generation effects autophagy-mediated protein breakdown, a highly regulated lysosomal pathway used for the degradation of non-myofibril cytosolic proteins and organelles in skeletal muscle [167]. RONS can directly affect this process as oxidative damage induced by H_2O_2 treatment of fibroblasts can result in an increase in the expression of key autophagy components such as LC3, beclin1, and increased formation of autophagosomes [176]. RONS may also alter the activity of the regulators of autophagy; for example, the inactivation of ATG4 can prevent the cleavage of LC3 during the generation of the autophagosome, which is an essential step in the process of autophagy [167, 177].

Furthermore, the regulation of the proteasomal degradation pathway can also be regulated by intracellular RONS. In vivo studies have demonstrated that increased RONS can promote muscle protein breakdown via increased activity of the proteasome system [178], [14] but also through the activation of calpains, specific proteases that are involved in the selective cleavage of target proteins [179].

13.8 Perspectives

Muscle atrophy and weakness, in the context of neuromuscular aging and a wide range of myopathies, has a significant effect on individuals with respect to independence and overall quality of life. There is ongoing research to develop both pharmacological and non-pharmacological therapeutic approaches to inhibit or prevent loss of skeletal muscle mass and function [180]. Age-related skeletal muscle atrophy is

a multifactorial process, involving a variety of metabolic processes and signaling pathways whose disruption ultimately result in skeletal muscle loss and functional deficits. The primary biochemical and molecular mechanisms responsible for muscle atrophy have not been fully identified. Considerable evidence in both humans and various organisms has shown that the myofibrillar redox environment can influence the activity of crucial pathways involved in biogenesis and degradation but also the regulation of excitation contraction coupling, making it an attractive target for interventional approaches. There is a wealth of scientific research from both human and animal studies that have described an altered redox environment within skeletal muscle with age, in particular increased oxidation of redox-sensitive proteins and macromolecules correlated with age-related atrophy. An altered redox environment has also been described in many age-related diseases including neurodegenerative disorders, neuromuscular diseases, and diabetes. However, whether disrupted redox signaling is the initial cause of disease, development or a consequence leading to disease progression has yet to be fully determined. To elucidate the role of redox homeostasis in age-related disease, particularly in neuromuscular integrity and function, the generation of tissue-specific knockout models and the development of sensitive tools for measuring RONS generation and the subsequent redox modifications and signaling roles are warranted. Identification of the precise signaling roles of endogenously generated RONS and the balance between RONS signaling and oxidative damage will increase our understanding of the role of redox homeostasis in skeletal muscle adaptation to exercise and maintaining neuromuscular integrity. Increased understanding of the precise molecular pathways that regulate the balance between adaptation and muscle growth compared with disuse and atrophy may reveal potential therapeutic targets for intervention and ultimately prevent sarcopenia in humans.

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Chapter 14
Disturbed Ca^{2+} Homeostasis in Muscle-Wasting Disorders

Guillermo Avila

Abstract Ca^{2+} is essential for proper structure and function of skeletal muscle. It not only activates contraction and force development but also participates in multiple signaling pathways. Low levels of Ca^{2+} restrain muscle regeneration by limiting the fusion of satellite cells. Ironically, sustained elevations of Ca^{2+} also result in muscle degeneration as this ion promotes high rates of protein breakdown. Moreover, transforming growth factors (TGFs) which are well known for controlling muscle growth also regulate Ca^{2+} channels. Thus, therapies focused on changing levels of Ca^{2+} and TGFs are promising for treating muscle-wasting disorders. Three principal systems govern the homeostasis of Ca^{2+}, namely, excitation-contraction (EC) coupling, excitation-coupled Ca^{2+} entry (ECCE), and store-operated Ca^{2+} entry (SOCE). Accordingly, alterations in these systems can lead to weakness and atrophy in many hereditary diseases, such as Brody disease, central core disease (CCD), tubular aggregate myopathy (TAM), myotonic dystrophy type 1 (MD1), oculopharyngeal muscular dystrophy (OPMD), and Duchenne muscular dystrophy (DMD). Here, the interrelationship between all these molecules and processes is reviewed.

Keywords EC coupling · Ca^{2+} channel · Myogenesis · Intracellular Ca^{2+} · Atrophy

14.1 Introduction

Numerous biological processes depend on the levels of intracellular Ca^{2+}. The neuromuscular transmission (NMT) is an emblematic example. It begins with the arrival of an action potential (AP) to the nerve terminal, with the ensued release and accumulation of acetylcholine (ACh) into the synaptic cleft. Subsequently, precise coordination of the gating of many types of ion channels (and transporters) results in a transitory increase in the levels of free myoplasmic Ca^{2+} ($[Ca^{2+}]_i$). More specifically, the influx of Na^+ through skeletal muscle ACh receptors depolarizes the

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membrane and thereby activates voltage-gated Na^+ channels, an AP is fired, and a process known as excitation-contraction (EC) coupling begins. During EC coupling, the voltage sensors of a voltage-gated Ca^{2+} channel ($Ca_V1.1$) activate the opening of ryanodine receptors (RyR1s, located in the sarcoplasmic reticulum or SR), which allows a massive release of Ca^{2+} to the cytosol. The resulting rise of $[Ca^{2+}]_i$ activates, in turn, not only the contractile machinery but also the SR Ca^{2+} ATPase (SERCA) that pumps Ca^{2+} back into the SR (reviewed recently in [1]).

Many human diseases course with skeletal muscle weakness, which (not surprisingly) can be explained by alterations in either NMT or EC coupling. Nevertheless, such modifications can also elicit a chronic loss of muscle mass. For example, by inhibiting the activity of the Ca^{2+}-calmodulin-dependent protein kinase (CamK). This kinase is important to not only stimulate the differentiation of precursor cells (myoblasts) [2] but also to induce transactivation of genes involved in hypertrophy. Apparently, CamK stimulates hypertrophy by inactivating a protein named glycogen synthase kinase 3 beta (GSK3β) [3], whose function is to limit the synthesis of proteins. Thus, by downregulating CamK, low levels of Ca^{2+} are well suited to generate atrophy. Paradoxically, a sustained rise of $[Ca^{2+}]_i$ also results in muscle wasting. This is because the amount of muscle mass depends on a balance between protein synthesis and degradation, and the elevated levels of Ca^{2+} can activate proteases and thereby promote the breakdown of proteins (Fig. 14.1) [4]. Accordingly, both agonists of the CamK signaling pathway and inhibitors of Ca^{2+}-dependent proteases represent intriguing candidates for treating the pathological loss of skeletal muscle (reviewed in [4, 5]). Herein, the interrelationship between all these physiological and pathological processes is reviewed. An emphasis is put on the role of Ca^{2+} as a critical node that manages the transition, from a healthy muscular structure to weakness and atrophy.

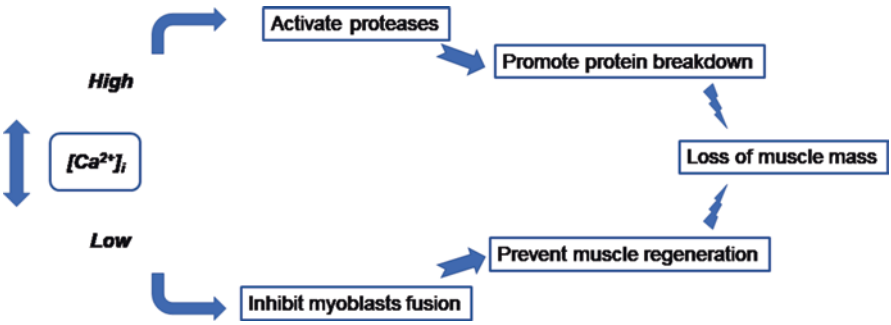

Fig. 14.1 The scheme depicts how pathological alterations of $[Ca^{2+}]_i$ can lead to atrophy. Changes in the levels of Ca^{2+}, in the up-and-down direction, activate two different signaling pathways that converge in promoting a significant loss of muscle mass. **High**: Sustained elevations of Ca^{2+} can activate a Ca^{2+}-dependent protease (calpain) and thereby result in the breakdown of proteins and atrophy. **Low**: On the other hand, a decrease in resting Ca^{2+} levels leads to an impaired formation of myotubes, preventing the proper regeneration of muscle and thus promoting the development of atrophy. See the text for further details

14.2 Dynamic Changes in Myoplasmic Ca^{2+}

The following three major physiological processes contribute to regulating the homeostasis of Ca^{2+}. They reflect the expression and activity of both Ca^{2+} channels and the SERCA pump.

14.2.1 Excitation-Contraction (EC) Coupling

EC coupling is the process by which an AP induces contraction and force development. A transitory increase in $[Ca^{2+}]_i$ (Ca^{2+} transient) is responsible for activating the contractile machinery, whose relaxation occurs as the Ca^{2+} levels return to normal values, thanks to the activity of SERCA. The source of Ca^{2+} for EC coupling is the SR, and it has been firmly established that extracellular Ca^{2+} is irrelevant for this process. For example, in the absence of extracellular Ca^{2+}, the skeletal muscle fiber contracts vigorously, for several minutes [6]. Additionally, the maximum levels of both $[Ca^{2+}]_i$ and contractile force can be elicited at membrane potentials where the influx of Ca^{2+} is practically null [7, 8]. Moreover, in 1973 Schneider and Chandler published what is known as the hypothesis of the physical link for EC coupling. It states that mobile particles embedded in the sarcolemma (voltage sensors) sense APs and mechanically activate the release of Ca^{2+} from the SR [9]. The molecular identity of voltage sensors was subsequently defined. They form part of a voltage-gated Ca^{2+} channel, also known as the dihydropyridine receptor (DHPR), or $Ca_V1.1$ [10, 11]. The junctional gap between transverse tubes of the sarcolemma (T-tubes) and terminal cisterns of the SR contains electron dense structures, termed "feet." They reflect the presence of the SR Ca^{2+} release channel, also known as RyR1 [12]. Indeed, mice knockout for the RyR1 gene lack feet [13]. Thus, $Ca_V1.1$ and RyR1 are both essential for EC coupling. Accordingly, they are also critical for survival [14–16].

14.2.2 Excitation-Coupled Ca^{2+} Entry (ECCE)

The Ca^{2+}-conducting activity of $Ca_V1.1$ is irrelevant for EC coupling [17]. This fact indirectly reinforces the concept that the SR is the only source of Ca^{2+} for this process (see Sect. 14.2.1). Nevertheless, it has been proposed that the entry of Ca^{2+} through $Ca_V1.1$ might participate in replenishing the SR during sustained depolarizations. A process known as excitation-coupled Ca^{2+} entry (ECCE, [18]) provides indirect support for this speculation. ECCE is a slow increase in the entry of Ca^{2+} in response to either sustained or repetitive depolarization (for review see [19]). A large amount of data suggests that in both, developing myotubes and adult muscle fibers, an entry of Ca^{2+} via $Ca_V1.1$ represents the underlying mechanism for ECCE [20–22].

The following direct evidence supports the notion that ECCE effectively contributes to SR Ca^{2+} loading. Robin and Allard (2015) reported that the SR Ca^{2+} loading is potentiated in response to an increase in the magnitude of Ca^{2+} current associated to ECCE. Moreover, they also found that Mn^{2+} is not only able to permeate during ECCE but also produces quenching of the fluo-5 N trapped in the SR [22]. Although these findings could be interpreted to suggest that ECCE is physiologically relevant, neither the development nor performance of skeletal muscle is altered in response to the elimination of Ca^{2+} influx via $Ca_V1.1$ [23]. Thus, the possibility that *a reduced* magnitude of ECCE be of pathophysiological relevance is practically null. Nevertheless, future work may lead to the exciting discovery that, conversely, *an increase* in ECCE leads to pathological symptoms.

14.2.3 Store-Operated Ca^{2+} Entry (SOCE)

SOCE is the process in which a decrease in the load of SR Ca^{2+} induces a protein of the SR to oligomerize and directly activate a Ca^{2+} channel of the sarcolemma: STIM is the SR protein, whereas Orai is the Ca^{2+} channel. Three isoforms of Orai have been identified in human, namely, Orai1, Orai2, and Orai3. They conform the well-known calcium release-activated Ca^{2+} channels (CRAC) [24]. STIM, on the other hand, consists of two isoforms, which have been detected in vertebrates (STIM1 and STIM2). The principal isoforms that underlie SOCE in skeletal muscle are STIM1 and Orai1 [25]. The C-terminal portion of STIM1 is cytosolic and presents domains critical for binding to—and activating—Orai1. On the other hand, the NH2-terminal segment of STIM1 is located in the lumen of the SR. It contains two regions that are critical for sensing the levels of luminal Ca^{2+}. More specifically, the following domains, EF-hand and sterile alpha-motif (SAM), are thought to constitute the sensor of Ca^{2+} (EF-SAM). Under normal levels of SR Ca^{2+} loading, the binding of Ca^{2+} to EF-SAM keeps STIM1 in its monomeric form. However, the EF-SAM conforms dimers and oligomers in response to depletion and thus promotes both binding of STIM1 to Orai1 and the subsequent entry of Ca^{2+} [21, 24, 26, 27].

It has been proposed that SOCE participates in refilling the SR of Ca^{2+}, but this idea is controversial. Evidently, an SR depletion is required for activating SOCE, but this condition is difficult to reach, not only physiologically but also experimentally [28]. The following evidence supports the view that SOCE, in effect, contributes to refilling the SR of Ca^{2+}. Mice knockout for myostatin (Sect. 14.3.3) develop a severe reduction in expression levels of STIM1 and Orai1, which correlates with an inhibition of SOCE and a faster SR depletion (induced by repetitive release of Ca^{2+}) [29]. Indeed, this tendency to readily exhaust the SR might explain why those mice deficient in myostatin also exhibit a significant muscle weakness (low specific force), in the face of an excessive muscle mass [30].

14.3 Myogenesis

14.3.1 Myogenesis Is Critical for Muscle Growth and Force Development

This is a brief explanation of how precursor cells contribute to the genesis and regeneration of skeletal muscle. The reader is encouraged to consult more extensive reviews on this topic [31–33]. During the embryonic development, precursor cells (termed myoblasts) fuse and form multinucleated cells, known as myotubes. The myoblasts withdraw from the cell cycle, adopt a spindle shape, and align with each other—forming a braid—and the fusion occurs. Subsequently, the myotubes are transformed into muscle fibers, through a maturation process that involves (among other things) the formation of T-tubes. The fusion of myoblasts is also known as "terminal differentiation" because it implies that DNA from the fused myoblasts will no longer replicate, and thereby the cell proliferation is arrested. In the adults, myotubes continually form. The corresponding precursor cells are known as satellite cells (SCs). Although not fully differentiated, proliferating myoblasts and SCs are committed to the myogenic lineage (i.e., they already express transcription factors of the MyoD family). Depending on specific conditions, precursor cells can be either mitotically quiescent or induced to proliferate. For example, injury stimulates SCs to proliferate, and the resulting colony provides for generating both a stock of quiescent cells and a significant number of fusion-competent myoblasts. The latter eventually will either form a new fiber or fuse into injured fibers contributing to healing [31–33].

In vitro, the fusion of myoblasts is often quantified as the "fusion index": that is, the number of nuclei per myotube, divided by the total number of nuclei per field of observation. The fusion index is crucial for in vivo conditions because the myofiber size and thereby the contractile strength depend on the number of nuclei in the fiber. Accordingly, it is well known that the number of nuclei in the myofiber declines during atrophy. Conversely, the restoration of muscle mass requires myonuclear accretion [34]. Remarkably, SCs also contribute to a robust neuromuscular junction (NMJ) [35, 36]. Indeed, the deterioration of NMJs, in aging, is more closely related to deficiencies in SCs and myogenesis rather than to denervation [36].

14.3.2 Role of Ca^{2+} in Skeletal Muscle Development

Myogenesis involves a dramatic change in phenotype which in turn depends on a coordinated activation of skeletal muscle-specific genes [37–39]. Apart from the expression of myogenic factors (e.g., MyoD, Myf5, Myf6, and myogenin), this process requires Ca^{2+}. More precisely, a Ca^{2+}-dependent signaling pathway that involves calmodulin and the family of transcription factors known as NFAT leads to the

fusion of myoblasts (for review see [5, 39, 40]). The recent discovery of a feedback mechanism by which SOCE and NFATc3 control the fusion of myoblasts highlights the relevance of this Ca^{2+}-dependent pathway [41].

Because myogenesis requires Ca^{2+}, a reduced entry of this ion tends to inhibit the proper regeneration of muscle. Ironically, however, sustained elevations of $[Ca^{2+}]_i$ also contribute to the degeneration of skeletal muscle (Fig. 14.1). This is because Ca^{2+}-dependent proteases lead to protein degradation (i.e., calpains, which contain Ca^{2+}-binding domains) [4]. Indeed, an increase in intracellular Ca^{2+} is frequently observed in both congenital myopathies and muscular dystrophies (see Sect. 14.4). Additionally, high rates of protein breakdown have been reported in many muscle-wasting diseases [42].

During myogenesis, the expression of several proteins involved in the homeostasis of Ca^{2+} is induced. An intricate relationship exists because Ca^{2+}, in turn, regulates the expression of at least two of these proteins (i.e., SERCA and $Ca_V1.1$) [43–45]. Therefore, dissecting the role of a specific protein in myogenesis is complicated. Nevertheless, the use of knockout animals has provided irrefutable proofs pointing to a leading role for $Ca_V1.1$ and RyR1. For example, it has been reported that dyspedic and dysgenic mice (i.e., RyR1 and $Ca_V1.1$ knockout) die both at birth. More interestingly, these two strains of mice also develop malformations, consisting in delayed development of skeletal muscle [14–16, 46]. Thus, RyR1 and $Ca_V1.1$ are both of paramount relevance for not only EC coupling (Sect. 14.2.1) but also myogenesis. On the other hand, a recent work elegantly showed that the Ca^{2+}-conducting activity of $Ca_V1.1$ is irrelevant for skeletal muscle development and function [23]. Thus, most likely this protein exerts its regulatory actions via mechanical control of RyR1 (as opposed to regulating the entry of Ca^{2+}, see Sect. 14.2).

In mice, the voltage-gated Ca^{2+} channel isoform $Ca_V3.2$ is expressed during embryonic development and then gradually disappears, after birth [47, 48]. In 2000, Biglenga et al. proposed that the entry of Ca^{2+} through this channel stimulates myogenesis [49]. More recently, this idea was tested and discarded because the fusion of myoblasts was unaltered by nickel (a $Ca_V3.2$ blocker) [50]. In addition to $Ca_V3.2$, both Orai1 (see Sect. 14.2.3) and a transient receptor potential channel (TRCP1) have also been proposed as necessary for myogenesis [51, 52].

14.3.3 Transforming Growth Factors Regulate Both Myogenesis and Ca^{2+} Channels

Several extracellular signaling factors participate in controlling distinct phases of myogenesis. For example, the hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) are both considered of critical relevance for SCs activation [53]. Myostatin (growth differentiation factor 8, GDF-8) is a member of the transforming growth factor-β (TGF-β) superfamily, and it has also proven essential to regulate myogenesis [54, 55]. The TGF-β superfamily includes many other types of

growth factors, which, similarly to myostatin, also inhibits the development of skeletal muscle. Specifically, in less than 24 h, the bone morphogenetic protein type 2 (BMP-2) and transforming growth factor β1 (TGF-β1) decrease both the expression of MyoD and myogenin. The effect on these transcription factors precedes a drastic inhibition of myotube formation (Fig. 14.2) [56], which saturates at nanomolar concentrations [57].

Because myogenesis requires Ca^{2+} (Sect. 14.3.2), it is possible that BMP-2 and TGF-ß1 arrest this process by interfering with the activity of Ca^{2+} channels. In support of this view, both growth factors also inhibit the functional expression of Ca_V3 channels (in semi-differentiated myotubes, see Fig. 14.2). Moreover, TGF-β1, but not BMP-2, also downregulates the activity of $Ca_V1.1$ [56]. Although these data suggest that $Ca_V1.1$ and Ca_V3 channels participate in myogenesis, a role for only $Ca_V1.1$ has been firmly established (see Sect. 14.3.2).

14.4 Role of Ca^{2+} in Diseases That Course with Skeletal Muscle Atrophy

The calcium ions are of paramount relevance in the context of muscle atrophy (Sect. 14.3.2). Thus, not surprisingly, the list of diseases in which alterations in the homeostasis of Ca^{2+} and skeletal muscle atrophy concur is vast. This section discusses examples where dysregulation of Ca^{2+} channels and SERCA has been observed. It also explains how such dysregulation contributes to understanding the corresponding loss of muscle mass. It is highly recommended to consult the following excellent reviews on these topics [58, 59].

14.4.1 Congenital Myopathies

14.4.1.1 Brody Disease

Brody disease is a congenital myopathy characterized by muscle cramping that usually manifests after exercise (especially in the cold) and is accompanied by impairment of muscle relaxation. Muscles from the legs, arms, and eyelids are principally affected, and they slowly return to relaxation if maintained at rest (reviewed in [60]). This disease is linked to mutations in the gene encoding the skeletal muscle SERCA (i.e., SERCA1) [61]. A related myopathy has also been observed but in the absence of SERCA mutations (termed Brody syndrome). Thus, in more general terms, these disorders are just referred to as "Brody myopathy." It has been reported that patients with advanced phases of this myopathy also show skeletal muscle weakness and atrophy (of both type I and type II fibers) [60, 62, 63].

A reduced SERCA activity is observed in muscle samples of Brody myopathy patients, and this alteration explains an increase in time needed for myoplasmic Ca^{2+}

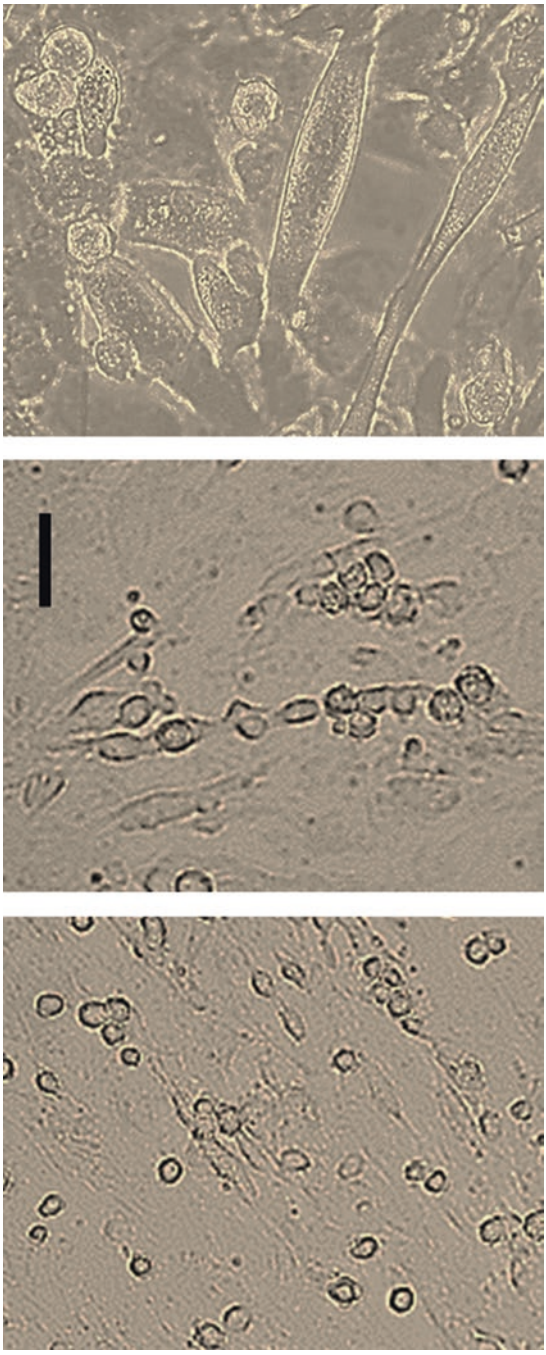

Fig. 14.2 TGF-β1 and BMP-2 inhibit myotube formation. Light-field images of myoblasts that were obtained from newborn mice and then kept 6 days under control differentiation conditions (upper panel) and the presence of either BMP-2 (5 nM, middle panel) or TGF-β1 (40 pM, lower panel). The scale bar represents 50 μm

extrusion after repetitive stimulation. Although this mechanism underlies the damaged muscle relaxation, stiffness, and cramping [64, 65], the primary functional defect responsible for the loss of SERCA activity remains unknown [60]. Likewise, the molecular basis underlying loss of muscle mass has yet to be elucidated. Because an increase in time needed for myoplasmic Ca^{2+} extrusion is ostensibly involved in this myopathy, it seems reasonable to speculate that an elevated level of $[Ca^{2+}]_i$ recruits Ca^{2+}-dependent proteases and thereby induces protein degradation (Fig. 14.1; see also Sect. 14.3.2). Dantrolene and verapamil, two inhibitors of EC coupling, are promising therapeutic agents for Brody myopathy. They limit the amount of Ca^{2+} released, and thereby the low Ca^{2+} pumping capacity readily restores the normal resting $[Ca^{2+}]_i$ levels, preventing Ca^{2+} overload ([65], discussed in [60]). Thus, in the near future, it will be interesting to investigate if these compounds also prevent the development of atrophy.

14.4.1.2 Central Core Disease

The following congenital myopathies have been related to mutations in the gene encoding RyR1: central core disease (CCD), multiminicore disease (MmD), core myopathies with rods, centronuclear myopathy (CNM), and congenital fiber-type disproportion (CFTD). They conform the also known as "RyR1-related congenital myopathies" (RyR1-RCM) [66, 67]. CCD was the first one being linked to RyR1, and thus the corresponding mutations have been more thoroughly investigated.

CCD is of early onset and courses with proximal weakness, wasting, and skeletal deformities. These symptoms can range from very mild to extremely severe. The diagnosis is based on the identification of areas located within the center of the myofiber, depleted of mitochondria and with poor oxidative enzymatic activity (for recent reviews, see [68, 69]).

Several CCD RyR1 mutant proteins exhibit an overactive or "leaky" behavior that depletes the SR of Ca^{2+} and thereby decreases the magnitude of the Ca^{2+} transient [43, 45, 70]. Another set of mutations, located nearby the pore leaning segment of RyR1 (i.e., exon 102, within the C-terminus region), results in mutant proteins with poor Ca^{2+} permeability. Thus, rather than being leaky, these "pore mutations" result in a functional uncoupling of SR Ca^{2+} release from the electrical stimulus (termed "EC uncoupling") [71–73]. A third mechanism indicates that certain CCD mutations induce a reduced expression level of RyR1 and thus also promote a lower magnitude of Ca^{2+} transients [74–77]. These three primary defects (i.e., leaky, Ca^{2+}-impermeable, and decreased expression) are not mutually exclusive. For example, it has been reported that the Y4864H mutation results in mutant RyR1 proteins that exhibit both, low expression level and altered functional properties (leaky behavior). Remarkably, this mutation also elicits a reduced magnitude of Ca^{2+} transients, and this defect is attributed to a modified gating of the channel (as opposed to a reduced number of Ca^{2+} release units) [77].

Although mutations located in many regions of the RyR1 result in leaky behavior, evidence exists suggesting that this alteration ultimately depends on a structural modification of the protein portion facing the lumen of the SR. In particular, it has been reported that the leak depends on a reduced threshold for store overload-induced Ca^{2+} release (SOICR) [78].

As reviewed above (Sect. 14.3.2), mice knockout for RyR1 exhibit several malformations, including a delayed development of skeletal muscle. Conceivably, these alterations could simply arise from the physical absence of RyR1. Nevertheless, the following evidence indicates that they are due to the inevitable loss of SR Ca^{2+} release. A point RyR1 mutation that renders Ca^{2+} impermeable channels (equivalent to I4897T in humans) also inhibits the fusion of C2C12 myoblasts [45]. Moreover, mice knock-in for the same mutation also exhibit a delayed development, which includes a reduced and amorphous skeletal muscle, and very small myotubes [72]. Thus, a reduced level of SR Ca^{2+} release is sufficient for disrupting myogenesis and thereby also contributes to explaining the atrophy seen in the corresponding CCD patients (Fig. 14.1).

On the contrary, in patients expressing leaky CCD mutations, the atrophy is likely due to a sustained increase in the levels of $[Ca^{2+}]_i$ [43, 45, 70]. More specifically, Ca^{2+}-dependent proteolysis [4] may result in increased rate of protein degradation [42] and thereby promote the corresponding loss of muscle mass (Fig. 14.1).

In a mouse model of CCD, the I4897T mutation (see above) was found to induce the development of endoplasmic reticulum stress, unfolded protein response, mitochondrial reactive oxygen species (ROS) production, muscle weakness, and atrophy. Currently, it is unclear how this Ca^{2+}-impermeable mutant protein results in all these alterations. Nevertheless, it is important to note that they were reverted by treatment with the chemical chaperone 4-phenylbutyrate (4-PBA) [79]. Similarly to 4-PBA, agonists of the G_s subgroup of G-protein-coupled receptors have also been reported to be of therapeutic potential in CCD [45, 80]. These findings are encouraging since no effective treatment exists for CCD.

14.4.1.3 Tubular Aggregate Myopathy

Tubular aggregate myopathy (TAM) is a condition characterized by the presence of "tubular aggregates," cramps, weakness, and myalgia. Such aggregates contain proteins of the SR and thereby are thought to represent structural alterations of this organelle. A genetic cause of the disease was recently found. Specifically, in 2013 Böhm and collaborators discovered a form of TAM that is inherited with an autosomal dominant pattern and is associated with mutations in the gene encoding STIM1 [81]. This finding was confirmed more recently [82–84]. Most of the naturally occurring mutations in STIM1 are punctual substitutions, and they are positioned within the NH2-terminal sequence, just where the EF-hand is located (Sect. 14.2.3). Accordingly, these mutations result in mutant proteins that exhibit an altered

capability to bind luminal Ca^{2+} and thereby also present constitutive oligomerization [81, 83, 85]. The principal role of STIM1 is to activate the entry of Ca^{2+} via Orai1 channels (during SOCE, Sect. 14.2.3). Thus, prominent levels of SOCE may represent an important functional defect of this myopathy. Indeed, TAM has also been linked to mutations in Orai1, and the corresponding mutant proteins allow an exacerbated influx of Ca^{2+} [86–88].

A TAM STIM1 mutation that consists of an extension of amino acids (I484RfsX21) was reported recently. Remarkably, it resides in the cytosolic part of the protein (C-terminal portion) and, in contrast to mutations of the lumen, it inhibits the entry of Ca^{2+} [84]. In addition, TAM has been linked to three different mutations in the gene encoding calsequestrin (CASQ1, which is responsible for Ca^{2+} storage in the SR). Interestingly, while all CASQ1 mutant proteins show a reduced ability to store Ca^{2+}, only two appear to stimulate SOCE [89]. These findings suggest that TAM, and the corresponding atrophy, can both arise from other pathophysiological mechanisms, in addition to elevated levels of SOCE.

14.4.2 Muscular Dystrophies

14.4.2.1 Myotonic Dystrophy Type 1 (MD1)

This disease is caused by the expansion of a CTG repeat in the gene encoding a protein kinase termed MDPK. Increased excitability, delayed relaxation, atrophy, and weakness represent the most common symptoms. The CTG-repeat expansion results in both lower MDPK protein levels and trapping of the corresponding mRNA into nuclear foci. Interestingly, muscle degeneration has been related to increased rates of myofibrillar protein breakdown [42], which in turn could be explained by an exacerbated activity of Ca^{2+}-dependent proteases [4]. Indeed, elevated levels of $[Ca^{2+}]_i$ have been observed in myotubes derived from both MD1 patients and DMPK knockout mice [90–92]. Nevertheless, it is important to note that a deficiency in DMPK has functional effects in neither cardiac nor skeletal muscle. Thus, the MD1 symptoms likely arise from toxic effects of the trapped transcripts, rather than to decreased levels of the protein [93]. Transcripts of at least both, transcription factors and alternative splicing factors can be trapped, which explains why in this myopathy the expression of multiple genes is altered. Remarkably, the trapping of mRNAs modifies not only the function but also the structure of the nuclei [94].

MD1 has also been associated with misregulated alternative splicing; for example, MD1 patients show repressed alternative splicing of exon 29 in $Ca_V1.1$. Of note, the degree of exon skipping correlates with the severity of muscle weakness, suggesting that the corresponding functional alteration in $Ca_V1.1$ contributes to exacerbating symptoms [95]. Additionally, the alternative splicing of both RyR1 and SERCA (1 and 2) is misregulated. Thus, aberrant splicing of the corresponding transcripts most likely also contribute (by affecting Ca^{2+}-dependent pathways) [92, 96].

14.4.2.2 OPMD

Oculopharyngeal muscular dystrophy, or OPMD, is a late-onset autosomal dominant congenital myopathy. The first symptoms begin between the fifth and sixth decades of life. They consist of progressive drooping of eyelids (ptosis), swallowing difficulty (dysphagia), muscle atrophy, and proximal upper and lower weakness. OPMD is linked to mutations in the gene encoding poly(A)-binding protein nuclear 1 (PABPN1). The OPMD mutations consist of an expansion of a tract that contains 10 alanines (to 12–17). The pathological hallmark is that the nuclei of skeletal muscle fibers develop aggregates or inclusions (termed intranuclear inclusions, INI), which contain a misfolded PABPN1 and sequester poly(A) RNA [97, 98]. This disease is also frequently accompanied by other severe symptoms, such as weakness and atrophy of the tongue, dysphonia, limitation of upward gaze, and facial muscle weakness [99].

Although the precise underlying mechanism is not yet clear, it has been proposed that the INIs generate toxic effects, likely by interfering with the cellular traffic of poly(A) RNA, and thus affecting gene expression [97, 98]. The expression of at least 202 genes is misregulated, as shown by microarray assays performed in muscle fibers from a mouse model of OPMD [100]. A recent study reported that an OPMD mutant protein (PABPN1-17A) promotes structural alterations of the nucleus, which contributes to explaining the wide range of genes whose expression is misregulated [101].

Interestingly, PABPN1 stimulates the fusion of myoblasts, and this property is missing in the PABPN1-17A mutant protein [101]. Thus, an altered capacity to regenerate muscle may explain the corresponding muscle atrophy and weakness in OPMD. In C2C12 myotubes, PABPN1-17A also elicits many alterations in the homeostasis of Ca^{2+} [101]. For example, it promotes a ~50% reduction of the magnitude of Ca^{2+} transients. This effect can be explained by parallel changes in the expression of RyR1 and SR Ca^{2+} content. In fibers from adult mice, however, this mutant protein is unable to modify the magnitude of Ca^{2+} transients [101]. This finding indirectly supports the notion that atrophy, due to inability to stimulate myogenesis (Fig. 14.1), likely represents the most significant pathophysiological consequence of PABPN1 mutant proteins [101–104].

14.4.2.3 Duchenne Muscular Dystrophy

The absence of dystrophin, a cytosolic protein that is critical for proper structure of the muscle, results in a genetic disorder known as Duchenne muscular dystrophy (DMD). This disease is characterized by shorter lifespan, cardiac involvement, and skeletal muscle degeneration and weakness. An increased structural fragility of muscle fibers and altered homeostasis of Ca^{2+} represent two relevant pathophysiological mechanisms. Indeed, an increased entry of Ca^{2+} (which promotes protein degradation and higher levels of ROS) has been proposed to explain the

corresponding atrophy [42, 105]. Accordingly, myotubes of mdx mice (a commonly used model of DMD) exhibit a higher activity of Ca^{2+} channels at resting membrane potentials, compared with controls. This hyperactivity is due to the presence of a mechano-transducing Ca^{2+} channel, which likely contributes to the high influx of Ca^{2+} [106, 107]. Although the identity of the corresponding stretch-activated Ca^{2+} channel(s) (SACs) has yet to be firmly established, members of the transient receptor potential channel (TRPC) family may be involved. TRPCs participate in muscle differentiation, and thus changes in their function/expression might also contribute to generating the corresponding loss of muscle mass. For a recent and comprehensive review, see [108].

An exacerbated SOCE has also been linked to DMD. For example, muscle fibers from mdx mice show not only increased levels of SOCE but also higher expression level of both Orai1 and STIM1 [109, 110]. Accordingly, it has been reported that the severity of this disease can be reduced by expressing a dominant negative Orai1, in two mouse models of DMD [111].

Like in many human myopathies, no effective treatment exists for DMD (other than palliatives focused on easing the symptoms). Thus, the search for a more effective treatment continues. With regard to "fixing" alterations in the homeostasis of Ca^{2+}, pharmacological approaches have been investigated. More precisely, the efforts have focused on using blockers of Ca^{2+} channels, as well as on regulating the activity and expression of SERCA (reviewed in [112, 113]). Knocking down the expression and activity of myostatin (see Sect. 14.3.2) also represents a promising therapy. This intervention is particularly beneficial to counteract muscle weakness and wasting, in not only DMD [114, 115] but also many other disorders [116].

14.5 Conclusions

In skeletal muscle fibers, much work has evolved in acquiring a deep knowledge of the mechanisms that control the homeostasis of Ca^{2+}, under both physiological and pathological conditions. Meanwhile, significant efforts have firmly established a pivotal role for Ca^{2+} in determining the amount of muscle mass. Accordingly, it is now generally accepted that this ion controls not only muscle mechanical properties but also the corresponding development, regeneration, atrophy, and hypertrophy. Therefore, treating wasting disorders with therapies based on a precise tune-up of the activity/expression of Ca^{2+} channels and transporters could eventually become a daily clinical practice.

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Part IV
Muscle Atrophy in Diseases and Aging

Chapter 15
Muscle Atrophy in Cancer

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Abstract Cancer is a prevalent disease with high mortality and morbidity. Muscle atrophy is a severe and disabling clinical condition that frequently accompanies cancer development such as muscle atrophy in pancreatic cancer, lung cancer, and bladder cancer. The majority of cancer patients are accompanied with cachexia. Cancer-associated cachexia is characterized by weight loss and muscle atrophy. Muscle wasting is a pivotal feature of cancer cachexia. Muscle atrophy refers to the reduction of muscle mass caused by muscle itself or the dysfunction of nervous system. Muscle atrophy causes serious clinical consequences such as physical impairment, poor life quality, reduced tolerance to treatments, and short survival. Although many reports have studied cancer-related muscle atrophy, there is still no clear understanding of it. Here we will describe the prevalence, mechanisms, pathophysiological effects, and current clinical treatments of muscle atrophy in cancer.

Keywords Muscle atrophy · Cancer · Cachexia

15.1 Introduction

Muscle atrophy is caused by muscle disease or neurological dysfunction. It refers to striated muscles dystrophy, which means that muscle fiber is thin and even disappears. Muscle atrophy has posed a great threat to patient health and brought a lot of inconvenience to patient life. The main cause of muscle atrophy is the imbalance of anabolic and catabolic processes. When protein breakdown rate exceeds protein synthesis rate, muscle atrophy happens [1]. Muscular atrophy is a common neuromuscular disorder with an incidence of 1 in 6000–10,000 births [2, 3]. Although researchers have made many progresses on the treatment of muscle atrophy, until now no effective therapy is applied on muscle atrophy patients. Exploring effective methods for muscle atrophy prevention and cure is highly needed.

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Recently, several studies have focused on muscle atrophy in clinical oncology. It is reported that cancer cachexia is closely associated with cancer type, tumor size, stage, and the use of anticancer drugs [4, 5]. Muscle atrophy emerges frequently in pancreatic cancer, lung cancer, and bladder cancer patients [6]. Cancer-related muscle atrophy is worsened by traditional treatment [7]. Many deleterious effects of drug treatment lead to worse outcomes [8–11]. Cancer cachexia is the major complication for cancer patients, which happens in 80% of cancer patients. Despite its clinical significance, most cancer cachexia is underdiagnosed [12, 13]. Cancer cachexia is featured with marked body weight decrease and muscle mass diminishment. Cachectic patients may lose up to 75% of skeletal muscle mass [14, 15]. Cancer cachexia-associated muscle atrophy is complex and multifactorial, the process of which is mediated by the interplay of tumor factors and host factors [16]. Therefore, exploring the underlying mechanisms of cancer cachexia is important for patient treatment.

At present, the measures to treat muscular atrophy include muscle physical exercise, nutritional interventions, and pharmacologic treatments. Muscle physical exercise has the abilities to reduce autophagy and mitophagy, enhance the disposal of damaged mitochondria, and improve muscle energy balance [17]. Muscle physical exercise has been shown to improve muscle mass and strength in mice model [18]. Nutritional intervention can provide adequate energy and nutrient supplement, and it helps to increase or stabilize muscle mass and body weight. Pharmacologic treatment, including appetite stimulants, agents targeting inflammation and agents targeting muscle catabolic pathways, can improve the muscular strength and endurance. However, these treatments do not achieve desired therapeutic effects. Better treatments are needed to be explored in future [19].

15.2 The Prevalence of Muscle Atrophy in Cancer

Cachexia is a prevalent symptom in hospital patients with cancer. Cachexia remains a great challenge in cancer treatment and causes up to 20% of cancer-related deaths [20]. In the United States, it has been estimated that cancer cachexia affects over 34 million people. A substantial number of patients suffering from cachexia manifest high proportion of muscle atrophy [21, 22]. According to the latest survey, approximately 5–5.7 million patients are likely to suffer from muscle atrophy caused by cachexia [23–26]. Studies have reported that up to 50% of cancer patients suffer from progressive atrophy of adipose tissue and skeletal muscle [27–29]. Muscle atrophy is an important component of the pathophysiology of cancer cachexia [30, 31].

The degree of cachexia is determined by cancer type. Cachexia frequently happens in gastrointestinal cancer and lung cancer [32, 33]. In addition to gastrointestinal and lung cancer, the mortality and morbidity of muscle atrophy are high in bladder cancer and pancreatic cancer [34, 35]. At present, there is not a certain treatment for muscle atrophy in the whole world. Before we find a good treatment, we should try our best to prevent its happening. Sarcopenia, a kind of muscle atrophy,

is highly prevalent among older patients with early stage colorectal cancer. According to the latest random survey, sarcopenia patients have significantly lower body mass index and skeletal muscle index compared to non-sarcopenia patients [36, 37].

15.3 The Mechanisms of Muscle Atrophy in Cancer

In normal human body, protein synthesis and protein degradation are kept in a relative balance state. But cancer cachexia breaks the balance [38, 39]. A study from Emery and Lund Holm showed that cancer cachexia-associated muscle atrophy mainly affected protein synthesis process, and the change of protein degradation was secondary [38]. It is known to all, there are two types of muscle, namely, fast muscle and slow muscle. Fast muscles include tibialis anterior and gastrocnemius, and slow muscles include soleus. Due to the protein oxidation changes in cachexia, fast muscles have a faster loss than slow muscles [28, 40]. In addition, dystrophin glycoprotein complex, which is a membrane structure associated with muscular dystrophy, plays an important role in cachexia-induced muscle atrophy.

Clarifying the signaling pathways involving in muscular dystrophy is important for therapeutic interventions [41–43]. PI3K/Akt pathway plays important roles in promoting protein synthesis and blocking protein degradation [44–46]. In addition, Akt/mTOR pathway controls the protein synthesis in cytoplasm [47]. In mechanism, Akt phosphorylates transcription factor FOXO which activates the transcription of Atrogin-1, MuRF1 [46], or autophagy-related gene LC3 [48]. Akt overexpressing mice exhibit muscle hypertrophy [49, 50], whereas Akt knockdown mice exhibit severe skeletal muscle atrophy [51]. Moreover, IGF-1/Akt pathway is important for muscle maintenance [52]. Consistent with this conclusion, Akt signaling defects related muscle atrophy is observed in different diseases or pathophysiological conditions, which include ALS [53–55], CKD [56, 57], diabetes [58], chronic hypoxia [59], statin-induced myopathy [60], sepsis [61, 62], burn injury [63], and aging [64]. In particular, some molecules behave as pro-trophic factors by reducing Akt signaling, which includes TNFα [65], TNF-related weak inducer of apoptosis [66], glucocorticoids [67, 68], angiotensin [69], and chemotherapy agents [11]. Besides, myostatin can activate Smad2/3 pathway and increase the expression of MAFBX/MuRF1 [70, 71]. Other studies have showed that the activation of Akt in Duchenne's muscular dystrophy promoted hypertrophy [72, 73], sarcolemma stability [74], and muscle fiber regeneration [75].

15.4 Control of Protein Synthesis in Cachexia

Protein synthesis in skeletal muscle is a conserved process which involves at least 13 factors in the initial stage of protein transcription, many of which are assembled from different subunits [76, 77]. There are two check points in the process of protein

synthesis. The first process is the binding of initiator methionyl tRNA to the 40S ribosomal subunit. The second process is eIF4F recruits 40S ribosomal subunit to mRNA through 5-cap structure recognition [78]. In cancer cachexia patients, the phosphorylation levels of both PKR and eIF2 are significantly enhanced compared with healthy people. Furthermore, there is an inverse proportion relationship between myosin expression and eIF2 phosphorylation [79]. Leucine also causes a reduction in the phosphorylation of eIF2, possibly by stimulating mTOR pathway. So nutritional supplements containing leucine will improve the muscle atrophy in cachectic cancer patients [78, 80].

15.5 Protein Degradation in Cachexia

Previous reports show that there are three major proteolytic pathways that affect proteins degradation in skeletal muscle. The first one is ubiquitin-proteasome system (UPS) which is composed of ubiquitin-activating enzyme (E1), ubiquitin-carrier protein (E2), and ubiquitin-conjugating enzymes (E3 or E3 protein ligase) [37]. The second one is lysosomal system which includes cysteine proteases cathepsins B, H, and L as well as aspartate protease cathepsin D. The last one is calcium-activated system [81–83]. Among them, ubiquitin-proteasome pathway plays a predominant role in the degradation of myofibrillar proteins, which is demonstrated not only in animal models with cancer cachexia but also in clinical cancer patients [84, 85].Transcription factor Foxo3 can affect both ubiquitin-proteasome pathway and lysosomal pathway in muscles through different mechanisms [86–88]. In some cases, patients showed an increased expression of cathepsin with no changes in the components of ubiquitin-proteasome pathway [89]. Myofibrillar protein is lost about 50% during atrophy, and myosin heavy chain is selectively targeted by the ubiquitin-proteasome pathway in cachectic state [90–92]. Furthermore, Atrogin-1 and Murf-1 protein are highly expressed in cancer cachexia-related muscle atrophy [93, 94].

15.6 Apoptosis in Skeletal Muscle

In addition to protein degradation, muscle cell apoptosis also plays a role in muscle atrophy. Apoptosis includes two processes: apoptosis in the early stage and metabolic abnormalities in the late stage [51–53]. The apoptosis-related proteins such as Bax, Bcl2, and Cleavd-caspase3 are increased in the process of skeletal muscle apoptosis.

During apoptosis, the cellular contents are enclosed as vesicles, which are finally eliminated by heterophagocytosis [95, 96]. In addition, the cell membrane fluidity and conformation are also changed in apoptosis cells. The morphological features are changed by proteolytic enzymes, which are also called caspases. These prote-

ases are activated by intrinsic pathways or extrinsic pathways. Intrinsic signals activate caspase-9 and then the downstream effectors such as caspase-3 and caspase-7. Next, intracellular substrates are degraded rapidly. Extrinsic signals activate specific death receptors on the cell surface, such as TNFα and Fas ligand. And then, the expression of Bcl-2 family members (Bax and Bcl-2) is altered [97, 98, 23].

15.7 The Pathophysiological Effects of Muscle Atrophy in Cancer

The main pathophysiological mechanism of muscle atrophy in cancer cachexia is inflammation-mediated abnormal muscle anabolism and catabolism, which disturbs the metabolism balance and leads to muscle-specific protein degradation [99].

Skeletal muscle is the most abundant tissue in the body of vertebrates and is involved in many important functions. Skeletal muscle mass represents a determinant of strength, endurance, and physical performance [99]. Skeletal muscle accounts for nearly half of whole-body protein mass [100]. In healthy individuals, skeletal muscle anabolic and catabolic processes are kept in a dynamic balance state, which means that muscle proteins are continuously synthesized; meanwhile the overall muscle mass is not changed [101, 102].The metabolic abnormalities in cancer cachexia are likely to be triggered by immune response and increased cytokines secretion. Tumor necrosis factor (TNF)-α is a primary catabolic trigger for skeletal muscle loss [100, 103–105]. In addition, tumor necrosis factor (TNF)-α can also attenuate bulbar muscular atrophy [106]. Besides, it has been reported that muscle-specific expression of insulin-like growth factor-1 (IGF-1) can promote muscle hypertrophy, increase physiological muscle strength, and ameliorate dystrophic phenomenon [107, 108]. IGF-1 plays pivotal roles in regulating cell proliferation [109–111], cell differentiation [112], myofiber growth [113, 114], and myofiber regeneration [113]. IGF-1 mainly effects PI3K/AKT pathway, which slows protein degradation and promotes protein synthesis [115, 116, 41]. In clinical patients, protein synthesis reduction, protein degradation increase, or a combination of both contributes to cancer cachexia-associated muscle wasting [101]. The phosphorylation of eukaryotic initiation factors leads to protein synthesis attenuation [117]. Adenosine triphosphate-dependent ubiquitin-proteasome proteolytic pathway plays a major role in muscle wasting and the breakdown of myofibrillar proteins.

Muscle atrophy in cancer leads to myofiber area reduction and muscle strength decrease. Through ordinary optical microscope or immunofluorescence staining, we can see that muscle tubular becomes smaller [118], which has also been observed in cancer patients with muscle atrophy. Muscle atrophy is a consequence of certain physiological processes such as aging; meanwhile, it is also a pathological process in cancer. Muscle atrophy represents a clinical feature of cachexia, which causes a lot of complications, like chronic heart failure, chronic obstructive pulmonary disease, chronic kidney disease, cancer, HIV, sepsis, immune disorders, and dystrophies [119, 120].

A reduced cross-sectional myofiber area with subsequent impaired strength is the main characteristic of muscle atrophy [121, 122]. During muscle atrophy, the loss of contractile proteins mainly affects type II fast fibers [122, 123], whereas, chronic heart failure patients have an increasing loss in type IIX fiber and type I fiber [124–126].

15.8 The Current Clinical Treatments of Muscle Atrophy in Cancer

The best treatment for muscle atrophy is to attenuate muscle mass loss and improve muscles repair and regeneration [127].

15.8.1 Muscle Physical Exercise

Physical exercise has been proposed as an important treatment for cachexia patients, which is demonstrated to improve life quality and reduce fatigue [128–131]. Notably, there are substantial differences between physical exercise and exercise modality [132]. Endurance training stimulates oxidative metabolism but has slight effects on muscle mass, whereas resistance training improves muscle hypertrophy through stimulating anabolism [133]. Moreover, physical exercise regulates cellular homeostasis and promotes muscle regeneration [134–136]. Experiments have proved that voluntary wheel running could prevent cachexia and increase the survival of tumor-bearing mice [137]. Furthermore, it has been demonstrated that resistance exercise could modulate the inflammatory response in tumor-bearing rats [138, 139].

Regular exercise therapy can reduce or mitigate paralysis sequelae significantly. However, inappropriate strength training can increase spasm. For example, using the affected hand to grip repeatedly will strengthen the flexor muscle coordination of the affected upper limbs; nonetheless, it will make it harder for hand function recovery [140, 141]. Actually, muscle atrophy is not only the problem of muscle weakness; mismatch also accounts for movement dysfunction. Therefore, when muscle physical exercise is applied, rehabilitation training and strength training should be differentiated [142].

15.8.2 Nutritional Intervention

Nutritional intervention is a mean to slow the progression of muscle atrophy. Adequate energy and nutrient supply can increase or stabilize muscle mass and body weight. But it is not suitable for severely ill patients [143, 144]. It is important

to design a rational strategy for early nutritional interventions [145–147]. Several hormonal treatments including insulin-like growth factor-1, anabolic steroids, β-adrenoceptor agonists, growth hormone, testosterone, and selective androgenic receptor modulators have been proposed to enhance muscle growth and function [148, 149]. But the limitations of these hormones in clinical application are obvious. Hormone treatment has serious side effects, so it is urgent to identify non-hormonal treatments for those patients who are in devastating conditions [150–152]. Studies has reported that HSPs treatment conferred protection on affected muscles in DMD patients [153].

Lung cancer patients with muscle atrophy have obvious hyperaminoacidemia, so protein intake is necessary to induce whole-body anabolism [154]. Another study reported that a high-protein formula was able to stimulate muscle protein anabolism in advanced cancer patients. Different from conventional nutritional supplement, high-protein formula contains abundant leucine, specific oligosaccharides, and fish oil [154, 155]. Meanwhile, protein anabolism should be maintained in a stable state. Once the steady state is broken, it may go to another direction. For example, a maladjustment was observed in cachectic pancreatic cancer patients. In cachectic patients only protein breakdown was reduced, while in control people, both protein breakdown and synthesis were modulated [156].

15.8.3 Pharmacologic Treatments

Pharmacologic treatment for cachexia-related muscle atrophy is still in the phase of assessment [157]. Several medicines have been tested to treat muscle atrophy [158]. Megestrol acetate can improve the appetites and body weights of cancer patients. Here, the weight gain is mostly due to fat and water increase rather than muscle increase [159]. Cannabinoids have also been used in muscle atrophy treatment. However, clinical trial showed that compared with placebo treatment, cannabinoids treatment did not have any better effects on cancer patients [160]. In addition, nonsteroidal anti-inflammatory drugs (NSAIDs) have been tested alone or in combination in muscle atrophy treatment. The study showed that NSAIDs could improve body weight or lean body mass [161]. Besides, multimodal cachexia intervention and thalidomide have been tested in cancer cachexia [162–164]. Further clinical investigation demonstrates that targeting cytokines may have some potential therapeutic effects on cancer cachexia [165–167, 143].

15.9 Perspective

Muscle atrophy in cancer is a prevalent symptom, which affects the physical health and spiritual health of patients. The prevalence of muscle atrophy in cancer is a worldwide tendency. Muscle atrophy in cancer has a high morbidity both in

newborn children and old man. In the last decade, we have acquired more understanding of the mechanisms in cancer-related skeletal muscle loss. However, there is still a long way to go in translating these knowledge into clinical therapy. What's more, mechanism elucidation and experimental model establishment are urgently needed. Here, we described the prevalence, mechanisms, pathophysiological effects, and current clinical treatments of muscle atrophy in cancer. In summary, cancer-related muscle atrophy is the result of abnormal metabolism. The pathophysiology of muscle atrophy in cancer is quite different from other diseases. At present, no effective therapies for cancer cachexia patients are available. For this reason, we need firstly implement strategies that are aimed to prevent or delay the disease. Another crucial point is the early diagnosis and treatment of muscle atrophy for cancer patients. We hope we can improve the survival rate of cancer patients and help them to live more independently in future.

Competing Financial Interests The authors declare no competing financial interests.

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Chapter 16
The Molecular Mechanisms and Prevention Principles of Muscle Atrophy in Aging

Yu Zhang, Xiangbin Pan, Yi Sun, Yong-jian Geng, Xi-Yong Yu, and Yangxin Li

Abstract Muscle atrophy in aging is characterized by progressive loss of muscle mass and function. Muscle mass is determined by the balance of synthesis and degradation of protein, which are regulated by several signaling pathways such as ubiquitin-proteasome system, autophagy-lysosome systems, oxidative stress, proinflammatory cytokines, hormones, and so on. Sufficient nutrition can enhance protein synthesis, while exercise can improve the quality of life in the elderly. This chapter will discuss the epidemiology, pathogenesis, as well as the current treatment for aging-induced muscular atrophy.

Keywords Muscle atrophy · Aging · Prevalence · Mechanisms · Pathophysiological effects · Treatments

16.1 Background

Muscle atrophy in aging, also known as sarcopenia, is a major public health problem, which can affect the quality of life and even shorten life span of the elderly [1–3]. To cure the disease, we have to know the mechanisms first. In adults, the normal muscle mass and function are maintained by activating signaling pathways that regulate protein synthesis and degradation. Despite the knowledge on

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mechanisms of muscle atrophy, the treatment options are very limited. Decreased athletic ability is a major factor contributing to the loss of muscle mass and reduced muscle strength in old people. So exercise plays a positive role in maintaining muscle mass and physiological functions. However, exercise is not the most suitable practice for all elderly people because they often have other chronic conditions such as kidney and heart failure, which limits their daily activity. Therefore, it is necessary to develop clinical interventions to help patients with sarcopenia.

The social and economic burden caused by sarcopenia is enormous; therefore, it is important to develop interventions to prevent or delay muscle atrophy. This chapter aims to discuss recent development in the prevalence, mechanisms, pathogenesis in sarcopenia, and the role of exercise and other interventions for preventing the development of muscle atrophy.

16.2 Prevalence of Aging-Related Muscle Atrophy

With continued growth of world population, aging occurs at an extraordinary speed, which causes a lot of problems on health care. Sarcopenia manifests as the loss of mass and strength of skeletal muscle associated with aging. About 40–50% of the population over the age of 80 suffers from sarcopenia, making it a major clinical disorder of the elderly and a main challenge for otherwise healthy aging population [4]. Among patients over 64, the prevalence of sarcopenia was 22.6% in women and 26.8% in men and rose to 31.0% and 52.9%, respectively, in those elderly over 80 [5]. Older age is associated with reduced mobility and can change body composition. Over time, old people tend to become more and more sedentary, leading to a vicious cycle of reduced mobility and physical activity levels [6]. Sarcopenia is associated with dyskinesia and muscular dysfunction in elderly over 60 [7]. In the FRAIL-HF study, 1-year survival rate was 89% in the non-weak group and 75% in infirm among patients with an average age of 80 ± 6 years [8].

The decrease in muscle strength is mainly due to the degradation of contractile protein, which can be detected by a reduction in muscle fibers' cross-sectional area (CSA). For instance, between 65 and 75 years of age, the CSA of muscle is reduced by up to 30%, and muscle strength is reduced by about 30–40% [9]. Though the prevalence of muscle atrophy in aging population is pretty high, there are no registered effective treatments currently. In order to fully study the mechanism of this muscle atrophy and seek effective treatment to prevent muscle loss, animal models has been used for preclinical research. By now, the aged animals were used widely although the cost was very high [4]. In addition, newer models such as high-fat diet [10] and senescence-accelerated mouse P8 (SAMP8) [11], hind limp unloading, and immobilization have been used to study mechanisms of muscle atrophy [12]. An important contributor leading to sarcopenia is the mutations of mitochondrial DNA accumulated with age that cause mitochondrial dysfunction [13]. The purpose of all these models is to better understand the pathogens of sarcopenia and to develop strategies to prevent muscle loss.

16.3 Mechanisms of Aging-Related Muscle Atrophy

This part discusses the latest research findings of mechanisms associated with muscle atrophy in healthy aging conditions (Fig. 16.1 in this chapter).

16.3.1 The Ubiquitin-Proteasome Systems and Aging-Related Muscle Atrophy

The ubiquitin-proteasome plays a major role in the turnover of muscle protein and is activated in most catabolic processes contributing to muscle atrophy. UPS consists of ubiquitin (Ub), ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin-protein ligase (E3), and 26S proteasome. The proteins have to be ubiquitinized prior to degradation by the proteasome. E1 and Ub combine to form the Ub-E1 complex, which is a process that consumes ATP. The Ub-E1 complex interacts with E2, which replaces El to form the Ub-E2 complex. Finally the proteins are transferred to E3. The procedure is repeated again until the target protein is connected with four to five ubiquitin molecules and then degraded in the 26S proteasome, resulting in degradation to polypeptides, and ubiquitin is released and is recycled for future use [14].

In general, muscle atrophy F-box (MAFbx) and muscle RING finger protein 1 (MuRF-1) are two main types of E3 ligases in UPS that are specifically expressed

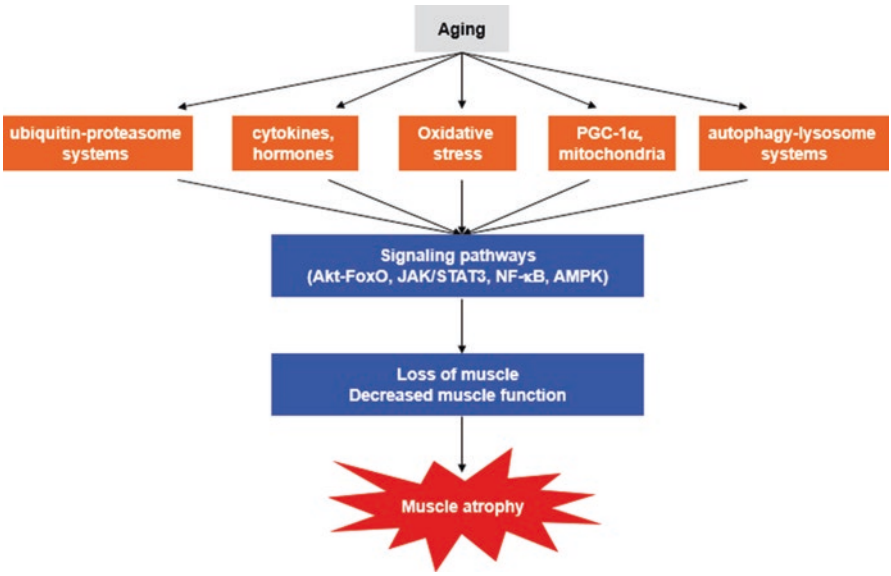

Fig. 16.1 The major mechanisms leading to the muscle atrophy in aging

in skeletal muscle. MAFbx participates in the formation of functional ligase complexes, ubiquitinates and degrades muscle differentiation proteins and eukaryotic translation initiation factor 3, and therefore plays an important role in the suppression of muscle protein synthesis. MuRF-1 also ubiquitinates and degrades troponin 1 [15] and myosin heavy chain [16]. Another E3 ubiquitin ligase is muscle RING finger protein 2 (MuRF-2). Like MuRF-1, MuRF-2 is also related to the ubiquitination of myofibrillar proteins, and MuRF-2 migrates to the nucleus and then causes ubiquitination [17, 18]. Serine/threonine kinase (AKT) [19, 20], extracellular signal-regulated kinase (ERK) [21, 22], inhibitor of κB kinases (IKKs) [23], and nuclear factor-κB (NF-κB) [24, 25] signaling pathways all regulate muscle degradation with UPS.

16.3.2 The Autophagy-Lysosome Systems and Aging-Related Muscle Atrophy

Autophagy is a process in which cells use lysosomes to degrade damaged organelles and excess or abnormal proteins in cells, which stabilizes the intracellular environment by balancing cell synthesis and catabolism. The autophagy lysosomal system is a key system for controlling muscle volume in catabolism [26]. However, the autophagy system also requires the most basic muscle fiber homeostasis, and its inhibition also leads to the degradation of muscle cells. Autophagy has maintained a low activity state in skeletal muscle tissue. Nevertheless, in pathological conditions such as oxidative stress, denervation, and fasting, skeletal muscle autophagy is significantly enhanced, resulting in protein degradation [27, 28].

The molecular mechanism of autophagy is complex and highly conserved, and the mTOR signaling pathway plays a major regulatory role. However, mTOR signaling pathway is not the main way to regulate the occurrence of autophagy in muscle tissue. Inhibition of mTOR in skeletal muscle cells can only slightly increase the degradation of proteins in myotubes [29]. In contrast, FoxO3, a transcriptional regulator, is a key gene that regulates autophagy in muscle. Some important autophagy genes such as *Bnip3*, *Gabarap*, *LG3*, and *Atgl2l* have been regulated by FoxO3 [30]. In particular, Bnip3 induces the formation of autophagosomea and contributes to FoxO3-induced autophagy. In addition, FoxO3 not only activates the autophagic/lysosomal pathway and the ubiquitin-proteasomal system but also regulates the two pathways independently. In addition, the p38/MAPK pathway also regulates the autophagy-related genes expression under oxidative stress [31].

The ubiquitin-proteasome systems and the autophagy-lysosome systems are both regulated equal to maintain normal organelles and protein composition in atrophic cells [29]. Proteasomes degrade short-lived proteins and myofibril [32, 33]; however, autophagy-lysosomes are thought to be able to control long-lived proteins and organelles [34, 35]. Therefore, it is necessary to further control the signal

transduction pathways which regulate the autophagy system and the related ubiquitin-proteasome system.

16.3.3 Oxidative Stress and Aging-Related Muscle Atrophy

Excessive accumulation of oxygen molecules of −1 valence state will form an oxidative stress state in the body. Reactive oxygen species (ROS) includes monovalent oxygen, peroxides, superoxide, hydroxyl radicals, and hypochlorous acid. Almost all types of cells in the body, such as smooth muscle cells, vascular endothelial cells, monocytes, skeletal muscle cells, and cardiomyocytes, can produce ROS. Excessive ROS can directly damage tissue or stimulate the body to generate more ROS and thus form a vicious circle. CHF, atrophy of limbs, atherosclerosis, diabetes, and cancer can all occur under certain pathological conditions [36]. It seems to be acknowledged that the main cause of muscle atrophy caused by unbalanced protein synthesis and degradation is oxidative stress [37]. Oxidative stress is thought to be a pathological state of redox imbalance. Furthermore, the generation of oxidants exceeds the resistance of the antioxidant defense system, and the generation of high ROS increases [38]. Increased oxidative stress in skeletal muscle during aging can lead to decreased mitochondrial function and molecular inflammation. These factors interact to induce apoptosis of muscle fibers and interfere with protein metabolism balance, which may be an important mechanism of senile muscular atrophy.

One view is that oxidative stress may lead to skeletal muscle atrophy in the following ways. First, Ca^{2+} overload and activation of calcium kinase; second, activation of cysteine protease and subsequent activation of the 20S proteasome system; and third, upregulation of gene expression of MAFbx and MuRF1 in mouse, followed by proteasome activation. In addition, it has recently been discovered that p38MAP kinase acts as a bridge between the autophagy gene and the ubiquitin-proteasome in oxidative stress and atrophic skeletal muscle and can stimulate the upregulation of these genes [39]. Fourth, ROS activates FoxO and NF-κB in the absence of mammalian atrophy [40].

It is reported that there are two sets of oxidant system in atrophic skeletal muscle as the main source of ROS, namely, NADPH oxidase and mitochondria, the former being predominant. After infusion of mice with AngII for a period of time, the ROS levels in the muscle were increased in parallel with the level of the NADPH oxidase subunit gp91phox [41]. Wei et al. [42] confirmed that AngII can significantly increase the activity of ROS production and NADPH oxidase in L6 myotubes, whereas these increasing effects can be interrupted by NADPH oxidase inhibitor apocynin and AT1 receptor blocker losartan. Recent studies have also found that mitochondrial-derived superoxide in skeletal muscle is also elevated in animal models of AngII perfusion [43], which demonstrates that AngII-induced oxidative stress may result in muscle atrophy in the mouse model, and the ROS originated from the two sets of oxidant systems (NADPH oxidase and mitochondrial system) mentioned in the previous paragraph all participate in AngII-induced oxidative stress.

Another view is that mice lacking mitochondrial superoxide dismutase in their muscles still have oxidative stress, but there is no obvious muscle loss, indicating that only oxidative stress is not enough to induce muscle atrophy [37]. Although the role of oxidative stress on disuse skeletal muscle atrophy could not be ignored, the causal relationship among them is not yet completely clear. Different research teams did similar experiments but got very different results. This shows that the researchers should focus on different species, different models of disuse, and different muscles, such as experimental animal mechanical ventilation, limb braking, and upper limb suspension. The patient's bed rest and unilateral lower extremity suspension are not the same as the muscle disuse atrophy caused by these conditions [44]. ROS exists in skeletal muscles and participates in the steady-state regulation of muscles as a major signal, which guarantees the normal physiological structure and function of skeletal muscle. However, the half-life of ROS in skeletal muscles is so short that it's difficult to determine their target substances directly and deviations often occur [45].

Oxidative stress is not related to all types of disused muscle atrophy. The degree of redox in different muscles and species fluctuates greatly. For example, oxidative stress is likely to have a causal relationship with diaphragmatic atrophy in mice, but whether it has been related to the disuse of a soleus muscle in HU mice has not yet been established. Little is known about the gastrocnemius, and the role in humans is even more unconfirmed. Experimental studies have shown that patients have a strong and rapid oxidative stress due to diaphragmatic atrophy induced by mechanical ventilation [46]. At this time, the extent of oxidative stress in the atrophic muscles of the limbs is weak and slow [47].

Decreased mitochondrial function and inflammation-induced apoptosis of muscle fibers and imbalanced protein metabolism may be the main mechanisms by which the number of muscle fibers decreases and existing muscle fibers shrink. Oxidative stress, decreased mitochondrial function, inflammatory response, and apoptosis have complex interrelationships at the cellular and molecular levels.

In brief, oxidative stress does have a certain responsibility for the occurrence of muscle atrophy, but it is still to be studied whether or not who is responsible for the relationship between the two. Because of the short duration of ROS, it may have different results because of the different timing, location, and nature of ROS. Moreover, different muscles, species, and models have different degrees of oxidative stress during disuse atrophy. There are too many variable factors in relevant experimental studies, and there are limitations in the means of monitoring relevant variables, so the original committee remains to be studied.

16.3.4 Proinflammatory Cytokines, Hormones, and Aging-Related Muscle Atrophy

16.3.4.1 Il-6

IL-6 is a cytokine with multiple immunoregulatory functions and is mainly produced by adipocytes, cardiomyocytes, and leukocytes. However, several studies have shown that skeletal muscle is also an important tissue organ that secretes IL-6. IL-6 is characterized by gradual loss of skeletal muscle tissue and is associated with diseases such as cachexia, aging, and muscular dystrophy. In skeletal muscle, IL-6 mainly activates the JAK/STAT3, ERK, and PI3K/Akt3 signaling pathways. Among them, STAT3 protein activation is the key to induce muscle degradation [48, 49].

16.3.4.2 TNF-α

TNF-α is a kind of multifunctional cytokine which plays an important role in immune, inflammation, and injury responses. The results of the study indicate that TNF-α can inhibit protein synthesis and accelerate its degradation [50]. In addition, elevated levels of TNF-α in the body are closely related to skeletal muscle protein degradation caused by aging or certain diseases like cancer, chronic obstructive pulmonary disease [51], and so on. TNF-α exerts multiple biological functions by binding two separate cell surface receptors, TNFR1 and TNFR2. The results show that TNF-α participates in the process of muscle protein degradation mainly through the TNFR1 receptor [52].

16.3.4.3 TWEAK

As a newcomer of the TNF superfamily, TWEAK is functionally similar to TNF-α, such as induction of apoptosis, promotion of inflammatory response, and regulation of immunity. TWEAK binds to its receptor Fn-14, which not only activates nuclear transcription factor NF-κB through TRAF6, upregulates MuRF-1 expression, induces muscle protein degradation [53, 54], but also enhances NADPH oxidase activity and promotes cell release of ROS [55]. TNF-α and TWEAK can also upregulate the expression of MAFbx and MuRF-1 by activating the p38MAPK and JAK/STAT3 signaling pathways [56, 57]. The cachectic phenotype can be induced by overexpressed TWEAK partially via the induction of the E3 ligase MuRF1 in pathological conditions [53].

16.3.4.4 Glucocorticoid

The adverse effects of glucocorticoids widely used in clinical practice could not be underestimated. It can reduce synthesis of muscle protein and accelerate the decomposition of protein and thus become the main hormone causing muscle atrophy. Atrophy may be related to its ability to induce the upregulation of MuRF-1 and MAFbx expression. Studies have shown that glucocorticoids and FOXO1 cooperate to induce MuRF-1 gene transcription [58]. Therefore, breakdown of muscle protein stimulated by glucocorticoid is mainly mediated by ubiquitin-proteasome-dependent proteolysis. In addition, the process may also involve calcium-dependent protein degradation.

16.3.4.5 Angiotensin II

In the renin-angiotensin system (RAS), angiotensin II is the one of the main effector molecules. It regulates the central nervous system, adrenal glands, blood vessels, and kidneys to maintain the body's water-sodium balance. The skeletal muscle atrophy caused by AngII includes the following mechanisms: Atrogin1/MAFbx, upregulation of E3 ligase encoded by MuRF-1, increased decomposition protein in ubiquitin-proteasome system, and increased active oxygen content [39].

16.3.5 PGC-1α, Mitochondria, and Aging-Related Muscle Atrophy

Energy transduction and oxidative metabolism pathways of mitochondria are essential to the function of skeletal muscle. One major effect of long-term muscle atrophy is reduction of mitochondria. Peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-lα) can not only promote the formation of mitochondria but also participate in the formation of slow muscle fibers, muscle fiber phenotype conversion, and other processes. PGC-1α and NFAT (activated T-cell nuclear factor) participate jointly in regulating the formation of oxidized type I muscle fibers [59]. Studies have shown that normal levels of PGC-1α cannot prevent atrophy, whereas overexpression of PGC-1α can protect skeletal muscles during the process of muscle atrophy, which may be related to inhibition of FOXO3 signaling [60]. As a highly conserved protein kinase, AMPK is related to the regulation of many physiological processes. Activation of AMPK not only promotes mitochondrial production via PGC-1α, activates TSC-2, or makes eEF-2 inactivated to inhibit mTOR- p70s6k pathway but also attenuates the translation of mRNA, which inevitably leads to reduction of protein synthesis [61]. Therefore, there is an urgent need to determine whether activation of AMPK may regulate PGC-1α without affecting protein synthesis. As a lot of proteins lost during the process of muscle atrophy,

activation of the AMPK pathway to enhance the inhibition of protein synthesis does not serve the original purpose of protecting skeletal muscles.

16.4 Pathophysiological Effects of Aging-Related Muscle Atrophy

16.4.1 Clinical Symptoms of Muscle Atrophy in Aging

With aging, the regeneration ability of tissue cells decreases, and the body will experience muscle atrophy and decreased strength or skeletal muscle atrophy, resulting in degenerative changes in muscle and function [9]. Irwin Rosenberg, a professor at the University of Tufts in the United States, first proposed muscular decay syndrome. It is a progressive systemic hypofunction syndrome with a series of changes, such as reduction of volume, quantity, and mass of skeletal muscle fiber, a decrease of skeletal muscle strength, and an increase in connective tissue and adipose tissue. The main clinical manifestations of patients are muscle weakness, muscle relaxation, decreased mobility, increased folds, reduced body mass and defatted body mass, explosive power and grip strength, and even decreased balance, difficulty standing, and lowering.

Epidemiological data show that the incidence of muscle attenuation in the elderly is high, which seriously affects the elderly's quality of life. In 2010, a working group of the elderly people with sarcopenia in Germany put forward the diagnostic criteria and grading of muscle attenuation syndrome for the first time. They proposed that muscle attenuation syndrome can be diagnosed by reduction of skeletal muscle volume, a decrease of skeletal muscle strength, and a decrease of limb and trunk motor ability. Two of them can be diagnosed as muscle attenuation syndrome, and all three are met with severe muscle attenuation syndrome [62]. Current research and clinical instrumental diagnostics mainly use computer tomography (CT) [63], nuclear magnetic resonance imaging (MRI), ultrasound, dual-energy X-ray absorption (DEXA) [64], bioelectrical impedance analysis (BIA), and other methods to measure the mass of skeletal muscle. The measurement of muscle strength and function of skeletal muscle mainly includes determination of pace, lower limb muscle strength, and grip strength [65, 66]. The combination of grip strength and lower limb muscle strength is a method for evaluating strength and function of skeletal muscle.

16.4.2 Histological Symptoms of Muscle Atrophy in Aging

Skeletal muscle is a terminally differentiated cell composed of multinuclear muscle fibers. Adult muscle fibers lose the ability to undergo mitosis, so skeletal muscle damage is mostly irreversible. From a histological point of view, muscle tissue is

one of the most complex structures in the organism. At the same time, muscle cells are the largest cells in the organism. Therefore, the study of the physiology and pathology of muscle and the method of histology in the fields of biology and clinical medicine occupies a very important position. Especially in clinical medicine, the diagnosis and treatment of neuromuscular disease is mainly based on the results of histological studies. Understanding the histological changes in muscle cell aging can more intuitively understand the characteristics of muscle cell aging and also provide a reference for a more in-depth study of aging and muscle function decline in histological research methods.

16.4.2.1 Structure Changes of Muscle Atrophy in Aging

Under normal circumstances, two types of muscle fibers compose the body's skeletal muscle, namely, fast muscle fibers (type II) and slow muscle fibers (type I).The proportion of the two fibers in different skeletal muscles of the body is different. For example, in the skeletal muscles that maintain the main body posture, the slow muscle fibers occupy a relatively high proportion, while in the exercise-oriented skeletal muscles, the proportion of fast muscle fibers is higher. During the process of human aging, muscle fiber structure will change which have been reported in detail. Autopsy analysis of human extraosseous muscles showed that type I and type II muscles were 50% less at the age of 90 than 20 [67]. In addition, at older ages, distribution of muscle fiber types to higher percentages converts type 1 muscle fibers more clearly than type II muscle fibers [68].

Studies have shown that sarcopenia is dominated by the reduction of fast myofibers [69]. However, other studies did not find any significant change in the type of muscle fiber composition with age [70, 71]. Histochemical analysis of muscle biopsies suggested that with age, the size of type II muscle fibers became smaller, while type I remained relatively unchanged in size. Although type II muscle fiber atrophy seems to be consistent with the muscle strength reduction during the aging process, the major factor in the loss of muscle strength is the decrease in muscle cross section during aging [67].

16.4.2.2 Changes of Myocyte Nuclear of Muscle Atrophy in Aging

Muscle fibers contain hundreds and thousands of muscle nuclei, and each myocyte nucleus controls a certain number gene expression of cytoplasmic bases, which is called the myocyte nuclear domain. Assume that the size of the muscle fibers changes, such as muscle fiber atrophy or muscle fiber hyperplasia, can be accompanied by changes in the number of muscle nuclei and myocyte nuclear domain [72]. Although animal models of changes in the number of muscle nuclei have been proposed, human experimental data show that there is usually no change in the number of muscle nuclei when muscles are atrophied [73]. The latest research showed that

the number of myocyte nuclei changed only when the mass increased significantly and there was no change when the mass was below 15% [74]. This theory is consistent with the notion that myocyte nuclei support cytoplasmic finite volume gene expression.

16.4.2.3 Changes of Muscle Satellite Cells of Muscle Atrophy in Aging

Skeletal satellite cell (SSCs) is a kind of adult stem cells distributed between the sarcolemma and basement membrane of muscle cells. Their location and arrangement are similar to satellites of muscle cells, so they are called satellite cells [75]. Skeletal satellite cells are undifferentiated muscle progenitor cells (MPCs) retained in muscle tissue of adult individuals, located on the basement membrane and basement membrane of muscle fibers. Among them, there is a potential for self-renewal such as differentiation and proliferation. The content of muscle satellite cells is relatively small, accounting for approximately 1% to 4% in adult skeletal muscle. In resting state, SSC also has less cytoplasm and organelles and has a higher ratio of nucleoplasms; its cell nucleus is smaller than that of myotubes, and its heterochromatin content is higher than that of muscle nuclei [76]. As the age increases, the abundance of SSC gradually decreases, and the potential of SSC to differentiate myogenicity and self-renewal remains, but the renewability decreases.

Satellite cells are usually stay in hibernation. With the influence of many external stimuli, satellite cells in the body are activated to enter the cell cycle, producing MPCs that multiply, differentiate, and fuse to form new muscle cells. After activation, the division mode of satellite cells follows that of stem cells, that is, two types of daughter cells are produced after cell division. One of them will remain as the source of cell division in the future and remain in the original state, and the other can be further differentiated into mature muscle fibers. During activation of satellite cell, numerous factors and cytokines are involved in the regulation of this process (e.g., FGF-2, HGF [77, 78], FGF, LIF, IL-6 [79, 80], IGF-1 [81, 82], SCF, and NO). However, it is still unclear whether these different growth factors affect the reformation of satellite cells, whether they affect the self-renewal of satellite cells, or whether they stimulate the expansion of the replicating myoblast bank alone. The self-renewing signaling pathways of muscle satellite cells are mainly Notch signaling pathway [83, 84] and Wnt/β-catenin signaling pathway [85, 86].

16.5 Current Clinical Treatments of Muscle Atrophy in Aging

The treatments consist of the nutritional support, exercise, drug, gene therapy, and cell therapy (Fig. 16.2 in this chapter).

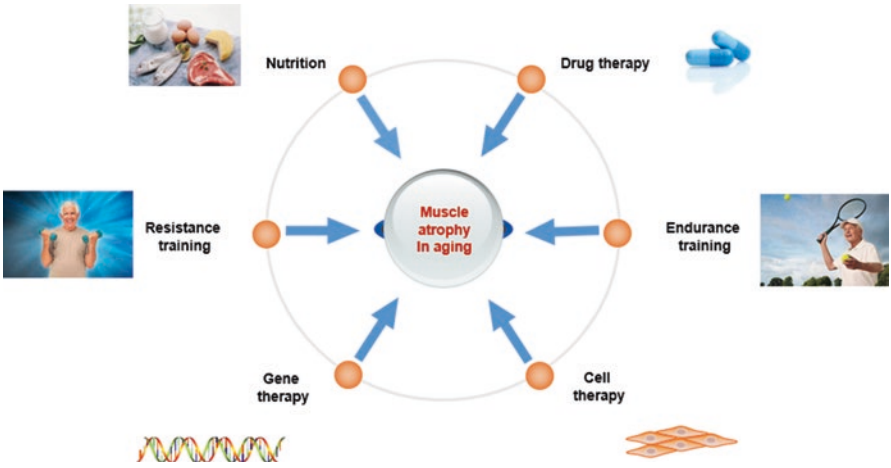

Fig. 16.2 The main strategies for the treatment of muscle atrophy in aging

16.5.1 Nutritional Support

Protein accounts for about 20% of the muscle mass. The balance of protein metabolism determines the amount of muscle. Therefore, the reduction of protein intake has a direct impact on sarcopenia. Nutritional support can improve the quality of life of malnourished people, such as the elderly and chronic wasting diseases, to a certain extent. Therefore, many researchers believe that nutritional interventions, especially the intake of protein and amino acids in the body, can directly promote muscle protein synthesis and prevent sarcopenia [87]. The recommended intake of dietary protein is 1.0–1.2 g per kilogram of mass per day [88]. The body's vitamin D is derived from the diet and the effect of UV on the skin, so strengthening protein intake and supplementation with vitamin D can improve strength and function of muscle in the elderly [89]. In addition, protein ingestion before sleep has been suggested as an effective way for increasing the anabolic response and to efficiently stimulate protein synthesis of muscle in the elderly [90]. However, a number of studies have shown that nutritional support could not effectively increase the muscle mass and improve the functional status of patients with sarcopenia [91, 92]. Therefore, nutritional interventions for patients with sarcopenia need a further study.

16.5.2 Exercise Training

Exercise training plays a positive role in maintaining the physiological functions. Many researches have confirmed that exercise improves mass and function of muscle in patients with sarcopenia significantly. After giving the elderly some exercise

training, their muscle mass and function have been significantly improved, and to a certain extent, the occurrence of falls and decreased mobility has been prevented [93].

16.5.2.1 Resistance Training

Resistance training is a kind of resistance exercise. The main purpose is to train the body's muscles. The traditional resistance training includes push-ups, dumbbells, and barbells. Resistance exercise can lead to a series of beneficial functional changes by promoting skeletal muscle anabolism and inhibiting catabolism [94]. With the development of the aging process, the quality and strength of human skeletal muscles will decline. Resistance training can convert type IIb muscle fibers into type IIa fibers, suggesting that resistance training may increase muscle aerobic capacity because type IIa muscle fibers have stronger aerobic oxidation properties than type IIb muscle fibers. It is reported that resistance exercise training can simultaneously increase satellite cell content [95] and extra strand and trapezius muscle fiber size [96], while some studies have reported that exercise induces a proportionate increase in myocyte nuclear content and induces muscle fiber hypertrophy [73]; however, there are still some studies that could not confirm these [97]. Horii [98] found that resistance training-induced changes in circulating C1q levels may be helpful to the prevention of fibrosis and atrophy of muscle via Wnt signaling in senescent mice. In short, resistance exercise is always a potent method to prevent the muscle mass loss during the aging process.

16.5.2.2 Endurance Training

Endurance exercise, also known as aerobic exercise, is the most important and basic exercise method for exercise prescription. Common aerobic sports include walking, jogging, walking, alternating stairs, swimming, cycling, power cycling, running, skipping, boating, water skiing, skiing, and ball sports. Endurance exercise produces good results mainly through improving cardiovascular health [99] and inhibiting proinflammatory cytokines [100]. However, recent studies shows that under various chronic conditions like cancer cachexia [101], cardiac cachexia [102], or diabetes [103], endurance exercise can also weaken atrophy of skeletal muscle. Besides that, endurance exercise can also enhance the mitochondrial function of skeletal muscle, which may be related to the enhancement of PGC-1α expression, but it has no significant effect on the size of skeletal muscle [104]. Moderate-intensity endurance training has the effect of accelerating the synthesis of fast-twitch skeletal muscle proteins in the aging body, which may have important potential in preventing and delaying sarcopenia's clinical approaches. Endurance training can not only reduce plasma-free amino acid levels but also increase the amount of protein in fast-twitch skeletal muscle and upregulate MHCII expression in skeletal muscle. This regulation may be mediated by the mTOR/p70S6K pathway [105, 106]. However, the specific mechanism is still not clear, and further research is needed.

16.5.2.3 Combination Training

Both resistance and endurance training can increase the contents of skeletal muscle, especially satellite cells of the type II muscle fiber. In addition, the active factors like MyoD, myogenin, Mrf4, and Myf5 that activate and proliferate the satellite cells also increase. More meaningfully, combining resistance and endurance training is more conducive to improve the body composition and fitness of the elderly than endurance or resistance exercise alone, but the mechanism remains to be studied. It is notable that performing strength and endurance training at the same time will bring a trading effect, which is called simultaneous training effect or interference effect compared to the exercise of strength and endurance alone. As the genetic and molecular mechanisms involved in the induction of resistance training and endurance training are different, therefore optimizing the design of the exercise program is needed.

16.5.3 Drug Therapy

Many researchers have applied different measures to treat sarcopenia based on known factors and underlying mechanisms, which have achieved certain results, such as the application of sex hormones (testosterone, etc.), growth hormone, growth hormone receptor modulators, nonsteroidal anti-inflammatory drugs (celecoxib), and so on. Although studies have shown that testosterone can increase LBM and improve function of skeletal muscle, celecoxib can significantly increase LBM and TNF-α [107]. However, the current related research is still in the initial stage of exploration. There is insufficient research data, especially clinical research data to support the efficacy of these drugs. Therefore, drug treatment for sarcopenia requires further experimental and clinical studies.

16.5.4 Gene Therapy

In gene therapy, viral or nonviral vectors are widely used to transport the target genes to adult cells. Currently, the viral can infect the skeletal muscle system systematically, but it has been completed only in mouse models, not in human system. Whereas, there are limitations to nonviral vectors once delivered into the recipient cells due to vector instability. There are two methods of gene therapy. One is the indirect method of introduction. The therapeutic effect is exerted by the secretion of the exogenous gene expression product. The second is the direct introduction method. The target gene would be combined with viral or nonviral vectors, or directly introduced into the target cells to express the desired functional protein in vivo and produce therapeutic effect. The direct method is easy to operate but lacks of specificity and targets during gene transfer. Rodgers [108], who created

AAVogen company, found that an adeno-associated virus can transport Smad7 into the muscle cells. As a signal protein, the Smad7 protein then obstructs another two signaling proteins named Smad2 and Smad3. Both proteins can be activated by myostatin and some other hormones causing muscle atrophy, and Smad7 can block muscle atrophy by blocking these signals.

16.5.5 Cell Therapy

Cell therapy meets a problem that peripheral environment does not continue to provide a sufficient number of cells when using committed cells or stem cells. The research of static satellite cell characterization is a promising study to improve the regeneration of muscle tissue. Skeletal muscle satellite cells serve as myogenic stem cells and play an important role in the repair and regeneration of skeletal muscle. Multiple signaling pathways are activated through the self-renewal of satellite cells. However, the self-renewal mechanism that skeletal muscle satellite cells proliferate and differentiate is still controversial and still needs further research to confirm. During quiescence, activation, and differentiation of satellite cells, miRNAs take part in the processes and thereby regulate the regeneration of muscle [109]. During the process of muscle regeneration, the expression change of several miRNAs seems similar to that observed in the processes of embryonic myogenesis and muscle regeneration, such as miR-1, miR-682, and miR-499 [110].

16.6 Perspective

Muscle atrophy is a common clinical syndrome characterized by skeletal muscle mass and its function decreasing. It often occurs in elderly people and not only increases the fall rate, disability rate, hospitalization rate, and even death rate but also increases the economic burden on individuals and society.

At present, the research on muscle atrophy in aging is still in an exploratory stage. The pathogenesis and even the diagnosis and treatment of the disease are still not very clear. It is of great significance to explore the effects of acute exercise and long-term exercise training on mitochondrial function, oxidative stress, apoptosis, and inflammation in the elderly. Except that, research on stem cells and gene therapy has changed the traditional view of human treatment of diseases, and humans have hoped for stem cell treatment for diseases that were previously difficult to treat in recently years. With further research on its cell characteristics, biological characteristics, differentiation potential, and differentiation mechanisms, there is a reason to believe that more incurable diseases will be treated with satellite cell transplantation.

It is essential to conduct further research using genetically manipulated animal models and animal models for sports, which helps further understanding the cellular

molecular mechanisms and prevention principles of muscle atrophy in aging, developing scientific exercise prescription interventions, improving skeletal muscle health of elderly people, and reducing the social and economic burden.

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Chapter 17
Muscular Atrophy in Cardiovascular Disease

Isadora Rebolho Sisto, Melina Hauck, and Rodrigo Della Méa Plentz

Abstract Currently, the number of chronic diseases has increased due to increasing in life expectancy of population. Among them, cardiovascular diseases (CVD) are the most prevalent and responsible for the high mortality and morbidity rates. Patients with CVD have metabolic, hemodynamic, and musculoskeletal changes. There is a debate regarding the correct term for musculoskeletal changes that affect this group of patients; therefore, we found in literature myopia, muscular atrophy, cardiac cachexia, and sarcopenia. However, although there is no standardization in relation to correct term, these musculoskeletal consequences directly affect the quality of life and are associated with a poor prognosis. In this way, the importance of prevention of muscular atrophy, but also of treatment for those patients with progressive muscle decline, is proven. We also emphasize the importance of a multiprofessional team, because therapeutic strategies are needed that are capable of delaying the onset or minimizing the consequences of skeletal muscle loss, from pharmacological management and nutrition to physical exercise.

Keywords Cardiovascular diseases · Myocardial ischemia · Stroke · Peripheral vascular diseases · Muscular atrophy · Cachexia cardiac · Sarcopenia · Myopenia

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17.1 Background

The number of chronic diseases, currently, has increased due to an increase in the population's life expectancy. Among them, cardiovascular diseases are disorders of the heart and blood vessels and include ischemic heart disease or coronary artery disease, cerebrovascular disease, and diseases of the aorta and arteries, such as hypertension and peripheral occlusive arterial disease [1]. Cardiovascular diseases account for 17.7 (31%) million deaths around the world [1]. Patients with cardiovascular diseases have metabolic, hemodynamic, and musculoskeletal abnormalities, which can lead to muscular loss, sarcopenia, and/or cardiac cachexia [2]. The loss of muscle mass may be a consequence of pathological changes, in the case of muscular dystrophies; due to the aging process, as in the case of sarcopenia; by simple disuse; or secondary to diseases inducing cardiac cachexia [3].

Sarcopenia is a progressive degradation of muscle mass originally observed during aging, with a prevalence of 5–10% in people over 65 years of age [4]. It is associated with an increased risk of fragility, worsening of quality of life, disability, falls, hospitalization, and even death [5]. The pathogenic loss of motoneurons during aging also contributes to the development of the disease [4]. Sarcopenia has a multifactorial etiology determined by changes in endocrine function, immobilization, impaired feeding, insulin resistance, denervation, and inflammation [6]. It can be classified into two forms, primary when it is related to age and the cause is aging and secondary when it is related to a disease [7]. When related to diseases, sarcopenia is associated with insufficiency of advanced organs, chronic inflammatory diseases, malignancies, and endocrine diseases that affect protein synthesis, proteolysis, neuromuscular integrity, and muscle fat content [7] and is characterized by the progressive and generalized loss of skeletal muscle mass and strength, with the risk of fragility and poor quality of life [8].

Cachexia is a complex and prevalent pathological condition, characterized by the patient's weight loss, as well as loss of body mass and adipose tissue [9]. It is usually related to chronic diseases, and its occurrence predicts the reduction of survival and poor prognosis. The pathophysiology of cardiac cachexia is multifactorial in nature. Several mechanisms, such as hormonal disorders, overexpression of proinflammatory cytokines, malabsorption, and reduction of food intake, are involved in the process of muscular atrophy [9].

Cardiovascular diseases are associated with a higher prevalence and increased risk of muscular atrophy and progression of these musculoskeletal disorders. Complementarily, to improve this symptomatology, and other variables, such as functional capacity and quality of life, the treatment encompasses a set of necessary and multi-professional actions to ensure a healthier lifestyle, which includes dietary change, pharmacological adherence, control of factors, as well as the use of physical therapy, psychosocial support, educational actions, and physical exercise, encompassing what we call rehabilitation [10–12].

17.2 Muscular Atrophy in Stroke

Stroke is characterized as a clinical condition due to a rapid loss of brain function because of interruption or hemorrhage of cerebral blood [13]. Spontaneous intracerebral hemorrhage occurs in 10–20% of all strokes, in-hospital mortality rate is quite high, and the main risk factor is chronic hypertension [14]. The ischemic stroke is characterized by an arterial occlusion that causes interruption of cerebral perfusion and oxygen and glucose supply, which generates a permanently infarcted tissue called the ischemic nucleus [15].

The size of ischemic nucleus will cause the greatest impact on the patient's life outcomes [15]. The collateral blood flow through the leptomeningeal vasculature is primarily responsible for limiting the extent of perfusion deficit and slowing the rate of ischemic progression [15]. The ischemic nucleus may be defined as cell death secondary to reduced cerebral blood flow, whereas the penumbra area is the site of cells with impaired functions that have not gone beyond the threshold for cell death [15].

Cerebrovascular dysfunction and brain stroke injuries are extensively studied unlike the systemic alterations and peripheral organ dysfunction [16]. Brain injury and subsequent interruption of the superior motor neuron pathway lead to contralateral upper limb paralysis [6]. Neurological deficits and restricted mobility are accompanied by muscular structural alterations [6]. There is a decrease in the number of motor units in hemiplegic musculature that persists in chronic phase of stroke [6]. There is loss and installation of a skeletal muscle remodeling process that can initiate significant physical but also metabolic consequences [16]. Thus, poststroke patients become susceptible to loss of muscle mass, a determinant factor for prolonged hospitalization, poor rehabilitation success, and long-term outcomes [16].

Stroke is the leading cause of disability in adults due to persistent neurological deficits that imbalance functional abilities and cause physical inactivity [17]. The World Health Organization (WHO) estimates that 15% of world population lives with some form of disability, of which 2–4% experience significant functional difficulties [18]. Current projections estimate an exponential increase in stroke impact on society over the next few years [15]. The progression and consequent reduction of poststroke muscle mass may be related to inactivity, reduced strength, and decreased aerobic capacity [16, 19]. However, it is likely that in addition to inactivity, other catabolic signals through neurohormonal overactivation, inflammatory cytokines, and free radical species further stimulate the conversion of muscle fibers and changes in body composition [16].

Risk factors for cardiovascular events can be grouped into three major groups: non-modifiable risk factors such as age, gender, and family history; modifiable risk factors with drugs, through drug therapy or surgical procedures; and modifiable behavioral risk factors, such as lifestyle changes, physical inactivity, and smoking [20]. Stand out among modifiable risk factors the hypertension, dyslipidemia, smoking, inactivity, obesity, and diabetes [13]. In addition, there are some factors that may influence the prognostic outcome, such as age, stroke severity, stroke subtypes, depression and physical function [13]. Recent study demon-

strated hyperglycemia is associated with length of stay and functional outcomes evaluated by Barthel [13].

17.2.1 Prevalence of Muscle Atrophy

Stroke is the leading cause of disability in adults; approximately 50% of poststroke patients have some degree of hemiparesis, making 30% incapable of walking without help, often resulting in long-term disability [19]. Recent clinical study showed prevalence of sarcopenia among poststroke patients ranges from 14% to 18% and is expected to increase over the next two decades [19]. In addition, more than 50% of the patients remain with some motor deficit making that costs for rehabilitation and daily support grow continuously [16].

17.2.2 Mechanisms of Muscle Atrophy

Muscular atrophy is predominantly responsible for poststroke weakness and not only motor control deficit due to neurological injury [21]. Sarcopenia is age-related loss of muscle mass and function, and skeletal muscle adaptations in the hemiparetic muscle currently characterize stroke-related sarcopenia [21]. A stroke-related sarcopenia has distinct characteristics such as rapid decline in muscle mass, structural muscle changes (altered muscle fiber type), a brain injury that determines bilateral differences in physical performance, loss of muscle mass not related to aging, and a catabolic signal that unbalances neurovegetative status [6].

Muscle weakness and atrophy observed in the upper limbs may possibly be associated with disuse and decreasing of contralateral and ipsilateral pathways [21]. Although weakness of paretic limb may be linked to impaired cortical activation, such deficit does not support the bilateral weakness often observed [21]. However, the mechanism that explains for such bilateral muscle weakness remains unclear. A combination of mechanisms, including immobilization, disuse, inflammation, and poststroke metabolic and neurovegetative imbalance, may result in loss of muscle mass and progress to stroke-related sarcopenia [4].

The acute ischemic event can induce a global stress response which generates local and systemic overstimulation of sympathetic nervous system, hypercortisolism, and activation of hypothalamic-pituitary-adrenal pathway [16, 22]. Damage in preganglionic inhibitory pathways of sympathetic nervous system can cause a sympathetic overflow and, consequently, a wide inflammatory and metabolic agitation [16, 22]. Sympathetic signaling stimulates catecholamines that can lead to catabolic stimulation, which triggers insulin resistance, protein degradation, and increased lipolysis [16]. There is also evidence of disturbances in the activation of cholinergic pathway of the vagus nerve and vagal reflexes, and reduction of heart rate variability is associated with negative functional outcomes [16].

There is a relation between sympathetic tonus and secretion of inflammatory cytokines, and catabolic cytokine TNF-alpha factor may be responsible for muscle mass reduction [16]. In the paretic lower limb were found increased levels of TNF-alpha mRNA in relation to the controls [19]. Sarcopenia may be also linked to increased skeletal muscle protein breakdown or reduced protein synthesis [19]. An increase of 40% in myostatin mRNA, a growth factor that negatively regulates muscle growth, was observed in the vastus lateralis muscle suggesting an imbalance between protein synthesis/degradation [19]. Recent experimental study with mouse stroke models [22] found catabolic activation in skeletal muscle due to increased apoptotic activation, a proteolytic breakdown of muscle tissue, and high levels of myostatin. These mechanisms resulted in a severe reduction in weight due to reduction of tissue mass and fat, as well as reduction of skeletal and myocardial muscle mass [22].

Inflammatory cytokines and catabolic overstimulation propagate functional muscle decline [16]. In addition to muscle atrophy, there are changes in capillarization, glucose use, proinflammatory cytokine activation, muscle fiber-type change, and endothelial dysfunction [22]. Many factors may contribute to reduction of fat (depletion of energetic reservoirs) and muscle (functional decline) mass and clinical manifestation of sarcopenia in poststroke patients [16]. Factors like physical and emotional stress, pain, spasms, and interruption of preganglionic inhibitory control in autonomic nervous system may explain all this sympathetic activation [22]. However, the complex process of metabolic and maladaptive adaptations that contribute to loss of muscle mass and development of sarcopenia, as well as its impact on functional capacity and other outcomes, is still poorly understood [16].

17.2.3 Pathophysiological Effects of Muscle Atrophy

Disability is usually attributed to brain injury; however, the skeletal muscle is the primary effector organ of poststroke disability [23]. Consequently, less attention is still given to relevant systemic effects, such as secondary alterations in muscle atrophy, metabolic and contractile capacity, and inflammation [23]. Figure 17.1 Synthesizes this effect. Muscle mass reduction is often observed in poststroke patients, and within 4 hours after brain damage, there is an initial reduction of motor neurons in the musculature of paretic limb that persists in chronic phase [4]. However, poststroke muscle dysfunction is a multifactorial phenomenon caused mainly by reduction of physical activity and achievement of compensatory motor patterns that lead to muscle weakness and atrophy [21]. It occurs also loss of muscle innervation which contributes to muscle weakness, inactivity, and immobilization resulting in muscular atrophy [4].

Hemiparesis causes muscular abnormalities with denervations, disuse, remodeling, and spasticity that can trigger a complex pattern of alterations of phenotypes and muscular atrophy [24]. There is a change in muscle fiber type I (slow-twitch) to muscle fiber type II (fast-twitch) and, consequently, a greater dependence on anaerobic metabolism [24]. This shift in muscle fibers is an important predictor of

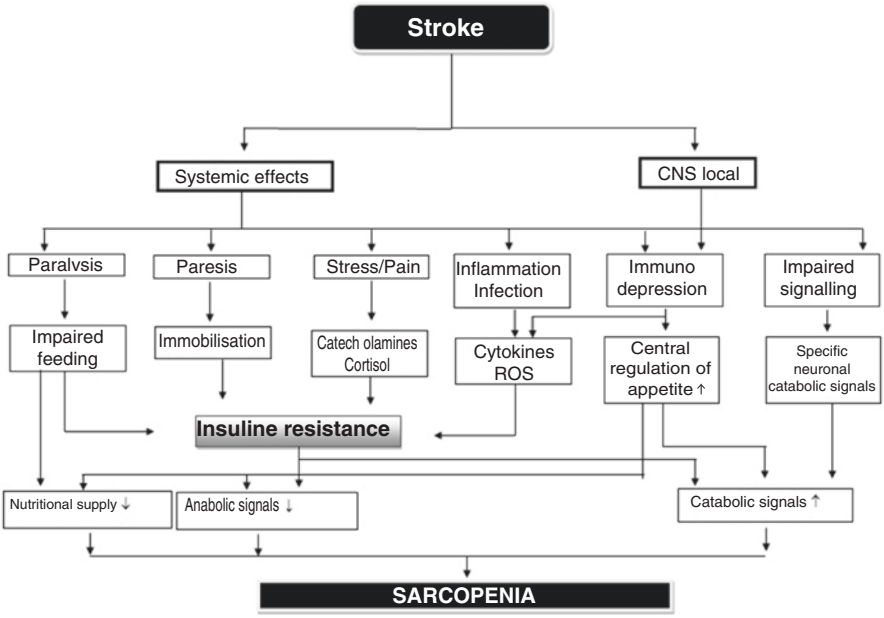


Fig. 17.1 Rationale scheme for stroke-related sarcopenia: signs of catabolic activation

imbalance in functional capacity, such as severe gait deficit [24]. The reduction of muscle mass and increased hemiparetic deficit during gait are independent factors for reduction of aerobic fitness, suggesting components of stroke-related sarcopenia directly influence functional capacity and fragility [19]. In a clinical study, Ryan et al. [17] showed an overall reduction of muscle mass in paretic lower limb. It occurs a reduction of area, volume, and muscular quality together with a subcutaneous increasing of fat around and within the muscle [19]. However, not only affected limb but also non-paretic upper limb changes in size and strength. A reduction in muscle mass of non-paretic upper limb can be observed in third week poststroke and muscle weakness in fourth week [4]. Similarly, it occurs a similar reduction of muscle mass in lower limbs of patients who are not able to walk [4]. Malnutrition is also a common problem in 49% of poststroke patients because of limited nutritional intakes of macronutrients and calories, which also contribute to sarcopenia [23].

17.2.4 Current Clinical Treatments for Muscle Atrophy

Physical activity in form of rehabilitation is part of standard poststroke treatment, and the prevention or recovery of muscle metabolic abnormalities will contribute substantially to this treatment [24]. Recent systematic review showed important reduction in the size and muscular strength of paretic upper limb after stroke and in

non-paretic upper limb [21]. However, results support practice of interventions with exercise to reverse decreasing in muscle mass and size that occurs in both upper limbs, paretic and non-paretic [21].

There is potential for strength training interventions to improve gait speed and strength in addition to muscle strength generation [21]. Clinical study [4] which showed improvement in strength of the paretic upper limb handshake after rehabilitation training may cause reduction of C-terminal agrin fragment (CAF), a potential marker of sarcopenia caused by degeneration of neuromuscular junctions in addition to increasing of muscle mass [4]. Declining of muscle mass in non-paretic muscle may be partly reversible, and exercise will contribute to the process of muscle mass restoration as well as recovery of structural and metabolic changes.

17.3 Muscular Atrophy in Peripheral Arterial Occlusive Disease

Peripheral arterial occlusive disease (PACD), or peripheral arterial disease, a systemic manifestation of atherosclerosis [25], is an atherosclerotic disease characterized by occlusion (blockage) or stenosis (narrowing) of the lumen of peripheral arteries that cause reduction of blood flow in lower limbs [26]. Atherosclerosis is a heterogeneous disease initiated by various pathophysiological pathways in the vascular wall and affects almost all blood vessels in the body [27]. The most affected arterial regions are the coronary vessels, carotid arteries, and arteries of lower limbs [27].

It is supposed that similarly to coronary atherosclerotic lesions, thrombus formation in peripheral vascular lumen is due to rupture or erosion of atheromatous plaque surface [27]. Inflammatory cells play an important role in the development of all stages of atherosclerosis. The cells accumulate in the intima layer of vascular wall in response to the presence of oxidized low-density lipoprotein (oxLDL) molecules [27]. Besides influencing foam cell formation, inflammatory cells are responsible for vulnerability and destabilization of atheromatous plaque due to release of metalloproteinases [27]. More severe clinical stages of PAOD are caused by more complex and extensive lesions in the arterial tree of lower limbs, composed of atherosclerotic lesions and apposition of unstable and vulnerable plaques thrombi [27]. In addition, not only the inflammatory cells but neovascularization of atherosclerotic lesions is responsible for development and progression of plaque.

The clinical symptoms of PAOD includes intermittent claudication, pain at rest, and reduction of muscle mass, which are due to reduced blood flow [25]. Some studies suggest that blood flow and oxygen delivery are not the only factors limiting the patients' function but a metabolic defect in the use of oxygen in skeletal muscle [25]. Thus, myopathy is present in skeletal muscles of patients with symptomatic PAOD and seems to be an important factor for pathophysiology of disease [25].

Patients in symptomatic PAOD stages have a high risk of lower limb amputation and other vascular problems such as myocardial infarction and stroke [27]. The use of statins, however, besides causing a reduction in number of cardiovascular events, can interfere in the activation and consequent release of proteases and cytokines and not only in the quantity of inflammatory cells [27]. However, despite the poor prognosis of patients with PAOD, cardiovascular risk management is still not desirable in most cases [26].

Cardiovascular risk reduction includes a variety of strategies such as control of blood lipids (statins), hypertension, diabetes, smoking cessation, weight reduction, and antiplatelet and antithrombotic therapy [26]. Medical treatment of intermittent claudication includes vasoactive drugs, such as phosphodiesterase inhibitors, which improve functional capacity [26]. Intravascular angioplasty improves blood supply and is generally indicated for patients with a more severe symptomatology and those who are not responsive to physical training or drug therapy [26]. One of the most effective treatments for improving exercise capacity and functional ability is supervised therapeutic exercise [26].

17.3.1 Prevalence of Muscle Atrophy

The prevalence of PAOD is growing around the world, with an estimated current prevalence of 200 million people [28]. In the United States, the vascular prevalence of the disease is 8.5 million people [29]. Risk factors include advanced age, smoking, hypertension, dyslipidemia, and diabetes [26]. The incidence and severity of atherosclerotic lesions also accelerate with advancing age; more than 20% of individuals over 75 years of age have PAOD [27]. In addition, the increasing numbers are mainly due to increase in the rate of obesity, incidence of diabetes, and smoking [28].

Although disease is associated with a high risk of morbidity and mortality, there is a significant reduction in number of leg amputations in patients with symptoms of intermittent claudication. This reduction can be attributed to the early detection of disease, preventive medical treatments, and increased endovascular revascularization [28]. Furthermore, possible change in genotype of iliofemoral atherosclerotic plaque, making it less destabilizing characteristics, due to treatment with statins, may be related to reduction of disease progression and improvement of vascular outcomes [28].

17.3.2 Mechanisms of Muscle Atrophy

Currently, it is suggested that myopathy is an important component of pathophysiology of PAOD, mainly due to dysfunctional bioenergetic system of mitochondria, increased oxidative damage, myofibrillar degeneration, and fibrosis of affected skeletal muscle [25, 29]. Microscopic assessments demonstrated that the muscle of

patients with PAOD has extensive myopathic changes that appear to be correlated with the severity of occlusive disease [25]. These changes include necrosis, phagocytosis, central nucleus, and endomysial fibrosis, which are accompanied by neuropathic alterations with evidence of significant myofibrillar denervation [25].

The structures of muscle fibers (myofibrils, mitochondria, nucleus, sarcolemma) and contractile elements are affected during myopathic process [25]. The sarcomere has extensive myofibrillar abnormalities, such as disorganization and fragmentation of Z-line and substantial disintegration of myofilaments [25]. There is a reduction in oxygen consumption by these myofibrils suggesting a defect in respiratory chain [25]. In addition, protein complexes (I, II, and IV), which compose respiratory chain, were significantly reduced in mitochondrial respiration together with enzymatic activities in patients with PAOD [25].

The mitochondria of muscles with occlusive disease demonstrated ultra-quantitative and qualitative abnormalities that make it primarily involved in myopathy [25]. Dysfunction in mitochondrial oxidative metabolism in PAOD may contribute to muscle dysfunction and exercise intolerance. During exercise, phosphocreatine (PCr) donates its highly energetic phosphate to maintain stable levels of adenosine triphosphate (ATP) [30]. At the same time that, ATP is broken down into adenosine diphosphate (ADP) and inorganic phosphate, making energy flow uninterrupted for the performance of muscle contraction [30]. At the end of the exercise, PCr levels are low, and ADP levels are high. When the muscle contractile stimulus is finished, both molecules (PCr and ADP) are restored to their basal rates by ATP produced primarily through muscle oxidative phosphorylation [30]. The oxidative phosphorylation is an energetic process that occurs exclusively in the mitochondria (respiratory chain); thus basal rates of postexercise PCr and ADP may be good indexes of muscular mitochondrial function [30].

The increasing of oxidative damage is associated with deterioration of the size and shape of gastrocnemius myofibrils, preferably type II fibers, whereas type I fibers persist and are less injured [29]. The change of myofibrillar phenotype together with defective mitochondria and neuropathy demonstrates the possible mechanisms for deficits presented by patients with occlusive muscular disease [29]. The effects of neuropathy in patients with PAOD include reduced nerve conduction velocity and decreased amplitude and increased duration of motor unit action potential [29]. All these alterations suggest that it is the mitochondrial energetic impairment, besides restriction of blood flow and oxygen, which compromises oxidative energetic production in the skeletal muscle, and consequently, it causes poor performance in exercise performed by patient with PAOD [25].

The combination of factors related to intensity and frequency of muscular ischemic insult along with oxidative stress may be the primary mechanism responsible for mitochondrial energetic deficit in chronically ischemic muscle [25]. The cycles of ischemia and reperfusion generate a cascade of inflammatory changes that induce the production of reactive oxygen species (ROS) in the skeletal muscle [30]. These daily events in long term result in morphological changes of contractile elements and mitochondria. There is an even greater reduction in mitochondrial energy levels and increase in ROS production by mitochondria [30]. A vicious cycle is created

and causes deterioration of mitochondrial function and damage to all myocyte structures, leading to development of myopathy which affects function and performance of patients' lower limbs with PAOD [30].

17.3.3 Pathophysiological Effects of Muscle Atrophy

The majority of PAOD patients are asymptomatic, and prevalence of intermittent claudication in this population is around 25–30%, and prevalence of critical limb ischemia is 1–3% [27]. There is also a decrease in muscle strength, endurance, and cardiorespiratory capacity ($PeakVO_2$), and maximum walking capacity is less than 50% of that observed in subjects of the same age and without DAOP [26]. The functional limitations associated with occlusive disease may be similar to those of severe heart failure [26]. These limitations have a major impact on quality of life, which makes disease associated with high levels of depression [26].

Intermittent claudication is the most commonly reported symptom and is typically described as a painful cramp, pain, or fatigue that affects the calf muscles and sometimes the thigh and gluteal muscles during walking or other forms of physical activity [26]. The symptom worsens with increased activity and is relieved only by the rest [26]. The metabolic demand of lower limbs during walking is exceeded at the limit of blood supply generating ischemia, exercise-induced discomfort, and pain in exercising leg [29]. Chronic ischemia-reperfusion of lower limbs causes biochemical and histological changes in the muscles of affected limb producing DAOP myopathy [29]. More severe symptoms are resting pain, non-healing skin ulcers, and tissue gangrene (necrosis), which are collectively referred to as critical ischemia of lower limbs [26].

The altered gait profile represents a decrease in the contribution of muscle strength in the ankle, knee, and hip joints [29]. There is a reduction of angular momentum curve (contraction) and maximum torque produced by plantar flexors which unbalance motor control strategies of patients with PAOD and may be related to neuropathy and myopathy [29].

17.3.4 Current Clinical Treatments for Muscle Atrophy

Currently, the importance of rehabilitation in treatment of PAOD patients is recognized, and the American College of Cardiology and the American Heart Association presents training programs as the primary treatment option for intermittent claudication [31]. Supervised walking training programs have a more positive impact, and there may be a significant improvement in walking distance (approximately 150%) [31]. The meta-analysis of Lane et al. (2014) showed that physical training programs with duration of 3–12 months improve in 5 min the average time of maximum treadmill walking [32]. Often there are changes in walking ability, together

with changes in the strength and endurance of the plantar flexors muscle group. Thus, training of plantar flexors has been shown to be an effective way of exercise to improve functional capacity of patients with PAOD [26].

The Exercise & Sports Science Australia presented an overview of exercise prescription for patients with PAOD [26]. Patients should aim to complete at least 6 months of physical training, starting with aerobic training, interval walking, or other aerobic exercises. The intensity should go up to moderate intermittent claudication and moderate intensity (effort perception rate in 3–4/10), with progression to vigorous intensity as tolerated (effort perception rate in 5–6/10). The duration should be as tolerated up to 40 min (excluding rest periods) with frequency of 3 sessions per week. Resistance training should be progressively increased (1RM60–80%, 6–8 exercise types), with 8–12 repetitions for 2–3 times with a frequency of 2 sessions per week on nonconsecutive days [26].

A correlation between improved walking ability and PeakVO_2 with supervised training was found, suggesting that benefits may be linked to an increase of oxygen supply to the muscle in motion and/or to an improvement in ability of muscle in movement to use oxygen [26]. However, physical training improves exercise tolerance in PAOD even without significant changes in blood flow possibly due to increase in muscle strength and various physiological adaptations, including changes in muscle metabolism and morphology [26, 31].

Myopathy had no therapies to prevent or reverse. In patients with severe symptomatic PAOD, the effects of a strategic focus on mitochondrial energy defects, and ROS production should be investigated [30]. Clinically relevant therapeutic modalities can prevent deterioration, as well as may generate a potential reversal of the already installed damage. In addition, medications in association with antioxidant agents capable of increasing mitochondrial metabolism may bring significant improvements in function of these patients [30].

17.4 Muscular Atrophy in Heart Failure

Heart failure (HF) is a major, highly prevalent public health problem characterized by the inability of the heart to meet the body's metabolic demands. It may result from disorders of the pericardium, myocardium, endocardium, heart valves or large vessels, or certain metabolic abnormalities, but most HF patients have symptoms due to impaired left ventricular (LV) myocardial function. HF may be associated with a broad spectrum of functional LV abnormalities, ranging from patients with normal LV size and preserved ejection fraction (EF) to severe dilation and/or reduced EF [33]. The main causes are myocardial ischemia, systemic arterial hypertension, dilated cardiomyopathy, Chagas' disease, and valvular disease [34, 35].

The manifestations of HF include dyspnea and fatigue, which may limit exercise tolerance, and fluid retention, which may lead to pulmonary and/or splenic congestion and/or peripheral edema. Some patients have exercise intolerance but little evidence of fluid retention, while others complain mainly of edema, dyspnea, or fatigue

[35]. After cardiac injury, in some cases HF is accompanied by low cardiac output, and several mechanisms are activated to perform this compensation by increasing inotropism and chronotropism; consequently several ventricular, molecular, structural, and functional alterations, known as cardiac remodeling, occur. This process is accompanied by cardiac and systemic inflammatory and neurohormonal activation, which adversely affects the heart in a vicious cycle and compromises different organs and systems [34, 36]. Therefore, in addition to affecting the cardiovascular system, HF causes pathological changes involving the system renal, neuroendocrine, immunological, musculoskeletal, hematological, gastrointestinal, and nutritional status [37].

HF has been characterized based on the classification proposed by the New York Heart Association composed of four classes with progression of symptoms. However, the symptomatology with the progression of the disease causes fatigue, dyspnea, and great limitation to the efforts. Although effort intolerance is associated with cardiovascular impairment, studies have shown that peripheral changes in skeletal muscle appear to have a stronger association with this condition [38]. In the terminal phase of HF, we can observe a series of consequences that affect the quality of life added to a poor prognosis, among them, muscular atrophy and/or cachexia. Cachexia is a prevalent pathological condition associated with chronic HF. Its occurrence predicts the reduction of survival, regardless of relevant variables, such as age, functional class of HF, ejection fraction, and physical capacity. Cachexia induces pathological changes in skeletal muscle structure and function, resulting in muscle atrophy and exercise intolerance and promoting functional abnormalities and fatigue [39].

Despite advances in the disease, patients with HF have a poor quality of life due to the many consequences of this syndrome, including musculoskeletal disorders. Still, the mortality of patients hospitalized with this syndrome in Brazil and in the world is still high. Thus, new studies are needed that seek alternatives to improve the quality of life, symptomatology, and life expectancy.

17.4.1 Prevalence of Muscle Atrophy

The prevalence of HF has been increasing in recent years worldwide [40] and is the common final pathway of most heart diseases [41]. Approximately 23 million people are carriers of HF in the world, and 2 million new cases are diagnosed each year [42]. It is the first cause of hospital admission in patients over 60 years of age in Brazil [41]. In the United States, about 550,000 new cases are diagnosed annually, being the fifth most frequent cause of hospitalization [42]. In Brazil, according to data from the Department of Informatics of the Unified Health System (DATASUS), approximately 238 thousand hospitalizations were performed per CI in 2012, with 26 thousand deaths occurring, accounting for a 9.5% mortality during hospitalization [43]. The Brazilian Registry of Acute Heart Failure (BREATHE) is the first national and multicenter registry of acute HF to include all regions of the country,

involving 51 public and private hospitals in 21 Brazilian cities, and identified an in-hospital mortality of 12.6% [44].

Statistical data from the United States estimate that 5.7 million Americans over 20 years of age have HF. It is expected to increase approximately 46% between 2012 and 2030, resulting in more than 8 million adults with HF [45]. Many comorbidities and consequences associated with HF worsen its prognosis, including musculoskeletal disorders. In the case of muscular atrophy, it is present in up to 68% of patients, and sarcopenia affects approximately 20% of older adults with HF [46]. To further complicate the problem, 10–15% of HF patients develop cardiac cachexia, a condition characterized by loss of body weight due to muscle wasting and the disappearance of adipose tissue [47].

17.4.2 Mechanisms of Muscle Atrophy

Patients with HF have a limitation in their ability to exercise. Although this intolerance to exercise is due to low cardiac output, the effects of skeletal muscle loss should not be overlooked. This muscle loss may occur due to sarcopenia with loss of muscle mass and function, which is common in the aging of the elderly population [48, 49] or in the form of cachexia which is associated with body weight loss [50]. Sarcopenia is associated with increased mortality regardless of age or other clinical and functional variables [51–53]. Similarly, the occurrence of cardiac cachexia predicts a reduction in survival, regardless of relevant variables, such as age, functional class of HF, ejection fraction, and physical capacity [50]. Sarcopenia in HF may ultimately progress to cardiac cachexia, associated with an extremely poor prognosis [50]. There is a debate regarding the terms sarcopenia and cachexia, suggesting that the term sarcopenia should be restricted only to healthy elderly. In this sense, recently the term "myopenia" has been suggested to describe muscle loss that meets the criteria of sarcopenia in patients with chronic disease; however, so far there is no standardized classification [54, 55].

It should be noted that there is also no standard definition of cardiac cachexia. In the past, low body weight was used to define cachexia, but low body weight would not classify a patient as cachectic. Already, in other studies, patients were classified according to body fat content, by lean tissue, or by anthropometric measurements [56]. Kotler [57] defined cachexia as "accelerated loss of skeletal muscle in the context of a chronic inflammatory response." It is believed that the process of developing sarcopenia in the HF patient is due to its shared pathophysiological pathways (Fig. 17.2) including the process of altered ingestion and absorption, malnutrition, inflammation, humoral factors, ubiquitin-proteasome system, myostatin signaling, apoptosis, and oxidative stress. These combined processes result in muscle abnormalities, changes in mitochondrial structure and function, increased oxidative stress, and multiple histological abnormalities in the skeletal muscle, leading to reduced exercise capacity [58, 59].

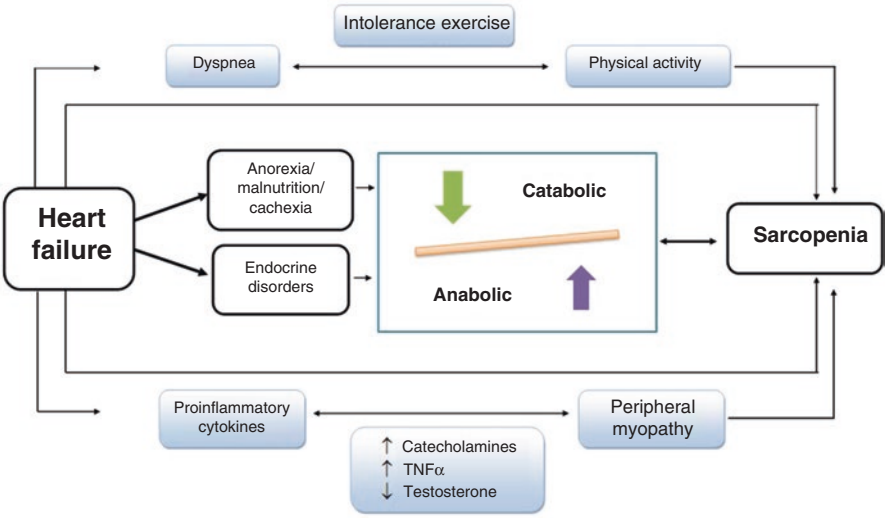


Fig. 17.2 Interaction and common pathways between sarcopenia and heart failure

The etiology of cardiac cachexia associated with HF is multifactorial, and the underlying pathophysiological mechanisms are not well established. However, some studies have shown that malnutrition, malabsorption, metabolic dysfunction, anabolic/catabolic imbalance, inflammatory/neurohormonal activation, and cell death play an important role in the pathogenesis of cardiac cachexia [60]. Proposed mechanisms include an anabolic/catabolic imbalance with increased myofibril degradation and myocyte apoptosis. Thus, the clinical effects include reduced muscle mass, strength, and consequently reduced exercise capacity [60].

Cachexia was recently defined as the loss of at least 5% of body weight over 12 months (or a body mass index <20 kg/m^2) in patients with chronic disease, such as HF, chronic obstructive pulmonary disease, chronic kidney disease or cancer, and at least three of the following clinical and laboratory criteria: decreased muscle strength, fatigue, anorexia, low fat-free mass index, and abnormal biochemistry, characterized by increased inflammatory markers [C-reactive protein, interleukin (IL-6)], anemia (Hb <12 g/dL), or low levels of serum albumin (<3.2 g/dL) [61]. While body weight loss defines cachexia, sarcopenia is not necessarily associated with changes in body weight because the decline in muscle mass may be masked by proportional increases in adipose tissue. Thus, imaging techniques, including dual-energy X-ray densitometry, computed tomography, or magnetic resonance imaging, are required to quantify muscle mass [62].

The skeletal muscle contains at least five proteolytic pathways that include the lysosomal, Ca^{2++}-dependent channel, ubiquitin-proteasome system (UPS), caspase, and matrix metalloproteinase. Among these pathways, there is convincing evidence that only the activation of the ubiquitin-proteasome system plays a key role in muscle loss. The adenosine triphosphate-dependent UPS pathway present in the nucleus

and cytosol is the major mechanism involved in the degradation of myofibril. The proteasome, a multi-subunit protein that degrades ubiquitin-conjugated proteins, is responsible for the degradation of intracellular compartment proteins [63].

The degradation of muscle protein in patients with HF has been attributed mainly to the overactivation of this pathway [64]. Since skeletal muscle structure is a matter of permanent changes, an anabolic/catabolic imbalance is needed to increase the degradation of myofibrils and apoptosis of myocytes. Looking at this imbalance, muscle wasting can be a consequence of reduced muscle anabolism, increased muscle catabolism, or both. Maintenance of balance depends largely on the balance between anabolic hormones and the type 1 insulin-like growth factor and the catabolic factors TNFα, interleukin-1β, interferon γ, myostatin, and glucocorticoids [65]. Therefore, muscle loss is a consequence of the imbalance of reduced protein synthesis and increased protein degradation, the latter associated mainly with an overactivation of the UPS system responsible for the elimination of damaged proteins [58]. Several mechanisms are involved, including UPS system activity, apoptosis, and fiber type change (Fig. 17.3) [66].

The UPS pathway involves a multiple-subunit protease that degrades ubiquitin-conjugated proteins through the action of three enzymes, the ubiquitin-activating enzyme, the ubiquitin-conjugating enzyme, and the ubiquitin (atrogin-1 and MuRF-1) ligases. The inducers of MuRF-1 expression are proinflammatory cytokines, such as TNF-α, interleukin-6, and interleukin-1β [64]. TNF is one of the major cytokines important for the development of catabolism, along with IL-1, IL-6, and

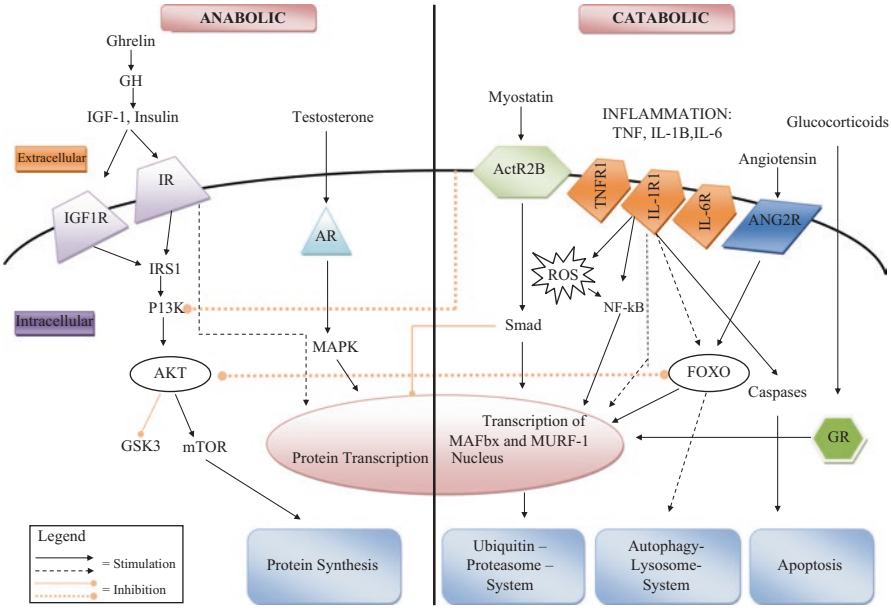


Fig. 17.3 Anabolic/catabolic imbalance affecting the endocrine and molecular pathways in muscle loss

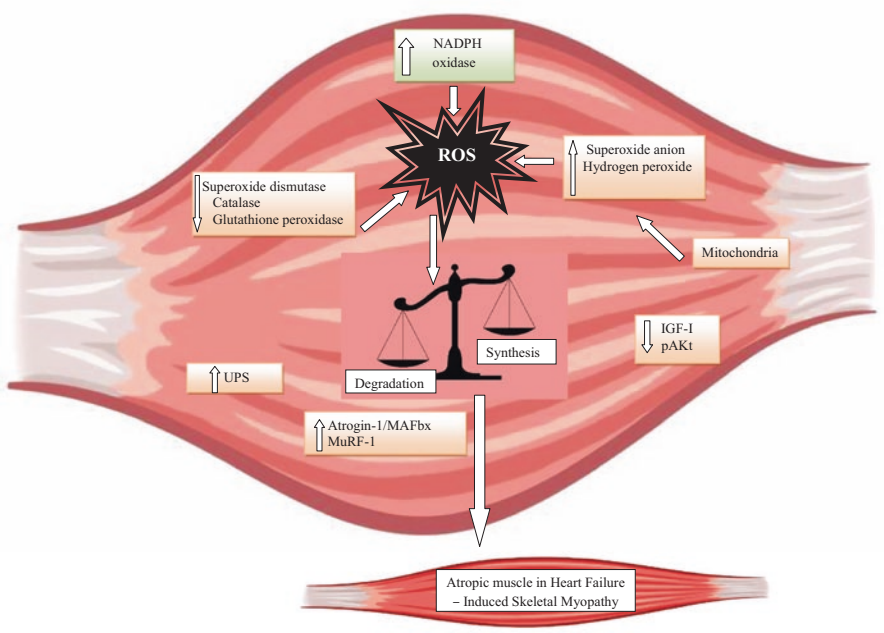


Fig. 17.4 Increased generation of reactive oxygen species, together with worsened antioxidant defense, leads to increased protein degradation and reduced protein synthesis in the skeletal muscle. Abbreviations: *Akt* protein kinase B, *atrogin-1/MAFbx* muscle atrophy F-box protein, *IGF-I* insulin-like growth factor-1, *MuRF-1* muscle RING-finger protein-1, *ROS* reactive oxygen species, *UPS* ubiquitin-proteasome system

the growth-transforming factor (TGF-β) [67]. Elevated levels of MuRF-1 were detected in the skeletal muscle of patients with CHF [68]. Similarly, elevation of TNF-α, IL-6, IL-1, norepinephrine, epinephrine, cortisol, angiotensin II, and aldosterone were found in cachectic patients with HF [67, 69]. Finally, IL-1, IL-6, and TNF-α are linked to UPS activation and can induce anorexia and lipolysis, contributing to weight loss [70] (Fig. 17.4).

17.4.3 Pathophysiological Effects of Muscle Atrophy

Muscle atrophy is present in up to 68% of patients with HF [14] and is an independent predictor of mortality [69]. Patients with advanced-stage HF exhibit multiple histological abnormalities in skeletal muscle and may be termed "cardiac skeletal myopathy" [71]. Thus, the clinical consequences of cachexia depend both on weight loss and systemic inflammation, which accompany the development of cachexia. Severe loss of body weight, even in the absence of systemic inflammation, is associated with deleterious effects in most organs and systems [37]. Systematically, loss

of three-compartment tissue, lean tissue, fat mass, and bone is found [67]. In skeletal muscles, an imbalance between synthesis and protein degradation leads to molecular changes and muscle atrophy, with decreased strength and impairment of daily activities [72, 73]. In healthy individuals there is a balanced distribution between type I (aerobic) fibers, type IIA fibers (both aerobic and anaerobic), and type IIB (mainly anaerobic) fibers [74]. In HF, a transition to type II fibers and reduced capillary density, as well as reduced cytochrome oxidase activity, are observed, but the mechanisms that lead to this change have not been clarified [75]. This modification, concomitant with reductions in the surface area of mitochondrial ridges, cytochrome C oxidase activity, and mitochondrial volume density, leads to a decrease in exercise tolerance [74, 76].

The aging process is accompanied by denervation and loss of fast motor units at a rate of 3% per year from the age of 60. Until the age of 80, it is estimated that 60% of the fibers are lost. In the sarcopenia process, type II fibers are more prone to atrophy than type I fibers [77]. Fatigue and muscle weakness are the two of the main symptoms experienced by patients with HF. The loss of lean body mass, which results mainly from the skeletal muscle protein atrophy, is one of the characteristics of cardiac cachexia [78].

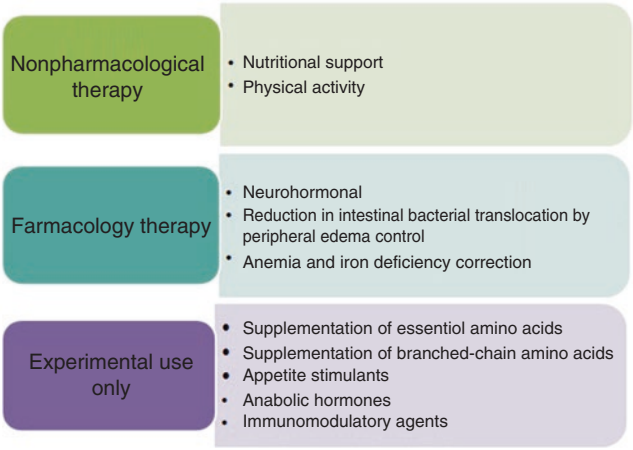
17.4.4 Current Clinical Treatments for Muscle Atrophy

Currently, there has been a better understanding of the pathophysiology of HF and its consequences, which allows a better understanding of the disease and the development of therapeutic actions. However, to date no drug therapy has been effective in stopping skeletal myopathy. Thus, multi-professional therapeutic strategies are needed that are capable of delaying the onset or minimizing the consequences of skeletal muscle loss [79].

Skeletal muscle loss may precede cachexia; therefore preventive strategies have been mainly directed toward the preservation of muscle mass [80]. Cachexia has a multifactorial origin, so prevention and treatment should include several approaches, as shown in Table 17.1 [53]. The approach may include nutritional support, neurohormonal blockade, reduction of intestinal bacterial translocation, treatment of anemia and iron deficiency, appetite stimulants, immunomodulatory agents, anabolic hormones, and physical exercise Schemes [53].

Nutrition considerations include avoiding excessive consumption of salt and liquids and restoring deficiencies in trace elements. The administration of omega-3 polyunsaturated fatty acids could be beneficial in some patients. High-calorie nutritional supplements may also be helpful. Drugs with potential benefit in the treatment of muscle loss in patients with HF include testosterone, ghrelin, recombinant human growth hormone, essential amino acids, and β2-adrenergic receptor agonists [81]. Possible future interventions are being studied, such as anti-inflammatory agents, appetite stimulants, proteolysis and apoptosis inhibitors, and specific hormone supplementation regimens being studied as possible future therapeutic options

Table 17.1 The perspectives for prevention and treatment in cardiac cachexia



[58]. The loss of muscle mass in patients with HF is a complex scenario, and no pharmacological treatment is effective in muscle loss. However, physical training is a non-pharmacological, effective, low-cost, and safe treatment that can help in this regard [81].

Aerobic exercise training is the therapeutic approach to neutralize skeletal muscle myopathy [66] and has shown improvements in functional capacity. These improvements are probably driven predominantly by peripheral mechanisms such as improved endothelial function, oxygen extraction, and skeletal muscle function [82]. Other studies have shown that exercise training, including aerobic and resistance exercises, improved strength, muscle mass, physical function, functional capacity, depression, and quality of life in patients with HF [68]. Another study evaluated the effects of regular physical exercise on local inflammatory parameters in skeletal muscle in patients with HF. Twenty patients were randomly assigned to a training group (n = 10) and control group (n = 10) submitted to 20 min of aerobic exercise. At baseline and after 6 months, serum samples and biopsies of the vastus lateralis muscle were collected. Serum TNFα, IL6, and I-1β levels were measured. It was observed that physical training was able to reduce local expression of TNFα, IL6, I-1β, and nitric oxide in the skeletal muscle. These local anti-inflammatory effects of aerobic exercise may attenuate the process of catabolic wear associated with the progression of HF [83].

A recent literature review evaluated the effects of aerobic training on skeletal myopathy induced by HF. It has been observed that the increase in the generation of reactive oxygen species, together with the deteriorated antioxidant defense, leads to an increase in protein degradation and reduction of protein synthesis in the skeletal muscle, and in this sense, aerobic exercise training neutralizes the mechanisms responsible by skeletal muscle atrophy in HF [79]. Patients with severe HF are intolerant to exercise; however, a promising alternative is the neuromuscular electrical

stimulation (NMES). According to the review by Saitoh [84], NMES is safe and beneficial in the outcomes of functional capacity, muscle strength, and quality of life compared to conventional aerobic exercise. In addition, NMES appears to have a beneficial effect on the proinflammatory cytokine, oxidative enzymatic activity, and anabolic and catabolic metabolism of proteins, which are the key molecular mechanism of muscle mass loss in HF patients.

Competing Financial Interests The authors declare no competing financial interests.

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Chapter 18
Muscle Atrophy in Chronic Kidney Disease

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Abstract The renal damage and loss of kidney function that characterize chronic kidney disease (CKD) cause several complex systemic alterations that affect muscular homeostasis, leading to loss of muscle mass and, ultimately, to muscle atrophy. CKD-induced muscle atrophy is highly prevalent and, in association with common CKD comorbidities, is responsible for the reduction of physical capacity, functional independence, and an increase in the number of hospitalizations and mortality rates. Thus, this chapter summarizes current knowledge about the complex interactions between CKD factors and the pathophysiological mechanisms that induce muscle atrophy that, despite growing interest, are not yet fully understood. The current treatments of CKD-induced muscle atrophy are multidisciplinary, including correction of metabolic acidosis, nutritional supplementation, reducing insulin resistance, administration of androgenic steroids, resisted and aerobic exercise, neuromuscular electrical stimulation, and inspiratory muscle training. However, further studies are still needed to strengthen the comprehension of CKD-induced muscle atrophy and the better treatment strategies.

Keywords Chronic kidney disease · Muscle atrophy · Pathophysiological mechanisms · Treatment

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18.1 Background

Chronic kidney disease (CKD) consists of renal damage and progressive and irreversible loss of kidney function (glomerular, tubular, and endocrine) [1] or glomerular filtration rate less than 60 ml/min/1.73m^2 for a period of 3 months or more [2]. Among the five stages of CKD, the last and most severe (terminal stage) is called chronic kidney failure (CKF), and the patients are extremely symptomatic, requiring replacement renal therapy (RRT) or renal transplantation, since the kidneys lose control of the internal environment [1].

The muscle fibers of chronic kidney patients have many abnormalities, possibly due to the adaptation of these cells due to an altered internal environment. These abnormalities include changes in capillaries, enzymes, and contractile proteins [3]. Myopathy is due to multifactorial causes [4]; however, it frequently occurs in uremic patients as a consequence of high serum calcium, urea, uric acid and creatinine levels, acidemia, carnitine low levels, and/or secondary hyperparathyroidism [5–7] and as result of disuse [4]. Still, patients with CKD in dialysis have a greater impairment of muscle mass in relation to those who do not undergo dialysis, where atrophy, particularly of type II fibers, has been demonstrated [3]. In addition to the aforementioned mechanisms, other pathways are involved in the process of muscle atrophy and sarcopenia of this patient and will be addressed in the subsequent topics of this chapter.

Atrophy is the primary mechanism for muscle weakness, and this is an important cause of reduced functional capacity of patients with CKD [4, 8]. In addition to involvement of the lower limbs, muscle weakness is also present in the respiratory muscles, compromising pulmonary function [9, 10]. Thus, the reduction in physical conditioning as a whole leads to worsening of quality of life and increased mortality in this population [11].

Regarding the prevalence, the rates of sarcopenia (characterized by the decline of mass and strength/or muscle function) [12] range from 6% to 10% among non-dialysis CKD patients and 4% to 64% among patients undergoing dialysis treatment. The wide variation is a direct consequence of the choice of the criteria that define sarcopenia, besides the demographic characteristics of the patients that are very variable [13, 14].

Still, Kim et al. (2014) performed a cross-sectional observational study evaluating 95 patients over the age of 50 in the final stage of CKD and found that sarcopenia was highly prevalent, being present in 37% of men and 29.3% of women [14].

Among the treatments available to attenuate and/or revert muscle atrophy are nutritional supplementation, correction of metabolic acidosis and reduction of insulin, administration of androgenic steroids [15], resisted and aerobic exercise [16], neuromuscular electrical stimulation [17], and inspiratory muscle training [18].

18.2 Pathophysiological Mechanisms of CKD–Induced Muscle Atrophy

CKD-induced muscle atrophy results from an imbalance between anabolic and catabolic processes that controls muscle homeostasis [19]. The loss of muscle homeostasis can result in impaired growth of new muscle fibers, suppression of protein synthesis, or stimulation of protein degradation [20]. Several factors that contribute to the loss of muscle homeostasis and consequent atrophy are altered in different degrees across the CKD phases until dialysis, caused by the loss of kidney function itself, comorbidities, complications, and treatments [21]. The factors include altered hormonal, immunological, and mitochondrial functions, alterations in progenitor cells and growth factors (insulin/*insulin-like growth factor-1 (IGF-1)*, myostatin), metabolic acidosis, malnutrition, physical inactivity, and angiotensin II excess [19].

Figure 18.1 shows a summary of the relations between factors that affect muscle homeostasis and the pathophysiological mechanisms of muscle atrophy in CKD. These relations are explored next.

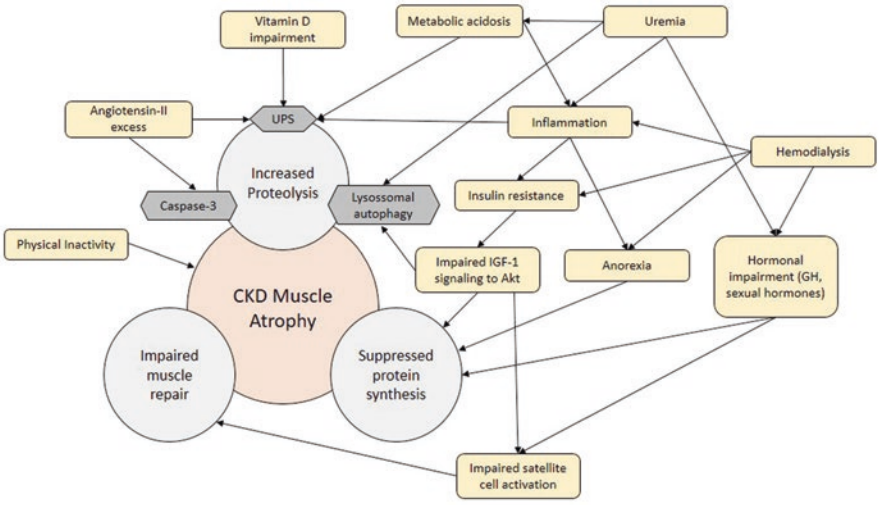

Fig. 18.1 Schematic representation of evidence-based relations between chronic kidney disease factors and muscle atrophy induction through activation of proteolytic pathways, suppression of protein synthesis, or muscle repair impairment

18.2.1 Protein Degradation

18.2.1.1 Ubiquitin–Proteasome System (UPS)

The UPS was identified as the main pathway of muscle proteolysis and is activated in CKD patients as well as in other chronic diseases [20, 22]. This system is responsible for the degradation of the majority of intracellular proteins [20]. Although there are other muscle degradation pathways, lysosomal and calcium-dependent, in catabolic conditions such as CKD, its contribution to muscle wasting is considerably less significant than UPS [23].

UPS activity is regulated in several steps and begins by marking proteins to be degraded [20, 24]. Proteins are marked by a covalent linkage of a ubiquitin-chain to lysine residues in the protein substrate. This connection is mediated by a sequence of enzymes. Firstly, a ubiquitin molecule is activated by the enzyme E1, at the cost of ATP, and subsequently transferred to the ubiquitin-carrier enzyme E2. After that conjugation, the ubiquitin can be recognized by the ubiquitin-ligand enzyme E3, which catalyzes the conjugation of ubiquitin to the protein substrate. The process is repeated until a polyubiquitin chain is formed that will be recognized and degraded by the proteasome, the major proteolytic enzyme that converts proteins into small peptides and amino acids [22–24].

The proteolysis by UPS can be activated by inflammation, reactive oxygen species, metabolic acidosis [20, 25], and insulin and/or IGF-1 signalization defects [26]. The forkhead transcription factors (FoxO) and the nuclear transcription factor *kappa B (Nf-κB)* were identified as the regulatory factors of the activation of two muscle-specific ubiquitin-ligand enzymes E3, namely, atrogin-1 – also known as muscle atrophy F-box (MAFbx) – and *muscle-specific ring finger 1* (MuRF1) [22, 26]. These E3 ligases specifically recognize and facilitate the protein degradation by UPS. FoxO1, FoxO3, and FoxO4 are present in the skeletal muscle, but FoxO1 was identified as the main mediator of muscle wasting in CKD [26, 27].

Myostatin and activin A, members of the family of *transforming growth factor-β (TGF-β)*, are also associated with protein loss in catabolic conditions [24]. The ligation of myostatin with its receptor involves the activation of the signaling pathway Smad2/Smad3 and phosphorylation of the protein kinase B (also called Akt) in the muscle, both finally leading to UPS activation. Low level of phosphorylated Akt is capable of reducing the phosphorylation of the family of transcription factors FoxO. It induces proteolysis, as FoxO transcription factors increase the expression of the ubiquitin-ligand E3 muscle-specific enzymes, MAFbx and MuRF1 [20, 28]. In the CKD model, pharmacological inhibition of myostatin prevents muscle atrophy, increasing the satellite cells function, improving the IGF-1 signalization, and suppressing the protein degradation [29].

18.2.1.2 Caspase-3 Proteolytic Pathway

Caspase-3 is a protease that participates in apoptosis [22]. Caspase-3 and UPS work together in the muscle proteolysis. Caspase-3 is involved in two ways. First, it is activated by catabolic conditions, as CKD, and acts to cleave complex structures of muscle proteins, yielding substrates to UPS [20]. It cleaves actomyosin in myofibrillar complexes, generating the 14 kDa actin fragment, in the insoluble portion of muscle tissue [20, 22, 23], this being considered a muscle wasting marker, even in early stages, in patients with CKD [23, 30, 31]. This cleavage is necessary since UPS degrades complex muscle structures (actomyosin and myofibrils) slowly, while it fast degrades monomeric composts of myosin or actin [20].

Second, Caspase-3 can stimulate the muscle degradation via UPS, directly stimulating the proteasome activity. Caspase-3 would act as cleaving-specific proteasome protein subunits, altering its conformation and increasing the number of proteins inserted in the proteolysis site of the proteasome [30].

18.2.1.3 Autophagy by Lysosome

The macro-autophagy system (here referred to as autophagy by lysosome) is activated in catabolic conditions as denervation, starvation, disuse, sepsis, and cancer [24, 26]. Autophagy is a homeostatic mechanism, used for degradation and recycling by the lysosome machinery of bulk cytoplasm, abnormal proteins or protein aggregates, and organelles, including mitochondria [20, 24]. The autophagy pathway begins with the formation of a phagophore around the targets of degradation in the cytoplasm. Autophagosome formation is stimulated by the reduction of phosphatidylinositol 3-kinase (PI3K) levels with the activation of the autophagy-related gene BECN1 [20].

Transcription factors FoxO, besides UPS activation, can also activate autophagy, with evidence showing stimulation of the production of a variety of autophagy-related genes [32]. It is therefore reasonable to consider that the activation of this system can, theoretically, cause cellular and protein loss in catabolic conditions as CKD, since CKD causes insulin resistance and suppresses the IGF-1/PI3K/Akt signaling, which could stimulate the autophagy system by lysosome [20, 33]. However, the lysosomal autophagy proteolytic pathway has not yet been rigorously investigated in CKD patients. A recent study points out that muscle loss in rat models of CKD is associated with autophagy activation. The uremic toxicity, but not acidification, induces the formation of autophagosomes in muscle culture. However, the increase of autophagy does not directly relate to myofibrillar protein cleavage. It is also perceived that the increase of autophagy leads to deterioration of mitochondrial function and reduction of ATP production [26].

18.2.2 Altered Muscle Growth and Repair

Besides proteolysis stimulation, CKD can modify the satellite cell (also known as muscle precursor cells) function, reducing the capacity of muscle growth and repair [15]. So far there is little evidence of this topic, but results from experimental studies show that CKD affects the proliferation and differentiation of satellite muscle cells, measured by the reduction of the myoblast determination protein 1 (MyoD) and myogenin levels [34, 35]. These myogenic cellular factors are released by satellite cells in response to muscle injury or growth factor changes (e.g., IGF-1) [35]. CKD can impair the release of these growth factors by the reduction of IGF-1 receptors signaling in satellite cells [20, 35]. Wang et al. (2009) showed that strength training in CKD models was capable of reversing the MyoD and myogenin suppression, possibly because physical exercise stimulates the local release of growth factors such as IGF-1 [34].

18.2.3 Suppression of Protein Synthesis

The suppression of protein synthesis can be considered a potential mechanism of CKD-induced muscle atrophy, as experimental models and patients with CKD show reduction of protein synthesis markers [36] and contractile muscle and mitochondrial proteins [37]. Attenuation of protein synthesis in CKD can be caused by malnutrition as a result of anorexia, as well as metabolic acidosis, uremia, and pro-inflammatory cytokines expression that can suppress the insulin/IGF-1 signaling to Akt through several mechanisms [38]. However, all these factors are also related to proteolysis stimuli, which seems to have a considerably bigger participation in CKD-induced muscle atrophy than the reduction of protein synthesis [20].

18.3 CKD Factors Related to Muscle Atrophy

18.3.1 Metabolic Acidosis

Metabolic acidosis is a common and prevalent complication among CKD patients, particularly in later stages [39, 40]. Chronic metabolic acidosis can cause several adverse effects in CKD patients including alteration in muscle metabolism, insulin resistance, protein-energy wasting, and hastening CKD progression [39]. It promotes muscle atrophy by stimulating UPS and reducing protein synthesis [15, 40], affecting the insulin/IGF-1 signaling pathway [20, 22].

18.3.2 Inflammation

Inflammation is an essential part of CKD and is linked to cardiovascular disease, muscle atrophy, and mortality [41]. Many factors can contribute to immune deregulation and inflammatory activation in CKD and are related to CKD itself, uremia, genetic and environmental factors, lifestyle, and diet. Clearly, the reduction of renal clearance contributes to the rise in cytokine levels and production [42]. With CKD progression, there is an increase in the reactive oxygen species production, mainly because of uremia, extracellular fluid volume fluctuations, and bio-incompatible dialysis devices [43]. The increased oxidative stress, in turn, increases the synthesis and release of pro-inflammatory cytokines, with deregulation of the immune system [44]. Metabolic acidosis is another cause of inflammation in CKD [45].

CKD presents high circulating levels of inflammatory markers, including C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). The inflammatory state of CKD has a connection with muscle atrophy since inflammatory cytokines act in the activation of the NFkβ pathway that stimulates MuRF-1, with consequent proteolysis by UPS. They also raise myostatin expression, which causes the inhibition of protein synthesis induced by insulin and alteration of IGF-1/Akt signaling [15, 20, 38, 46]. Furthermore CKD-related systemic inflammation impairs the hypothalamic responses to appetite-regulating hormones, leptin, ghrelin, and melanocortin, resulting in persistent activation of anorexigenic neural pathways. The resulting anorexia limits the nutritional ingestion of amino acids, possibly reducing IGF-1 concentrations with consequent protein synthesis impairment [38].

18.3.3 Angiotensin II Alteration

The renin-angiotensin system is activated in many catabolic conditions, including CKD. Despite the poor expression of angiotensin II receptors in adult muscle fibers, angiotensin II contributes directly and indirectly to muscle atrophy. The increase of angiotensin II reduces the pool of satellite cells and the regenerative muscle capacity, besides increasing proteolysis by Caspase-3 and UPS pathway activation. Angiotensin II also affects intermediary molecules such as IL-6 that impairs the insulin/IGF-1 signaling and reduces Akt phosphorylation and activates the TGF-β pathway [15, 19, 40].

18.3.4 Vitamin D Impairment

Besides the well-known effect of vitamin D as a bone regulator, recent studies show that it can be also important for muscle maintenance and regeneration [47]. Vitamin D deficiency can induce muscle wasting, acting primarily via UPS [48] reducing the

muscle strength in CKD patients [49]. Both deficiency and insufficiency of vitamin D are common, with a frequency higher than 80% in CKD and end-stage renal disease (ESRD) patients [50, 51]. Vitamin D deficiency seems to increase with the CKD progression [52]. Among factors associated with vitamin D deficiency/insufficiency in CKD patients are age, female sex, proteinuria, low level of physical activity, diabetes, body adiposity, low vitamin D synthesis in the skin, and low tubular reabsorption of vitamin D, in addition to the need for peritoneal dialysis or hemodialysis [47].

18.3.5 Hormonal Alterations

18.3.5.1 Sexual Hormones

Reduction of production/availability of anabolic hormones is another factor related to muscle atrophy [19]. Testosterone is an anabolic hormone that plays an essential role in muscle hypertrophy. This hormone facilitates the muscle anabolism, promoting nitrogen retention, stimulating fractioned protein synthesis, inhibiting muscle degradation, and enhancing the efficiency of amino acid reuse by the muscles [53]. Hypogonadism in men, with consequent testosterone deficiency, is a common alteration in CKD, with prevalence varying between 30% in mild and moderate levels of CKD, until more than 50% in ESRD [53, 54]. Reduced testosterone levels in CKD patients' serum were associated with muscle mass and strength reduction [53, 55]. This condition arises mainly because of the lack of clearance of prolactin and uremic inhibition of luteinizing hormone signaling [53, 55] and can be aggravated by common CKD comorbidities, such as obesity, hypertension, and diabetes mellitus [19]. The potential mechanisms by which the testosterone reduction can cause muscle catabolism include alteration in IGF-1 signaling and increased myostatin levels [15, 22].

Little is known about differences between men and women about CKD-induced muscle atrophy; however, women can present higher levels of muscle wasting than men. CKD women exhibit estrogen deficiency even in the earliest stages of the disease [15], and reduced estrogen levels are associated with a reduction in muscle strength and function [54].

18.3.5.2 Growth Hormone

Growth hormone (GH), IGF-1, and insulin are important factors for muscle mass gain. GH is the main promoter of body growth in children and exerts anabolic effects in adults, acting in protein synthesis stimuli, protein degradation reduction, improvement of fatty acid mobilization, and increased gluconeogenesis, IGF-1 being the main mediator of these actions [56]. Both uremia and inflammatory status seem to contribute to GH resistance in CKD [57]. Also, abnormalities in the GH/IGF-1 physiological axis have been described as potential causes of increased protein catabolism and CKD-induced muscle atrophy [15, 56].

18.3.6 Physical Inactivity

CKD and dialysis patients present a reduced level of physical activity [15, 58], with higher inactivity in advanced stages of the disease [58]. Physical inactivity is considered an important factor that impairs exercise capacity, functional independence, and muscle atrophy [59]. Moreover, there is a positive association between the physical activity level and muscle mass in hemodialysis patients [60]. The physical activity reduction, with consequent muscle wasting, is a crucial factor in the prognosis of hemodialysis patients [61].

18.3.7 Hemodialysis

Hemodialysis consists in an RRT capable of ensuring the survival of ESRD patients. Although the life of this patient is maintained with acceptable quality, hemodialysis for long periods contributes to a series of complications, including cardiovascular diseases, a tendency to bleeding, gonadal dystrophy, malnutrition, insulin resistance, immunological deficiency, chronic inflammation, anemia, and muscle atrophy [62]. Considering these complications it can be observed that most of them were already independently approached in this chapter because of their relation with proteolysis increase or protein synthesis reduction in CKD patients, explaining why ESRD patients in hemodialysis show more severe muscle atrophy than predialytic CKD patients [4, 22].

18.4 Clinical Implications of CKD-Induced Muscle Wasting

Previously to approaching the clinical implications of CKD-induced muscle atrophy, it is necessary to introduce the concept of sarcopenia. The term sarcopenia was initially used to describe the age-related muscle mass decline [63]; however, strength and function impairment is frequently associated with muscle wasting. Thus, nowadays, sarcopenia refers to loss of muscle mass and strength or function reduction [64] and is considered a powerful morbidity and mortality predictor in dialysis patients [40].

Muscle atrophy is responsible for an important reduction in physical function of CKD patients [8], and among the most frequent muscle atrophy-related clinical implications are peripheral [8] and respiratory [9, 10] muscle strength reduction, increasing muscle weakness and fatigue [40, 65], reduction of functional capacity [10] and functional independence of this patient [66] leading to a sedentary lifestyle [67], impairment of quality of life [68], and increased episodes of hospitalization and elevated mortality [69].

Johansen et al. (2003) assessed the cross-sectional area and muscle strength of ankle dorsiflexors, as well as the walk speed of 38 patients with CKD in dialysis, and compared with healthy controls, paired by age and sex [8]. Among the findings, this study identified that CKD patients showed less contractile muscle area, although the total cross-sectional area showed no difference between groups. Also, muscle strength and walk speed were lower in CKD patients than in controls. Thus, the correlations between contractile muscle area, strength, and walk speed support the argument that muscle atrophy and resultant weakness are important causes of physical function impairment in this population.

Respiratory muscles are also compromised by CKD, and this is probably associated with peripheral muscle strength reduction [70]. Schardong et al. (2008) verified in a cross-sectional study with 30 CKD patients in dialysis that the inspiratory and expiratory muscle strength was below the predicted levels. Equally, the forced vital capacity (FVC) and the forced expiratory volume in the first second (FEV_1) were altered, pointing to an impairment of lung function [9].

Muscle weakness, defined as the failure to produce strength [71], is a prevalent clinical manifestation in patients with CKD, as many studies showed that muscle strength is found to be reduced in these patients [8, 72].

Fatigue, in its turn, defined as the failure to sustain muscle strength or power [71], is also considered a common finding in dialysis patients. Although still little understood, many mechanisms are listed as causes of perceived fatigue during exercise [40], including muscle wasting and weakness [73].

On the other hand, Fahal (1997) showed that dialysis patients and healthy individuals have similar fatigability. However, in a sub-analysis by nutritional status, undernourished patients have higher fatigue in comparison with the well-nourished group. Despite these findings, current evidence showed that muscle abnormalities in uremic patients happen even with an adequate nutritional intake [40]; thus, further research is needed to highlight the mechanisms of muscle fatigue in CKD patients [74].

Concerning functional capacity, Dipp et al. (2010) evaluated 30 ESRD and verified through the 6-minute walking test (6MWT) that they walked a distance shorter than that stipulated by the prediction equations [10]. The distance covered in the 6MWT is an independent predictor or mortality for CKD, since every 100 meters covered, there is a protective factor of 5.3% in survival [75]. Also, Dipp et al. (2010) verified that these patients have expiratory muscle weakness, with a positive correlation between functional capacity reduction and maximum expiratory pressure [10].

Moreover, Martinson et al. (2014), in a longitudinal study with 105 CKD patients in dialysis, showed through magnetic resonance imaging of thigh muscles and 6MWT that an elevated percentage of body fat is associated with low functional capacity [68]. In the other hand, a higher percentage of muscle mass is associated with better physical function and quality of life [68].

About mortality, Ysoyama et al. (2014) assessed the relation between muscle mass, strength, and mortality in a cohort of 330 CKD patients [69]. According to the authors, even though the muscle mass and strength reduction are prevalent conditions among CKD patients, they are not congruent, that is, they are not always

associated. Among the individuals evaluated only 20% were sarcopenic. A quarter (24%) has reduced muscle mass and appropriate muscle strength, 15% have reduced muscle strength and adequate muscle mass, and 41% were within normal range in both. Also, the same study points out that these two measures (muscle mass and strength) are strong predictors of mortality when considered independently; however, muscle strength showed a stronger association with mortality [69].

18.5 Current Clinical Treatments

In order to attenuate or even revert the process of muscle atrophy, nutritional supplementation and correction of metabolic acidosis are necessary. Other strategies such as reducing insulin resistance, administration of androgenic steroids, resisted and aerobic exercise, neuromuscular electrical stimulation, and inspiratory muscle training should also be considered to avoid progression of sarcopenia.

18.5.1 Nutritional Supplementation

Low protein diets (0.6–0.8 g/kg/day) have been recommended for patients with glomerular filtration rate < 45 ml/min/1,73m^2, since they seem to delay the progression of CKD to CKF, because they attenuate uremia [76].

On the other hand, patients in RRT require a higher protein intake (> 1.2 g /kg/day), because there it is not necessary to protect renal function after the initiation of dialysis and the dialysis treatment itself is responsible for stimulating protein catabolism [77].

18.5.2 Correction of Metabolic Acidosis Through Alkaline Therapy

Metabolic acidosis induces muscle loss by stimulating glucocorticoid adrenal secretion [78]. Evidence indicates that the correction of this disorder has beneficial effects on nutritional parameters in patients with CKD [77] besides preventing the progression of the disease [79].

Oral bicarbonate supplementation has been suggested to maintain serum levels within the normal range [80], since low concentrations are associated with high mortality in patients with CKF [81]. The consensus is that alkaline therapy should be administered to achieve a plasma concentration of HCO_3 > 22 mmol/L, independently of the cause of metabolic acidosis [82].

18.5.3 Reduction of Insulin Resistance

There is a close relationship between the altered signaling of insulin/IGF-1 ratio and catabolic conditions that stimulate muscle protein degradation according to experimental studies in animal models [83]. Activation of Caspase-3 and UPS is probably involved in this process and stimulates protein muscle catabolism; however, to date, there are no studies in humans identifying sensitizers of insulin as a treatment strategy [23].

18.5.4 Administration of Androgenic Steroids

Low plasma concentrations of testosterone may contribute to muscle loss [84] since they modify IGF-1 signaling and increase the concentration of myostatin (protein that suppresses muscle growth) [85].

Androgenic steroids such as nandrolone decanoate, a synthetic derivative of testosterone increase muscle mass in healthy adults and in patients with CKD. Macdonald et al. (2007) in a clinical trial (II phase) with 54 patients in stage 5 of CKD observed that nandrolone when given once a week (100 mg for 24 weeks) induced an increase in appendiceal mass without any fluid overload. However, this dose was not tolerated by women as a result of side effects (virilization) [86].

18.5.5 Aerobic and Resisted Exercise

Studies regarding physical training in patients with CKD confirm substantial improvement in leg muscle size [87] and muscle power. These, in turn, correspond to the morphological changes in capillary density [88], in the improvement of oxidative metabolism [89], in muscle mitochondrial biogenesis [90], and in the reduction of systemic inflammation [91].

Kouidi et al. (1998) performed a combined exercise program for 6 months in patients with end-stage CKD and, through a muscle biopsy of vastus lateralis, found that training significantly improved muscle atrophy (increase of 51% in type II fibers), thus reflecting an overall increase in physical performance (increase of 48% in VO_2 peak) [92].

Regarding the functional variables, systematic reviews [16, 93–95] that evaluated the effect of aerobic and resisted exercise in patients with CKD show benefits in muscle strength, functional capacity, cardiac dimensions, and also in patients' quality of life.

The exercise prescription should be individualized and according to the assessment of the patient's physical capacity. Aerobic and resisted exercises are recommended, but flexibility exercises and those aimed at improving balance can be included in the training program according to need [66].

Aerobic exercise can be performed using a cycle ergometer (even during dialysis), walking, or swimming. A weekly frequency of 1–2 times/week, with intensity between 55% and 70% of maximal heart rate or 11–13 points on the Borg effort scale (6–20-point scale), is recommended. The progression of training should be made according to the patient's response to 3–5 times/week, with intensity between 55% and 90% of maximal heart rate or 11–16 points on the Borg effort scale. The ideal exercise time is at least 20 min/day or shots of 3–5 min when interval exercise [96, 97].

Regarding resistance exercise, it is advised that it be performed in several muscle groups, contemplating agonist and antagonistic muscles. The weekly frequency indicated is 2 times/week, and exercises should be performed at 60–70% of the maximum repetition one test (1RM), or 5RMs of each movement may be performed. Initially, 1 set of 10–15 repetitions is recommended, but progression of training should be done until 2–4 sets are achieved. Also, 8–10 different exercises for the main muscle groups are indicated, respecting intervals of 2–3 minutes of rest between the sets [96, 97].

Finally, when choosing to perform the exercises during hemodialysis, these should be performed until the second hour of the dialysis session to ensure hemodynamic stability of the patient [93].

18.5.6 Neuromuscular Electrical Stimulation

Neuromuscular electrical stimulation (NMES) is an alternative to conventional physical exercise, and it should be encouraged especially for those patients who are more debilitated, where voluntary exercise is not feasible. Vigorous and involuntary muscle contractions were applied by Schardong et al. (2017) in a randomized clinical trial using an 8-week (3 times/week) protocol in patients with CKF and during hemodialysis [17]. The exercise through NMES was performed in isometric form on the quadriceps muscle and with the following stimulation parameters: 80 Hz, 400 μs, 10s contraction time, rest time ranging from 50 to 20s, application time of 20–34 min, and intensity at motor threshold level tolerated by the patient. Among the findings, the authors observed a protective effect for quadriceps muscle atrophy when assessed by ultrasonography in the intervention group. The same did not occur with the control group, who did not perform any type of exercise. In addition, the group that received the NMES had an increase in the muscle strength of the lower limbs and in the number of repetitions in the sit-and-stand test [17].

18.5.7 Inspiratory Muscle Training

Inspiratory muscle training (IMT) is among the treatment resources for patients with CKD that aim to improve the performance of respiratory muscles [98], since they have significant weakness when compared to normal values for healthy individuals

[10]. In this way, it can be a useful tool, since the strengthening of the respiratory muscles slows down complications resulting in the loss of muscle mass [99].

The IMT should be applied with a fixed load to ensure a strong activation of the inspiratory muscles [100]. This may result in effects such as modification of respiratory muscle phenotype, in addition to increasing strength and endurance [101].

Medeiros et al. (2017) through a systematic review and meta-analysis of randomized clinical trials found that IMT improved the inspiratory muscle strength of CKD in hemodialysis when compared to sham training or control, with a significant effect of 22 cmH_2O (95% CI 16–29) [18]. In addition, the authors found benefits in pulmonary function, functional capacity, and quality of life of these patients. The studies included in this review used the following training parameters: adjusted load between 15% and 60% of maximal inspiratory pressure for 20–60 min or 3 sets of 10–15 breaths, 3 times/week, for 6–12 weeks [18]. Despite the positive results, we emphasize that the reviewed studies are heterogeneous and present important methodological limitations.

18.6 Perspective

The pathways leading to muscle atrophy and therefore sarcopenia in CKD are complex and involve several mechanisms and associated factors. In addition, the loss of muscle mass is progressive, leading to a sedentary lifestyle and worsening quality of life. Associated with other comorbidities and cardiovascular complications that are frequent in this population, muscle atrophy is responsible for the reduction of physical capacity, functional independence, and an increase in the number of hospitalizations and mortality rates.

In this sense, preventive measures are necessary, aiming at the individualized evaluation of these patients, still in the early stages of the disease, addressing an early identification of functional alterations that may precede the muscular atrophy process. Identifying these individuals, the clinical treatment and rehabilitation strategies discussed in this chapter should be traced and performed by a multiprofessional team in order to mitigate, delay, or even revert these manifestations, thus providing a better quality of life and survival for this population.

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Chapter 19
Sarcopenia in Liver Disease: Current Evidence and Issues to Be Resolved

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Abstract Sarcopenia is a common clinical symptom in aging and patients with wasting diseases, characterized by a decreased skeletal muscle mass. As a consequence of lifestyle change, the nonalcoholic fatty liver disease (NAFLD) presents a rising trend. In the past three decades, increasing evidence has proved that sarcopenia is related to NAFLD. In this chapter, we will summarize the emerging evidence of the predictive role of sarcopenia in NAFLD and review the diagnosis value, feasible mechanism, and therapy strategies of sarcopenia in NAFLD. Sarcopenia is a potential risk factor for NAFLD, and targeting sarcopenia can benefit NAFLD to some extent.

Keywords Sarcopenia · Nonalcoholic fatty liver disease · Liver fibrosis

19.1 Background

Skeletal muscle is the major component of the mammalian motor system, with the function of secretory, mechanical, and supporting activities [1]. Similar to bone, the weight of muscle peaks at about 45–50 years old and then gradually decreases at a rate of 1–2% per year [2–4]. This kind of typical changes in human body composition related to aging is a progressive loss of muscle mass and strength, called sarcopenia [5, 6]. Sarcopenia is one of the most common types of muscle atrophy in aging population, strongly associated with senescence and malnutrition [7–12].

Sarcopenia is defined as reduced skeletal muscle mass, which is a common complication of most liver disease patients. It is observed in up to 60% of patients with

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end-stage liver disease (ESLD) [13, 14]. Nonalcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease, which refers to hepatic steatosis that is not caused by significant alcohol consumption or other causes of liver disease. In Western Europe and the United States, about 64 and 52 million people suffered from NAFLD, respectively [15, 16]. NAFLD is classified into different degrees, from the "benign" called simple steatosis (overall 20–30% prevalence) to steatohepatitis (NASH, 2–5% prevalence) and fibrosis [17]. Regarded as a metabolism disease, NAFLD shares amounts of pathophysiology process with sarcopenia. For example, both the liver and muscle are target organs for insulin action, and insulin resistance is known as a key factor in the pathophysiology for both NAFLD and sarcopenia.

During the last four decades, researchers have made a lot of efforts to investigate the relationship between sarcopenia and NAFLD [18–22]. In this chapter, we will give an introduction of involvement of sarcopenia in liver disease, including the pathology, diagnosis, and management of NAFLD associated with sarcopenia [23].

19.2 Current Proof in the Relationship Between Sarcopenia and Chronic Liver Disease

19.2.1 Sarcopenia as an Independent Predictor of NAFLD

Compelling evidence have shown the connection of sarcopenia and NAFLD [24]. To confirm the relationship between sarcopenia and NAFLD, the Korean Sarcopenic Obesity Study (KSOS) was conducted. The researchers built a cohort including 452 apparently healthy adults to perform a prospective observational cohort study and explore the correlation of sarcopenia and NAFLD with cardiometabolic risk factors. They found that after adjusting for confounding factors (insulin resistance and inflammation), the risk of NAFLD increased in patients with low muscle weight [25]. The next study showed that all these relationships happened among people of different sexes, although age group and menopausal status have an effect on it; and further confirmation of this relationship was required [26].

Another research group carried out a cross-sectional study in representative samples of the Korean population in 2015. In addition, based on the existence of liver fibrosis in patients with NAFLD, further stratification was carried out to preliminarily study the connection between sarcopenia and the progression of NAFLD. The data showed that regardless of condition of obesity or metabolic control, sarcopenia was associated with increased risks of NAFLD and advanced fibrosis [27]. They further stratified the sample according to the grade of liver fibrosis and continuously studied the relationship between sarcopenia and NAFLD-related cirrhosis. Interestingly, they found that sarcopenia was associated with significant liver fibrosis in subjects with NAFLD, and the association is independent of obesity and insulin resistance when comparing patients with fibrosis and NAFLD patients without

fibrosis [28]. A rough analysis based on another NAFLD cohort showed that sarcopenia was related to NAFLD, with an OR of 3.82 (95% CI, 1.58–9.25), which was confirmed by biological systems.

In practice, NAFLD patients are often associated with other metabolic diseases. Yoshitaka Hashimoto et al. focused on the patient with type 2 diabetes mellitus and assessed the correlation between skeletal muscle mass index and hepatic steatosis. They draw a conclusion that mass of skeletal muscle was negatively related to hepatic steatosis in patient with type 2 diabetes mellitus which was consistent with previous results [29]. Similarly, worsening fibrosis was found related to increased prevalence of sarcopenia, independent of IR and obesity. Furthermore, the presence of fibrosis was 22% in nonsarcopenic patients compared to 60% in those with sarcopenia.

19.2.2 Sarcopenia in Prediction of Chronic Liver Disease and Its Complication

Liver cirrhosis is the end stage of liver disease characterized by the destruction of hepatic lobules. Among the multitudinous etiologies of cirrhosis, nonalcoholic steatohepatitis (NASH) is the most familiar one with increasing incidence year by year. Liver cirrhosis accompanied with sarcopenia is very common; the estimated prevalence of sarcopenia in subject with liver fibrosis is 40–70% [30]. The incidence is 50–70% in men slightly higher than that in women [31, 32]. A Canadian study showed that sarcopenia was associated with both visceral obesity and IR [33]. The median survival time of the patients with sarcopenia (19 ± 6 months) was shorter than that of nonsarcopenia patients (34 ± 11 months) (P = 0.005). They also observed L3 skeletal muscle index was not relative to Child–Pugh scores ($r = -0.14$; $P = 0.1$) and Model for End-Stage Liver Disease (MELD) (r = −0.07; P = 0.5) [33]. Another study revealed the median survival was 16 ± 6 months and 28 ± 3 months, respectively, in patients suffering from concurrent cirrhosis and HCC with or without sarcopenia [34]. The 1-year probability of survival in patients with sarcopenia was significantly lower compared to that of patients without sarcopenia as a conclusion of multiple results from different groups (85% vs 97%, P = 0.01 [35]; 52% vs 82%, P = 0.003 [34]; 53% vs 83%, P = 0.005 [33]; 63% vs 79%, P = 0.04) [36].

Sarcopenia is not only associated with the survival of patients with cirrhosis but also has a suggestive role on the complications of cirrhosis. Sepsis is one of the leading causes of death in cirrhosis patients. In patients with sarcopenia, the death rate associated with sepsis is 22%, higher than that of nonsarcopenia (P = 0.02). In earlier studies, however, no difference was found in the frequency of sepsis-related deaths in patients with or without sarcopenia. Hormones and biochemical changes and circulating endotoxins and other factors leading to sarcopenia in patients also impaired immune function and increased the risk of infection [37, 38]. In addition, patients with refractory ascites are particularly prone to malnutrition and sarcopenia,

as increased ascites increases the static energy consumption, while the food intake is reduced by increased abdominal pressure. The treatment of refractory ascites by transjugular intrahepatic portosystemic shunt (TIPS) has been proven to improve refractory ascites of patients with dystrophic liver cirrhosis, which will ameliorate the sepsis recurrence. Other complications including hepatic encephalopathy are also related to sarcopenia. Previous study has confirmed a higher incidence of hepatic encephalopathy in patients with reduced muscle mass and muscle contraction force [39]. The increase of ammonia content in peripheral blood of patients with sarcopenia may be one of the reasons [40]. Therefore, it is recommended to include sarcopenia into the evaluation system for prediction and prognosis of the patients with cirrhosis. Sarcopenia alone or in combination with conventional prognostic systems has shown promise for cirrhosis prognosis. How to include an objective assessment of sarcopenia with conventional scores to optimize the prediction outcome for patients with cirrhosis requires further researches [41, 42].

Liver transplantation (LT) is considered as the only cure for current end-stage liver disease, and the occurrence of sarcopenia is also closely related to its therapeutic effect [43–45]. By observing a cohort from the United States, researchers found that 59% patients have sarcopenia during LT evaluation. CT scan was performed on 59 patients with pre-transplant sarcopenia at 6 months posttransplant, and 56 (95%) remained sarcopenic, and a large proportion of patients would continue to remain sarcopenic in 1 year. Meanwhile they found that obesity was an independent predictor of pre-transplant sarcopenia ($P = 0.00001$, odds ratio [OR] 0.22) in cirrhotic patients [43, 46, 47].

19.3 Emerging Mechanism in Sarcopenia with NAFLD

Finding the common pathological process between sarcopenia and NAFLD is a key strategy to analyze their correlation in the mechanism. The current study mostly focuses on the insulin resistance, inflammation response, vitamin D, oxidative stress, decreased physical activity, and other possible mechanisms.

Insulin Resistance Insulin resistance (IR) is a common pathophysiological mechanism between sarcopenia and NAFLD [48–50]. In NAFLD patients with insulin resistance, the liver and adipose tissue are less sensitive to insulin. When adipose tissue becomes resistant to the antilipolytic effect of insulin, fat decomposition increases and free fatty acids (FFA) are released [23, 51]. The increased levels of triglycerides in the liver caused by IR are the main factors leading to liver steatosis. First, insulin cannot inhibit the lipolysis of adipose tissue by hormone-sensitive lipase, leading to FFA influx and subsequent absorption by the liver. Second, IR-associated hyperinsulinemia and hyperglycemia are upregulated by membrane-associated transcription factors sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP). Third, hyperinsulinemia directly inhibits β-oxidation. These phenomena together promote

the FFA accumulation in the liver and the hepatic triglyceride accumulation and steatosis through esterification [52, 53].

Study showed that even NAFLD patients without obesity have increased concentration of FFA and Adipo-IR compared to the control group [54, 55]. FFA enriched in the liver inhibits growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis, which has protective effect in age-related muscle loss and muscle regeneration [56]. In addition, IR is accompanied with compensatory hyperinsulinemia, which leads to glucose production disruption, decreased glycogen synthesis, increased lipolysis, and/or increased fat intake. Triglyceride (TG) transfer changes and inhibits β-oxidation, which caused TG accumulation in muscle tissue.

The gluconeogenesis is caused by IR-aggravated muscle protein loss and muscle degradation. IR itself can be a contributing factor to age-related muscle mass loss and leads to sarcopenia directly [57]. As an important mean of maintaining muscle mass and muscle metabolism, autophagy or lysosomal degradation is inactivated by IR through mammalian target of rapamycin (mTOR) pathway [53]. Collectively, these are how IR reduces muscle mass and leads to sarcopenia. Interestingly, the study found a negative correlation between IR and muscle mass, while IR was directly related to hepatic fat accumulation. These results support the common understanding of pathophysiological basis underlying the IR-mediated pathogenesis. Consistent with this view, the metabolic syndrome (MS) associated with IR should also be associated with NAFLD and sarcopenia [25].

Sarcopenia is associated with adverse glucose metabolism disorder, and the evidences indicate that low muscle mass can predict diabetes susceptibility. Given the increase in the prevalence of obesity, there is an urgent need for further research in developing control strategy of obesity and metabolic effects of sarcopenic disorders. Similarly, myosteatosis has also been shown to be related to IR. Synergistic effects of sarcopenia and obesity can lead to severer IR and metabolic disorders. In this regard, sarcopenia is also a factor that contributes to the onset of NAFLD by promoting IR.

Inflammation Chronic inflammation and oxidative stress are essential processes in the development of and liver fibrosis followed directly. NASH is accompanied with an inflammatory reaction that occurs in the absence of pathogens or external antigens belonging to sterile inflammation. Lipid-induced hepatocyte stress, damage, and cell death could be the reason of sterile inflammation. Fatty acid oxidation (FAO) in the liver enhances the production of oxygen free radicals, causing lipid peroxidation and inducing pro-inflammatory cytokine synthesis. For example, transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) are the most common factors of NAFLD. Furthermore, these cytokines stimulate protein degradation and metabolism, resulting in muscle mass loss and sarcopenia. These cytokines support both the recruitment of T cells and development of specific immune response against antigens. They activate synthesis of each other and stimulate IL-6 secretion. Although these cytokines show highest levels and activities in acute diseases like sepsis and are upregulated in trauma or after surgery, they also play key roles in NAFLD and infections which lead to loss of muscle cells and

acceleration of muscle protein breakdown, contributing to sarcopenia. Inflammation markers in circulation, including CRP, TNF-α, and interleukins (IL-6 in particular), are closely related to the occurrence of sarcopenia.

Vitamin D Low vitamin D levels have been reported to be involved in the pathogenesis of both sarcopenia and NAFLD [58]. NAFLD and vitamin D deficiency are associated with insulin resistance, obesity, type 2 diabetes mellitus, and cardiovascular disease. Many studies exploring the relationship have emerged over the past few years. Recent animal studies have shown that vitamin D is of critical importance in the production of pro-inflammatory cytokines and consequently regulates oxidative stress, hepatocyte apoptosis, and even hepatic fibrosis, although the mechanism of the association between vitamin D and NAFLD is not fully understood. The insulin receptor in pancreatic β-cells and in peripheral target organ (including the liver) is induced by vitamin D by activating vitamin D response elements (VDREs) in the human insulin receptor (hIR) gene promoter [59]. VDR is a receptor for 1α, 25-dihydroxy-vitamin D3 (1α, 25-(OH)2-VD3), activated from vitamin D3, and has a significant effect on calcium–phosphate homeostasis and bone metabolism but also on other physiological functions, including immunomodulation, cell growth, and differentiation. The effect of vitamin D on insulin sensitivity changes was mediated by vitamin D receptor (VDR) by improving systemic inflammation [60–62]. VDR in skeletal muscle can also be activated by vitamin D, which mediates muscle genesis, skeletal muscle growth, and inflammation. Results from animal studies prove vitamin D deficiency myofibrinolysis is increased with vitamin D deficiency. Lower levels of vitamin D were associated with lower muscle strength, poor muscle function, and increased muscle loss. People with muscular dystrophy have significantly lower levels of vitamin D. Vitamin D supplements may improve muscle strength and function in muscular dystrophy patients.

Decreased Physical Activity The decrease in physical activity and the atrophy of muscles cross-promote each other. In addition, the decrease of physical activity is one of the main reasons which lead to IR and metabolic diseases. Patients with muscle atrophy, due to limited mobility, tend to live sedentary lifestyles and lack exercise [63, 64]. A sedentary lifestyle can increase the risks of obesity, metabolism diseases, and NAFLD, which has been well proven. It is speculated that this sedentary lifestyle will lead to a decrease in energy expenditure, which consequently leads to obesity and liver fat. In fact, studies have shown that in patients with sarcopenia, the amount of fat increases, as well as the body composition and the level of CRP, which further increased the risk of NAFLD [65].

Myokines and Myostatins Skeletal muscle is considered as an endocrine organ. Myokines are defined as the peptides that are produced, expressed, and released by muscle fibers, including cytokines and other peptides with autocrine, paracrine, or endocrine effects. Muscle-derived hormones provide a new thought to build the communication between skeletal muscle and other organs, such as the adipose tissue, liver, pancreas, bones, and brain [66]. IL-6, one of the many myokines, appears to have systemic effects on the liver mediating crosstalk between intestinal L cells

and pancreatic islets. Activation of IL-6/STAT3 pathway subsequently downregulates lipogenic genes but upregulates fatty acid oxidation-associated genes in the liver of interleukin-10-deficient mice [67]. Moreover, increased muscle peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1 alpha (PGC-1α) expression protects mice from sarcopenia and metabolic disease and prolongs their lifespan [68]. The PGC-1α-dependent myokine irisin drives brown-fat-like development and causes a significant increase in total body energy expenditure whereby reducing body weight and thus obesity and IR [69]. Serum irisin concentrations were downregulated in the patient with NAFLD and inversely associated with the triglyceride contents in the liver and liver enzymes in obese adult [70]. The downstream signal transduction pathway activated by irisin involves the peroxisome proliferator-activated receptors α (PPARα), which are of vital importance in fatty acid β-oxidation in the liver [71]. FGF21 regulated by PPARα reduces hepatic steatosis and leads to reduced lipogenic gene expression and possibly the rate of fatty acid and triglyceride synthesis [72]. Myokines also significantly blunt insulin-stimulated glucose uptake and may participate in the occurrence of IR in the liver [73]. Therefore, it is determined that the protective effect of muscle on NAFLD will disappear mediated by hormone secretion when sarcopenia occurred.

Myostatin (also known as growth differentiation factor 8, GDF-8) is a member of TGF-β superfamily, with an inhibiting effect in protein synthesis and regeneration [74, 75]. In skeletal muscle, myostatin can activate mediated autophagy proteolysis and ubiquitin proteasome pathway which are two main pathways of skeletal muscle protein hydrolysis [76]. Myostatin also increases the quality of adipose tissue, leading to decreased adiponectin production [77–79]. The receptor of myostatin expressed on hepatic stellate cells. In hepatocytes, myostatin inhibits hepatocyte proliferation and insulin-stimulated glucose uptake [73]. Increased serum myostatin level is related to poor prognosis in liver cirrhosis patients [80]. This may be a potential link between sarcopenia and liver disease. But there is still doubt about which one is the consequence.

Other Daniel Cabrera et al. found that the American Lifestyle-Induced Obesity Syndrome (ALIOS) diet-induced NAFLD mouse showed decreased muscle fiber diameter and myosin heavy chain (MHC) protein levels. Serum insulin-like growth factor-1 (IGF-1) was detected decreased, which is an anabolic hormone essential for muscle homeostasis without increase of inflammatory mediators. Since leptin in the brain can stimulate the production of IGF-1 in the liver, a later study explored the relationship between sarcopenia and NAFLD based on this regulatory mechanism [81].

Metabolic disturbances, inadequate dietary intake, and malabsorption are also involved in the pathogenesis in the end stage of NAFLD. After entering the stage of cirrhosis of the liver, because of glycogen synthesis and storage damage in the cirrhotic liver tissue, fat and muscle catabolism glycosylation of noncarbohydrate sources are promoted [82]. About 15% to 30% of patients with cirrhosis are in a highly catabolic state. Only if ensured adequate protein intake, it usually causes muscle atrophy [83]. The cause of highly catabolic state is unknown. The cause may include the activation of the sympathetic nervous system through the hypermeta-

bolic pathway, the displacement of the gut bacteria, or systemic inflammation. At the same time, sepsis will exacerbate energy consumption in patients with cirrhosis and accelerate protein degradation. As a consequence of portosystemic shunt, the lack of cholestasis, and intestinal bacterial overgrowth, malabsorption of nutrients is a possible cause of muscle loss [36].

19.4 Diagnosis and Management of Sarcopenia in Liver Disease

The Asian Working Group for Sarcopenia (AWGS) (AWGS/grip criteria) and European Working Group on Sarcopenia in Older People (EWGSOP) (EWGSOP/grip criteria) are always used in the diagnosis of sarcopenia in patient with chronic liver disease [84–87]. EWGSOP/grip criteria found that age-related muscle volume reduction is related to low muscle strength and/or physical performance [88–90]. Low muscle mass and decreased muscle function (muscle strength or properties) are used as a screening test, according to the diagnostic criteria of EWGSOP in 2010, it means establishing a diagnosis requires meeting criteria 1 and criteria 2 or criteria 3 at the same time [91, 92]. Different from EWGSOP/grip criteria, the patients are also diagnosed as sarcopenic by both muscle strength (handgrip strength) and physical performance (usual gait speed) as the instruction of AWGS/grip criteria [85]. Due to differences in body size, lifestyles, ethnicities, and cultural backgrounds, each criterion describes the cutoff value used for Asian and European populations by detail. The cutoff threshold for calf circumference is 33 cm, and that of hand grip strength in male and female are 32 kg and 22 kg, respectively [87]. Psoas muscle thickness and total muscle and adipose tissue cross-sectional area at the level of the third lumbar vertebra (L3) transverse processes are always commonly used for measuring muscle mass imaging with computed tomography (CT) or magnetic resonance imaging [93–95]. In the diagnosis and screening of sarcopenia in patients with chronic liver disease (CHD), scientists have made many attempts and explorations. Some scholars have found that serum BCAA and albumin levels are significantly associated with handgrip strength and PSI (psoas index) in patients without BCAA granule supplement, though the contact strength is weak [96, 97]. The reduction of BCAA level as a manifestation of CHL progress may play a role in the muscle atrophy associated with primary disease. Researchers are still looking for highly sensitive and noninvasive markers to improve diagnostic efficiency.

19.5 Method of Reversing Sarcopenia of Cirrhosis

Because muscle reduction is associated with adverse outcomes of liver cirrhosis, limited data has shown that increased muscle mass can improve survival of patients with liver cirrhosis after transplantation. Therefore, reversing muscle mass

reduction is a key measure for patients with cirrhosis [98]. According to the physiopathological mechanism of sarcopenia, the method of managing sarcopenia was built by considering nutritional status, physical activity, ammonia, and hormones [99]. Guidelines and consensus statement put forward basic concept. The present therapeutic strategies for sarcopenia in cirrhosis include exercise and nutrition therapy, supplemental hormone therapy, and mechanistic targeted treatments.

19.5.1 Exercise and Nutrition Therapy

Vast solid evidences have identified the positive effects of exercise, whereas, unfortunately, this "panacea" has not been applied properly. Smart selection of exercise type is important to ensure maximum benefit to the patients [100]. Resistance exercise (RE) can stimulate muscle protein synthesis (MPS) which has the potential to modulate muscle mass gain [101]. Different from RE, endurance exercise (EE) may improve the exercise capacity and muscle strength. Only few studies have been conducted to assess the benefit of patients undergoing exercise training in combination with RE and EE by far, so the benefits still remain unclarified. It is still not possible to predict whether a synthetic metabolic nutrient resistance will be observed during exercise. The mechanical stimuli activate mTOR signaling in muscle through a PLD-dependent increase of phosphatidic acid (PA) [102]. The current exercise guidelines for patients with chronic diseases recommend that individuals perform 150 min of moderate physical activity per week, and two times a week for endurance and flexibility training. Due to the limitations of exercise capacity, these guidelines may not be feasible in most patients with cirrhosis. It is still advocated that the exercise experts should assess the patient's motor ability and clinical status and formulate the individualized exercise prescription [64, 103, 104]. But all the studies were carried out in the patients or animal models without cirrhosis, and it was not clear whether the responses were tested in patients with cirrhosis or not. For example, studies have shown that hyperammonemia leads to decreased muscle function without affecting the muscle mass and that hyperammonemia impairs skeletal muscle strength and increases muscle fatigue. These suggest that blood ammonia may also affect the therapeutic value of exercise for muscle atrophy in the liver disease model, different from that in the simple sarcopenia model [105].

Because the lack of nutrition in patients is an important cause of sarcopenia, which is mainly due to insufficient intake of total calories and protein, thus guidelines and consensus statements recommend frequent feeding. Oral rehydration is the best way to supplement, and enteral or parenteral nutrition is applied if necessary [106–108]. There are numerous strategies for extra nutrition through high-calorie feeding and/or enteral feeding provided by different studies [109–111].

In terms of nutrition, the two main problems are the plan and time of nutrition supply. Study indicated that giving patients late-night food is a feasible intervention to reverse the reduction of synthetic metabolism and muscle atrophy in patients with cirrhosis and can improve the life quality of patients with cirrhosis. The long-term

benefits and the value on lifespan were critically evaluated. The subsequent meta-analysis was disappointing, and nutritional supplements for patients with alcoholic hepatitis and liver cirrhosis demonstrated no improvement in survival rate. The exact mechanism of the protective effect of supplemental nutrition on muscle loss is unclear, which allows us to consider other factors that contribute to such uncertainty. As a form of resistance to synthetic metabolism, the nutritional problem of cirrhosis may not be compromised by supplementing energy alone. We need to consider the effects of impaired mitochondrial function on nutrition management. Other clinical symptoms, including encephalopathy and septicemia, and how to improve the life quality are also needed to be considered in future studies.

Protein supplementation is another way to improve the supply of essential amino acids. However, liver cirrhosis and high blood ammonia may accelerate the decomposition of amino acid. This results in ammonia accumulated in skeletal muscle, which damages the protein synthesis and further increases the autophagy. These are not benefits to reverse sarcopenia. In the selection of protein sources, plant proteins have an advantage over animal protein, which are rich in branched-chain amino acid (BCAA) rather than aromatic amino acids [112–114]. For example, leucine is particularly an important activator of mTORC1 via the Rag small GTPases and a plethora of regulatory proteins, leading to decreased autophagy and protein synthesis, which is the protection mechanism against loss of muscle. Confirmed results have provided direct evidence on interference of the molecules in skeletal muscle during cirrhosis [115, 116]. A single oral BCAA mixture enriched with leucine (BCAA/LEU) can impair mTOR1 signaling, autophagy, and GCN2 activation in cirrhotic patients without altering myostatin expression [117]. Combined with in vivo and in vitro data of, hyperammonemia is considered as the mediator of hepato-muscular axis and BCAA supplement is beneficial for cirrhosis [118, 119].

19.5.2 Supplemental Hormone Therapy

Both sarcopenia and low testosterone have been found associated with poor prognosis in men with cirrhosis, independent of the Model for End-Stage Liver Disease (MELD) score. Testosterone and growth hormones are used to improve nutritional status and muscle mass in cirrhosis patients, but the clinical benefits remain to be verified [120–123]. Anabolic androgenic steroid oxandrolone shows an improvement in nutritional status, body composition, and muscle function, as well as the non-muscle beneficial effects such as the ameliorating condition of the original disease in men with cirrhosis. But unfortunately, testosterone treatment can significantly reduce the mortality of patients (16% vs. 25.5%, $p = 0.352$). Even though research suggests that low testosterone has its advantages in predicting mortality in men with advanced liver disease than sarcopenia [124], it still needs to be addressed whether testosterone is continuously effective in improving the prognosis in liver cirrhosis patients with sarcopenia.

19.5.3 Other Potential Strategies

According to the documented mechanism mentioned above, the scientists propose treatment strategies for the corresponding targets, which require preclinical trials to clarify the effect. Myostatin antagonists, antioxidants, mitochondrial protectants, and direct mTORC1 activators may benefit skeletal muscle protein turnover but are not adequately evaluated [117, 118, 125].

Hyperammonemia could be another common concern in both sarcopenia and end-stage liver disease. Current methods for decreasing plasma ammonia include nonabsorbable disaccharides and antibiotics by preventing the production of ammonia. In the treatment of patients with liver cirrhosis, the main purpose of lowering blood ammonia originally is to cure hepatic encephalopathy; however, the latest views suggest that blood concentration of ammonia is completely not associated with the severity of hepatic encephalopathy [126]. Since it takes a long time for serum ammonia to affect the muscles, lowering blood ammonia in the short term does not reduce muscle blood ammonia concentration. The changes of high blood ammonia on signal pathway activation and metabolism cannot be reversed. Loss of muscle mass and function can be saved only by long-term, continuous ammonia-lowering therapies, or by targeting lower levels of ammonia in the skeletal muscle. Supplemental BCAA are used as a therapy in patients with cirrhosis, especially in the patients with hepatic encephalopathy (HE) [127–129]. The oral dosage of BCAA can enhance the metabolism of muscle ammonia, reducing the ammonia content in muscle. However, this method may also temporarily increase the concentration of arterial ammonia, which may be due to the external metabolism of glutamine (GLN). The contents of GLN in skeletal muscle can be maintained by parenteral α-KG supplemental after surgery. GLN synthesis may exert adverse effects of catabolism stimulation by BCAA in skeletal muscle. Thus, reducing the use of α-KG and other drugs that promote GLN synthesis should be considered [130–132].

19.6 Challenges in Study on Sarcopenia in Liver Disease

Sarcopenia is a common manifestation of chronic liver diseases. On one hand liver disease accompanied with sarcopenia adds the burden of the disease; on the other hand, sarcopenia can become a potential monitor of liver diseases and its complications. Although the researches have drawn a similar conclusion of correlation between sarcopenia and NAFLD and put forward the possible mechanism, there remain questions to be addressed. Firstly, some researchers have pointed out that it needs to pay attention to the diagnostic criteria of NAFLD used in studies. Skeletal muscle index (SMI) is the most commonly used index for assessing sarcopenia (SMI = total appendicular skeletal muscle mass [kg]/body mass index [kg/m2]). NAFLD is diagnosed by noninvasive evaluation methods, such as NAFLD liver fat

score and liver attenuation index (LAI). NAFLD patients are likely to be more obese, which affects the score of SMI. Moreover, there is no uniform standard to the choice of cutoff point in NAFLD diagnosis [133]. Hence, it is indispensable to build a research based on biopsy-proven or imaging-defined fatty liver. Secondly, the analysis results of the above data used adjustment variable in the logistic model. Some exposed factors such as IR, obesity, and low vitamin D, which will affect the results, are not included, though the researchers adjusted for other variables. The effect of these moderators should be considered deliberately. Meanwhile it is clear that lifestyles, ethnicities, and cultural background have a great influence on IR, which is the important component in the formation of either NAFLD or sarcopenia. Multicenter large-scale trials need to put into practice for formulating feasible and effective primary intervention strategies. Thirdly, the evidence shows a significant correlation between sex and the occurrence of sarcopenia in patient with NAFLD, which maybe a consequence of sex hormone. But there are no individualized treatment options for male and female. Lastly, we are still not certain about whether NAFLD is a cause or a consequence of IR. In conclusion, sarcopenia is a promising early warning factor for chronic liver disease, especially NAFLD, whereas lots of issues will need to be discussed in future studies.

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Part V
Diagnosis, Drugs and Promising Agents of Muscle Atrophy

Chapter 20
Muscle Atrophy Measurement as Assessment Method for Low Back Pain Patients

Elżbieta Skorupska

Abstract Low back pain is one of the most common pain disorders defined as pain, muscle tension, or stiffness localized below the costal margin and above the inferior gluteal folds, sometimes with accompanying leg pain. The meaning of the symptomatic atrophy of paraspinal muscles and some pelvic muscles has been proved. Nowadays, a need for new diagnostic tools for specific examination of low back pain patients is posited, and it has been proposed that magnetic resonance imaging assessment toward muscle atrophy may provide some additional information enabling the subclassification of that group of patients.

Keywords Low back-related leg pain · Multifidus · Muscle atrophy · Gluteus muscles

20.1 Background

Low back pain (LBP) is one of the most common pain disorders that may concern around 54–90% of people throughout their lives [1]. It is commonly defined as pain, muscle tension, or stiffness localized below the costal margin and above the inferior gluteal folds, with around 25–57% of cases suffering additionally from accompanying leg pain [2]. Most of the LBP cases are classified as non-specific type, namely, pain unattributed to a recognizable pathology (e.g., infection, tumor, osteoporosis, rheumatoid arthritis, fracture, or inflammation). Currently, the multidimensional nature of LBP has been underlined and indicated as a possible explanation for the discrepancies in study results. Different classifications and ways of subgrouping LBP patients are available, and they can be organized into five categories: (i) clinical features, (ii) pathoanatomical source of pain, (iii) treatment-based

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approach, (iv) screening tools and clinical prediction rules, and (v) pain mechanism [3]. The last – mechanism-based – classification is thought to be the most promising for the classification of LBP cases toward effective pain treatment. Nevertheless, it is impossible to classify LBP by any system objectively based on the routinely used clinical examination, radiographic and laboratory data, or questionnaires. Nowadays, a need for new diagnostic procedures has been posited. While some authors propose the utility of different nonstandard procedures (such as small invasive, high-tech imaging under control, or guided injection procedures) to confirm some LBP subtypes, others indicate simply the MRI assessment toward muscle atrophy to be of great use [4, 5].

Evidence from epidemiologic studies suggests that the lumbar spine structures are associated with the development and progression of LBP, and the atrophy of specific muscles has been assumed as an unheralded symptom of LBP. Until the end of the previous century, only a few authors detailed the role of muscle atrophy in the etiology of LBP [6–10]. During the last decade, however, more and more authors assumed the interaction between the atrophy of specific muscles, LBP, and spinal pathology to be well documented [4, 9–25]. Moreover, deficits in trunk and hip muscle strength [26, 27], endurance [28, 29], and motor control [30–32] have been identified in LBP individuals. Still, it is unknown if these deficits are the cause or the effect of LBP, and it has been suggested that LBP develops as a result of inactivity [33], denervation [34], inflammation [35], or injury [36]. What is important, the disturbed patterns of muscle fiber activation and histopathological changes indicate the atrophy independent of aging [37].

Generally, inactivity atrophy is usually associated with short-term or long-term immobilization, either of whole or part of the body, or with a limited use of muscles owing to a decreased physical activity. More precisely, if the LBP patient is sparing the symptomatic side or any improper functioning of the muscles responsible for trunk and pelvis stability develops, then muscle atrophy can be observed. Such situations are considered to be one of the main contributors to chronic low back pain [38–42]. Another possibility of muscle atrophy development among LBP cases is atrophy secondary to a direct muscle injury, infection, congenital myopathy, or inflammatory disease. Among histopathological changes, the following have been distinguished: muscle fiber degeneration, scattered chronic inflammatory cells, fibrosis, and focal areas of fatty tissue between degenerated fibers [43]. The next type of atrophy, which seems quite common among LBP patients, is neurogenic atrophy. It may occur as a result of an indirect muscle damage due to nerve injury (e.g., nerve root compression), which can provoke some metabolic changes in the sympathetic nervous system, then the metabolic activity of the musculoskeletal system, vasoconstriction, and – finally – atrophy [44].

20.2 Imaging Techniques Used to Assess Signs of Muscle Structure Degeneration

To assess muscle structure characteristics, different evaluation techniques are used, but the most frequently applied ones include computed tomography (CT) [14, 45, 46], ultrasound imaging (US) [47–50], and magnetic resonance imaging (MRI) [51, 52]. US images can show strong artefacts that may be inadequately handled by automatic segmentation software. As for computed tomography and magnetic resonance imaging, MRI is indicated superior to CT for soft-tissue segmentation for two reasons: (i) problematic radiation exposure, especially if multiple sessions are necessary, and (ii) the fact that CT provides only an indirect assessment of muscle quality (muscle density) [53]. Moreover, MRI shows the adipose tissue directly, which allows to detect even subtle variations in both muscle morphometry and tissue composition [54]. However, tissue morphometric analyses require image segmentation, and – depending on the localization – manual assessment can be necessary. It seems that for soft-tissue segmentation, the MRI assessment can give the most reliable results. Among the newest techniques, chemical shift-based water-fat separation methods, like the multipoint Dixon fat mapping MRI technique, are recommended for quantitative evaluation of fatty degeneration in patients with lumbar disc pathology [55–57].

All available data for LBP cases mainly concern the MRI assessment toward three major signs of muscle degeneration detected on imaging: (i) a decrease in the size of cross-sectional area of a muscle, (ii) a decrease in radiographic density, and (iii) an increase in the amount of fat deposits.

Cross-sectional area (CSA) of a muscle can be measured either by computer tomography, magnetic resonance imaging, or ultrasound. It has been proposed that the CSA measurement by means of MRI can be directly correlated with the clinical measure of muscle strength [58, 59]. The CSA can be measured either as total CSA or functional CSA. These two can be further used to calculate the atrophy ratio (functional CSA/total CSA) serving as an indicator of muscle composition. Side-to-side difference in atrophy ratio, CSA asymmetry (as a percentage), and fat CSA to total CSA ratio can be additionally measured [60]. It is difficult to point out the most appropriate spinal level for lumbar spine examinations because data vary significantly among studies. For a single-level CSA measurement, the L4–L5 vertebral space is recommended because it has been proved to be affected 6–9 times more frequently than any other spinal level [61]. Within a single level, the CSA should be measured at the center of the intervertebral disc, at the middle of the lamina, at the superior/inferior endplate, or at the center of the vertebral body. More precise but time-consuming is the cross section of up to 11 levels between L1 and S1 [48, 51, 60, 62, 63].

The second characteristic of muscle degeneration, namely, muscle density (MD), has been described either as a mean attenuation coefficient or as muscle fat infiltration (fat between muscle fibers and within muscle cells) [64–66]. Muscle density is associated with poor metabolic function and may be indicative of muscle function impairment [67, 68]. Additionally, MD measurement may also reflect the compactness of muscle fibers or the amount of protein within muscle and other potential soft-tissue elements not segmented from muscle such as tendons, blood vessels, aponeuroses, and fascia.

The third sign of muscle degeneration, namely, fatty infiltration and accumulation, may vary depending on the patient's state. It may also reflect the aging process or a late stage of muscular degeneration. Thus, for LBP cases relevant data should be interpreted separately for the lean tissue and fat. The most commonly recommended methods of fatty infiltration measurement in a noninvasive manner are MRI, MR spectroscopy, or US [51]. The methods used to assess fatty infiltration can be classified as either visual semiquantitative or quantitative measurement techniques [69]. The visual semiquantitative assessment of fatty infiltration can be simply graded by standard criteria used in adults – 0 (no fat), 1 (slight infiltration), and 2 (severe infiltration) – if present at one or more lumbar levels proposed in a commonly used five-point semiquantitative scale: (grade 0) normal, (grade 1) some fatty streaks, (grade 2) less than 50% fatty muscle, (grade 3) as much fat as muscle, and (grade 4) more fat than muscle [70, 71]. However, Kalichman et al. [72] adapted that scale and proposed a more quantitative assessment: grade 1, a normal muscle condition, fatty infiltration up to 10% of the muscle's CSA; grade 2, moderate muscle degeneration, 10–50% of fatty infiltration; and grade 3, severe muscle degeneration, >50% of fatty infiltration. Both methods provide a numerical scale for fat content, which favors MRI over CT for both acute and chronic LBP [69].

The fourth sign of muscle degeneration considered for the purpose of muscle atrophy assessment is muscle volume (MV). It has been claimed that the accuracy of the CSA measurement depends on the body area and does not always reflect MV. Although the CSA measurement is faster – and thus more widely used in research studies – its results are not always representative for bigger muscles [73, 74]. What is more, it is difficult to define and then reproduce the optimal level to carry out the measurement [75–78]. Thus, the CSA assessment seems quite good for erector spinae but not for pelvic muscles [75–78]. However, normative MV values for pelvic muscles are lacking. Moreover, similar MV of dominant and nondominant side (pelvis and lower extremity muscles) in healthy humans and symptomatic pelvic muscle atrophy for LBP $^{+}/_{-}$ leg pain have been confirmed [4, 79–82]. The MV measurement can be based on the MRI [73, 83], CT [74, 84], US [85, 86], or bioelectrical impedance analysis [87, 88]. However, once again MRI is the most commonly recommended. The MV measurement necessitates manual segmentation because of the pelvic muscle anatomy [89]. The manual MV measurement requires both more time to outline several CSAs and some practical training of raters to get reliable results. Currently, different methods are used, but the most commonly recommended for LBP cases is the method based on interpolation and deformation of a parametric-specific object [73, 83, 90–92]. It requires fewer axial images to assess

muscle geometry, thanks to parametric ellipses using basic dimension of muscle contours. The method has been used for gluteal, tensor fascia lata, and sartorius muscles, and its satisfactory accuracy using approximately 5–6 slices (average volume error of 2.4%) has been reported [89].

Fatty infiltration can be also measured by quantitative methods, and once again some proposals are available. The first one is the ratio of fat CSA to total CSA as an indicator of muscle composition (or fatty infiltration), and the second one is the signal intensity used as an indicator of fatty infiltration, where a higher mean signal intensity value reflects more fat content in the muscle [93–98].

For musculoskeletal applications, automated methods are commonly used [99–101]. However, automated methods are desirable if the contrast of the evaluated tissue is high or if the border between the two muscles is clear. Otherwise, the manual segmentation – which is susceptible to human error to a higher degree than automated or semiautomated methods – is required [102, 103]. Moreover, each measurement has to be conducted at least twice (repeated analysis) by the same rater (intra-rater reliability) or different raters (inter-rater reliability) [100]. The high-quality and reliable results of muscle atrophy assessment require anatomical preknowledge, appropriate muscle segmentation algorithm, and specific imaging modality, namely, two-dimensional or three-dimensional data sets with high contrast between the tissue classes and high spatial resolution to avoid partial volume effects with the low image noise [104, 105].

20.3 Current State of Knowledge About Muscle Atrophy and Low Back Pain Correlation

20.3.1 Paraspinal Extensors

The first muscles to be examined toward atrophic changes due to or leading to LBP were lumbar extensors. They are considered to be dynamic stabilizers, thanks to providing stability to the motion of spinal units. Muscle force imbalance may lead to kinetic instability of the spine or, e.g., changes in the orientation of the facet joint structures. Multifidus muscle (MF) has been an obvious choice for first observations of the link between muscle atrophy, lumbar spine degenerations, and LBP symptoms due to its unique feature – unilateral and segmental innervation pattern [106, 48]. It is known that the CSA of paraspinal muscles is symmetrical for the right and left side in normal (without LBP) individuals [107–109].

Additionally, it has been confirmed that all paraspinal muscles (except multifidus) with multisegmental innervation presented the maximum relative atrophy at the level below the pathology due to disuse or inflammation process [110, 111]. Quite uniquely, the subjects with disc pathology presented unilateral multifidus atrophy above the pathological disc [112]. Histological studies confirmed that disc herniation provoked MF changes of both sides. However, they were more severe on

the symptomatic side than on the opposite one. Both Type I (slow-twitch oxidative) and Type II (Type IIX/MHC-2X fibers, "fast-twitch glycolytic") fibers presented a symptomatic decrease in the size together with structural changes. Generally, a variety of pathological findings, such as fiber-type grouping, small angulated fibers, group atrophy, moth-eaten appearance, intermyofibrillar network irregularity on nicotinamide adenine dinucleotide tetrazolium reductase-stained biopsy specimens, and internal nuclei, has been confirmed [113, 114]. An assumption that multifidus atrophy appears to be level- and side-specific led to the development of studies focused on paraspinal atrophy measurement in relation to LBP and radiculopathy symptoms [4, 10, 22, 47, 115–117]. Additionally, localized spinal trauma, disc herniation, or spinal nerve lesion confirmed by electromyographic, histological, or radiographic measurements and their correlation with muscle atrophy coexistence have been examined [21, 34, 55, 112, 115, 118, 119].

The atrophic changes of MF have been confirmed in around 77–80% of LBP cases, especially at the L5–S1 level [4, 18, 47]. Next, different subtypes of LBP were investigated, and it has been confirmed that the facet joint osteoarthritis or spondylolisthesis can provoke muscle density changes on a specific level [69]. Some authors stated that the muscle atrophic changes depend on the duration of neural compression after disc herniation, which can influence segment-specific degenerative changes in lumbar multifidus and erector spinae [120]. That assumption was supported by some studies where the experimentally inflicted disc, nerve root lesions, and nerve root avulsion were followed by muscle atrophy [34, 121]. Then, it has been confirmed that both specific and non-specific LBP equally presented a decrease in both multifidus and paraspinal muscles in chronic LBP compared to healthy controls [122]. Additionally, it has been indicated that CSA among non-specific chronic LBP differs depending on a muscle and MF gets decreased, whereas erector spinae (ES) remains unaltered [14, 45–49]. Then, the meaning of the symptoms duration was checked, and muscle atrophy has been confirmed for acute, chronic, and recurrent LBP [22, 123, 24]. As it was expected, different CSA results were observed. The muscle size reduction in the acute phase was explained by disuse caused by pain or an inhibition along a long-loop reflex to protect impaired muscles at the symptomatic level [10]. For the chronic phase, there is generally a moderate evidence of MF decrease at different levels, but the CSA results of paraspinal muscles and the erector spinae muscle were less conclusive [14, 45–50]. It has been proposed that symptomatic muscle atrophy for chronic LBP is either caused by pain inhibition together with compensatory hypertrophy of the non-painful side or related to degenerative changes of the lumbar disc [124]. However, when the subjects were divided into chronic and recurrent LBP subgroups, the chronic one presented atrophy of the erector spinae, whereas the recurrent one did not. This allows to hypothesize that atrophy develops over some prolonged period of time or that muscle size recovery can be taking place during symptom remission [46, 51]. To summarize all the available data on the atrophy of paraspinal muscles for acute, chronic, and recurrent LBP, it seems that the results are conflicting and there is little evidence of the paraspinal lumbar muscle size and composition changes [23, 22].

It has been also proposed to check the utility of more precise observations of the relationship between specific vertebral levels and MF muscle atrophy, which confirmed lowered CSA mainly more caudally [14, 47–50]. Moreover, L5 atrophy was larger compared to L4, and it was explained by an anatomically bigger size of MF at the L5 level [14]. Thus, the atrophy of a bigger muscular mass is more visible and less questionable. What is more, the atrophy at one level may lead to local muscle weakness and spine instability, which can provoke further instability of the adjacent vertebral levels resulting in atrophy development.

Additionally, it has been indicated that the results can be influenced by the fact that some cases presented MF muscle CSA reduction and increased fatty infiltration on multiple levels, but side-specific in relation to chronic LBP symptoms [98]. Moreover, the situation is complicated owing to the fact that paraspinal muscle asymmetry can be observed among asymptomatic subjects. Thus, the idea that the level- and side-specific MF atrophy can be used as a marker for localizing the site of painful lumbar pathology has been questioned [10, 121, 125–128].

The next proposition for possible diagnostic utility of MF atrophy [47] considered a 10% or greater asymmetry in multifidus CSA as an indicator of potential spinal abnormality. Unfortunately, the fact that paraspinal muscle asymmetry greater than 10% is quite common in adults without LBP history limited the idea of a simple link between the results of muscle CSA asymmetry and pain or specific lumbar pathology [95]. Next proposal considered fatty infiltration. Some authors thought the following to be worth considering, namely, that conflicting or confusing study results can be related to common fatty infiltration at the lower part of the lumbar spine [18] or the occurrence of muscle pseudoatrophy observed within weeks after denervation. Moreover, fatty infiltration in the lumbar multifidus is common in adults and strongly associated with LBP [18]. However, the mechanisms of intramuscular fatty infiltration are not clear. Some meaning of the altered differentiation of fibroblasts after paraspinal muscle inflammation has been suggested [129]. Additionally, it has been confirmed that some fat replacement of erector spinae was associated with reduced intervertebral disc height [130].

Battie et al. [131] stated that MF atrophy measurement in patients with symptom duration of less than 6 weeks to localize specific lumbar disc or nerve root pathology should be focused on the MF composition, i.e., fatty infiltration, rather than CSA measurement. A similar observation was confirmed by Goubert et al. [132], who examined the influence of continued pain complaints on muscle structure and function. They confirmed a smaller fat CSA and a lower amount of fatty infiltration in recurrent LBP and noncontinuous chronic LBP compared to continuous chronic LBP without any differences in total CSA or muscle CSA. The authors stated that recurrent LBP, noncontinuous chronic LBP, and continuous chronic LBP are part of a complete spectrum of LBP complaints in which each subgroup is marked by different muscle characteristics [132]. Moreover, it has been posited that multifidus fatty infiltration should be associated with neural injury rather than lumbar stenosis and can be used as a prognostic factor of functional performance in spinal stenosis [48].

Currently, there are no studies reporting fatty infiltration of lumbar paraspinal muscles in acute non-specific LBP, and the data from chronic LBP remains conflicting. Some authors confirmed an increased fatty infiltration in MF [48] or ES [46], whereas others did not find it in any paraspinal muscles [14]. The same was confirmed for recurrent non-specific LBP, where for the subjects in remission, no fatty infiltration was revealed despite – interestingly – an increased muscle fat index, which can reflect an increased relative amount of intramuscular lipids in the lean muscle tissue [51].

Moreover, some authors underlined the need to consider age differences, which can lead to the misinterpretation of study results of fatty infiltration among LBP. Based on the comparison between younger and older LBP subject in reference to healthy controls, the authors stated that fat content increases with age [14, 45, 52, 93, 133].

Generally, it can be summarized that fat content is supposed to be a result of aging, long-lasting inactivity, or long-lasting LBP. Moreover, fatty infiltration is thought to be a sign of muscle atrophy [18, 20], but it should be underlined that the replacement of muscle with fat may not significantly alter muscle CSA [51].

20.3.2 Psoas Muscle

The multifidus muscle together with other paraspinal and trunk muscles plays an important role in lumbar segmental stability, for which the strengthening of deep and superficial stabilizer muscles and their co-coordination are necessary [134]. In lumbar segmental stability, hip muscles are also thought to be crucial. Among them, the psoas muscle as a significant hip flexor is of a particular interest. However, similar to MF atrophy measurement, contradictory results of psoas CSA in LBP patients are reported in the literature [23, 14, 9, 135]. It has been confirmed that any decrease in CSA can lead to a loss of proper biomechanics and thereby to LBP [110, 118, 136–138]. Interestingly, an association between facet orientation and tropism and the asymmetric parameters of paraspinal and psoas muscles in patients with chronic low back pain have been confirmed [139]. Next findings were that the psoas size is changeable and depends on the age and sex. Fatty infiltration and psoas CSA decrease for older patients, and a larger psoas relative CSA and a lower multifidus fatty infiltration among stenosis patients with high functional performance have been confirmed. Moreover, selective atrophy of multifidus and an increase in the CSA of psoas and abdominal muscles for patients with prolonged bed rest have been observed [140]. However, the nature of psoas atrophy as regards LBP is not clear, and longitudinal studies are needed to understand this relationship.

20.3.3 Gluteal Muscles

The gluteal muscles are three buttock muscles, namely, gluteus maximus, gluteus medius, and gluteus minimus, and the link between the atrophy of these muscles and LBP has been made by only a few authors [141]. The currently available results allow to hypothesize that muscle atrophy needs some specific conditions for development independent of the LBP occurrence. Firstly, the idea of a possible utility of gluteal muscle atrophy measurement among LBP cases was based on the studies which confirmed the weakness of these muscles, asymmetry in strength [26, 142, 143], as well as a different recruitment pattern of gluteal muscles during, e.g., prolonged sitting or standing [42, 141]. Additionally, the biomechanical studies showed that gluteal muscle impairment depends on its specific role and a certain type of activity can influence each specific gluteal muscle separately, e.g., gluteus medius is involved in pelvis stability, as well as trunk stability during running [143]. Interestingly, that muscle has been indicated among other gluteal muscles as the one of the most interest for the possible atrophy measurement among LBP cases. Some authors suggested that the gluteus medius muscle probably plays a diagnostic role due to its common atrophy in chronic LBP and thus that it could serve as a predictor of chronic non-specific LBP presence when compared to the controls. Moreover, gluteus medius weakness was noted among LBP pregnant women [144, 145]. However, it is unclear whether the initial gluteus medius muscle weakness is the cause or the consequence of LBP and how this observed dysfunction should be managed [146, 42].

The connection between the gluteus maximus muscle and LBP is based on the muscle's importance for load lifting from a fully flexed position [147–149]. Thus, this fact is commonly associated with LBP, where lifting is thought to be one of the important factors causing LBP, especially due to disc herniation [150–154]. Another hypothetical explanation is the connection of gluteus maximus via proximal attachment with thoracolumbar fascia, whose meaning for LBP development is widely known and both structures (muscle and fascia) are activated during spinal extension. Moreover, it has been confirmed that gluteus maximus has a tendency to fatigue among LBP patients compared to healthy subjects and alterations in gluteus maximus strength symmetry have been observed for women with LBP history [155]. The first studies concerning muscle atrophy measurement denied the occurrence of gluteus maximus atrophy among chronic LBP women [45]. However, the most recent data indicate that the atrophy of gluteal muscles is characteristic for more than 50% of LBP with leg pain cases and a certain variability of gluteus maximus CSA among LBP women depending on age and number of back pain-related medical visits has been confirmed [82, 81]. Although these studies indicate some meaning of gluteal muscles for LBP, it is unclear what proportion of LBP population as compared to healthy controls is involved, as well as which muscles are subject to atrophy. There is no data on gluteus

minimus atrophy among LBP cases, except for one study concerning LBLP cases [81]. Further research on gluteal muscle atrophy and its link with LBP, with subgrouping the sample into age, sex, and LBP subtypes, is recommended.

20.4 Current State of Knowledge About Muscle Atrophy Among Low Back-Related Leg Pain Subjects

Low back-related leg pain (LBLP) is considered among 23–57% of cases suffering from LBP. There are a few subtypes of LBLP given in the literature, e.g., lumbosacral radicular syndrome defined as sciatic neuralgia and atypical leg pain (also called pseudoradicular), motion segment, sacroiliac joint syndrome, or facet joint pain [156, 157]. However, all of LBLP subtypes have a very similar clinical picture but completely different pain mechanisms, which requires a different treatment approach. This situation causes lots of controversy and leads to failed therapies because the widely used diagnostic procedures such as neurological bedside examination, MRI, and Lasègue sign interpretation do not allow to objectively subclassify LBLP patients [158–162]. It has been posited that the MRI assessment toward muscle atrophy would have some diagnostic utility [4]. However, only a few studies concerning symptomatic muscle atrophy in patients with LBLP are available. Apart from Skorupska et al. [81], who were first to prove symptomatic pelvic muscle atrophy among LBLP cases, most of them were focused on the multifidus muscle [4, 163, 117, 34, 115].

The occurrence of MF atrophy related to nerve root denervation or dorsal ramus injury among LBLP cases has been confirmed and observed in 20–60% of cases [4, 23, 163–165]. More recently, when a particular spine level (L4–L5 and L5–S1) was considered, the atrophy has been confirmed in around 80% of subjects [4]. Although a significant correlation between lumbar MF muscle atrophy and leg pain (radicular and non-radicular) has been proved, the authors stated that there is no significant dependency between muscle atrophy and radiculopathy symptoms, nerve root compression, herniated nucleus pulposus, and a number of degenerated discs. Moreover, muscle degeneration was usually bilateral and multilevel, even in patients with a single nerve root irritation. Additionally, fatty infiltration and fibrous tissue replacement of multifidus were also shown to be associated with leg pain [4].

It seems that MF atrophy has little importance when it comes to diagnosing LBLP subjects. Firstly, it would be difficult to assess whether MF atrophy is due to LBP or LBLP. Secondly, asymmetric MF atrophy and bilateral and multilevel MF degeneration have been confirmed even in patients with a single nerve root irritation. This gives too much variance which could lead to questionable diagnostic conclusions. Thus, the confirmation of atrophy in other muscles related to different subtypes of LBLP could have a possible differential diagnostic value [10, 166, 14, 167, 22, 118, 47]. Similar to LBP, some links can be found between LBLP and pelvic muscles. The symptomatic side weakness of the gluteus and piriformis mus-

cles has been confirmed for pregnant women with pseudoradicular leg pain [144], as well in subjects with sciatic or sciatic-like pain [38, 80]. Interestingly, Skorupska et al. [81] confirmed that more than 50% of LBLP patients presented a smaller volume of the symptomatic side for gluteus maximus, gluteus minimus, and piriformis, but not for gluteus medius, which seems to be important for LBP. The results are quite valid due to a big sample, manual measurement, and muscle volume calculation not limited to CSA measurement only.

One of the possible explanations for symptomatic pelvic muscle atrophy can be the neurogenic type of atrophy due to nerve compression, which provokes metabolic changes in the sympathetic nervous system, then the metabolic activity of the musculoskeletal system, vasoconstriction, and – finally – atrophy [44]. That kind of muscle atrophy was confirmed for rats with neuropathic pain, which can develop in some chronic state cases of every neurogenic pain [168]. If the same can be confirmed for humans, it would be of great help because – due to a completely different treatment approach in LBLP – an objective tool for distinguishing neuropathic LBLP is nowadays indicated as the most important. The proportion of patients with neuropathic pain as a component ranges from 8% in patients with pain restricted to the lumbar area to 15% in patients with pain radiating proximally, 39% in patients with pain radiating below the knee without neurological signs, and 80% in patients with pain radiating toward the foot in a dermatomal distribution with neurological signs corresponding to typical radiculopathy [169].

Another possible explanation for pelvic muscle atrophy among LBLP patients can be inactivity due to changes in balance between muscle fiber apoptosis and regeneration [170, 171]. This type of muscle atrophy can be observed as a result of the patient sparing the symptomatic leg or due to an improper functioning of the muscles responsible for trunk and pelvis stability. With high probability, it can be observed among patients with lumbosacral radiculopathy, who commonly develop analgesic posture, or sacroiliac joint syndrome cases, where the gluteus maximus together with the quadratus lumborum has a crucial meaning for lumbopelvic stabilization [172, 142].

The results of healthy subjects suggest some diagnostic utility of muscle atrophy measurement among LBLP cases. The side-to-side comparison performed for gluteus group and piriformis muscles revealed nonsignificant differences under 1.24%, except for gluteus medius (3% and 2.61%; $p<0.05$) [81]. Additionally, it has been confirmed that the normative value of gluteus medius and gluteus minimus muscle volume has no age, gender, and dominant leg dependency [173]. It is not known what could possibly influence the gluteus medius results of the control group. Further studies concerning the reliability of MV measurement for gluteus medius and gluteus minimus of healthy subjects are necessary [81].

The meaning of the piriformis muscle for LBLP symptoms has been posited in two ways: firstly, due to the widely known and described piriformis syndrome, which is a pain state due to sciatic nerve entrapment, and – secondly – because of the importance of piriformis hyperactivity in the sacroiliac joint syndrome or myofascial pain involved in LBLP symptoms. There is no available data concerning the meaning of piriformis atrophy in LBP cases apart from the study by Skorupska et al.

concerning low back-related leg pain subjects, which confirmed piriformis atrophy for more than 50% of cases. However, neither the possible mechanism nor the clinical meaning of the observed atrophy is clear. The anatomical variation, changes of the sciatic nerve position relative to the piriformis muscle, as well as the muscle hypertrophy in MRI studies have been also presented [174–178]. Interestingly, the confirmation of the asymmetry in the size of the piriformis muscle has been suggested as a predictor of good surgery outcome for piriformis syndrome patients [179]. However, some authors reported symptomatic piriformis muscle atrophy and fatty infiltration dependent on the botulin toxin (BT) treatment, which correlated with both the number of BT injections and the timescale between the start of the treatment and the MRI examination. It has been suggested that the MRI measurement of piriformis atrophy and fatty infiltration may enable the prediction of a possible BT effect for piriformis syndrome symptoms and allow the assessment of the remaining muscle mass with a view to additional injections [180].

20.5 Summary

Generally, it should be remembered that the choice of applied techniques (US, CT, and MRI) to investigate CSA and fatty infiltration can influence the results of a study, e.g., magnetic resonance spectroscopy could reveal increased metabolic fat content, whereas conventional MRI using a semiquantitative visual grading system might not reveal such differences. Every technique has its disadvantages which should be considered when planning a study. Computed tomography is not good for muscle investigation due to its poor ability to differentiate soft-tissue types. The type of the applied MRI sequence is important for appropriate sensitivity of muscle measurement. Additionally, new techniques such as opposed-phase magnetic resonance, Dixon, and proton magnetic resonance spectroscopy should be considered because they allow to quantify fat fraction in tissues. The possible utility of the lumbar and pelvic muscle size measurement for LBP cases is concerned in three ways. First is for the diagnostic purpose as a new direction which would allow to subgroup LBP sample objectively. This is the most important thing that has been underlined by many authors involved in the LBP studies. The second aim of muscle atrophy measurement is to use it as a predictor of LBP occurrence. However, the data are conflicting, and some authors argue that neither muscle CSA nor fatty infiltration in the paraspinal musculature can be used as a predictor of future LBP, thus leaving a number of questions unanswered [60, 122, 181–183]. The third way to use muscle atrophy measurement is to complete the LBP treatment strategy and to observe therapy results.

However, there are no simple and reliable measurement methods, as well as high-quality research studies focused on the association between paraspinal and pelvic muscle degeneration, spinal pathology, and LBP. It is necessary to establish the norm with respect to sex, age, and perhaps some specific LBP subtypes. Then, it could be easier to identify pathological deviation in muscle degeneration param-

eters. Additionally, when study methodology is planned and study results are interpreted, all mentioned suggestions should be considered:

Suggestions for future studies:

1. There is a strong need to establish uniform methods for evaluating degenerative changes of paraspinal muscles.
2. It is important to check the role of paraspinal muscles in the development of LBP over different time periods and in different LBP and LBLP subtypes.
3. The relationship of the psoas and possibly quadratus lumborum muscles with LBP should be checked in case-control or longitudinal studies.
4. There has been limited investigation into the role of the size and fatty infiltration of all four paraspinal muscles.
5. Uniformly used MRI parameters are worth establishing by specifying the weighting or magnetic field strength.
6. The quantitative measurements providing greater precision and reliability than qualitative assessments should be favored in future studies.
7. The age should be taken into account as a confounding factor when investigating fat content.
8. Age should be used as a covariate in studies evaluating the association between paraspinal muscles, spinal degeneration, and LBP.
9. The patient population included in the study should be clearly defined as acute, chronic, or recurrent LBP and specific and non-specific types. Due to many different definitions, it should be clearly included every time in the group description.
10. The information about unilateral or bilateral LBP symptom occurrence should be provided. Hence, it is recommended that for unilateral complaints each side should be examined separately, and if pain occurred bilaterally, mean values of both sides should be averaged only if no significant side differences occur, which should be also reported.
11. For LBLP studies, the information about symptoms duration, level of leg pain, and possible pain mechanisms, especially neuropathic, is recommended.
12. In every study on unilateral pain, both symptomatic and asymptomatic sides should be considered.

Important facts for low back pain muscle measurement to be considered during result analysis:

1. Men have a larger CSA and higher density of paraspinal muscles than women.
2. Men show lower fatty infiltration in paraspinal muscles than women.
3. Paraspinal muscle CSA and density are higher in men than in women.
4. Younger individuals have a higher density than older ones.
5. Individuals with less weight have a higher density of paraspinal muscles than those who are overweight.
6. Women show greater fatty infiltration, regardless of weight or body mass index.
7. For adolescents, the visual assessment of fatty infiltration is unsatisfactory and should be interpreted with caution.

8. The amount of intramuscular fat significantly increases in the lower lumbar segments for the multifidus and erector spinae muscles compared with the upper lumbar segments.
9. Paraspinal muscle asymmetry >10% is commonly found in men without LBP history.
10. The subjects in a supine position (the most common for MRI) can present muscles with small amounts of flattening because of the body weight. In an upright position, the human body needs a minimum of muscular activity to stabilize the spine, which might affect the lumbar muscle size. Comparing study results where different examination positions are applied could lead to bias.
11. Both CSA and quality of paraspinal muscles decrease with age.

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Chapter 21
Drugs of Muscle Wasting and Their Therapeutic Targets

Kunihiro Sakuma and Akihiko Yamaguchi

Abstract Muscle wasting and weakness such as cachexia, atrophy, and sarcopenia are characterized by marked decreases in the protein content, myonuclear number, muscle fiber size, and muscle strength. This chapter focuses on the recent advances of pharmacological approach for attenuating muscle wasting.

A myostatin-inhibiting approach is very intriguing to prevent sarcopenia but not muscular dystrophy in humans. Supplementation with ghrelin is also an important candidate to combat sarcopenia as well as cachexia. Treatment with soy isoflavone, trichostatin A (TSA), and cyclooxygenase 2 (Cox2) inhibitors seems to be effective modulators attenuating muscle wasting, although further systematic research is needed on this treatment in particular concerning side effects.

Keywords Muscle wasting · Sarcopenia · Myostatin · Ghrelin · Soy isoflavone

21.1 Introduction

Skeletal muscle tissue accounts for almost half of the human body mass. Muscle contractions of the skeletal muscle enable to move the body and maintain homeostasis. Any deterioration in the contractile, material, and metabolic properties of the skeletal muscle has a marked effect on human health. Muscle wasting and weakness such as cachexia, atrophy, and sarcopenia are characterized by marked decreases in the protein content, myonuclear number, muscle fiber size, and muscle strength [1–3]. In addition, it is also associated with an increased risk of death. Muscle wasting elicits a poor functional status and reduces quality of life. Up to one-third of all cancer patients directly die because of cachexia and not from cancer. Different types

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of molecular triggers/catabolic factors such as pro-inflammatory cytokines and myostatin seem to involve muscle wasting [4, 5]. In contrast, several studies recently suggested a functional defect in autophagy-dependent signaling in sarcopenic mice and humans [6–8]. Such a condition accumulates the denaturing protein, and non-functional mitochondria eventually results in the atrophy of sarcopenic muscle fibers because of the deterioration of homeostasis.

To attenuate various forms of muscle wasting, many researchers have investigated exercise-based, supplemental, and pharmacological approaches. For example, the combination of resistance training and amino acid-containing supplements is thought to effectively prevent sarcopenia [9, 10]. In addition, myostatin inhibition for sarcopenic patients was successful in phase II trials [11], but the effect on muscular dystrophy is unclear [12]. The administrations of ghrelin and acetate megestate have shown good results against cancer cachexia [13]. The trial of an angiotensin-converting enzyme (ACE) inhibitor for chronic heart failure (CHF) patients is recommended [14]. Furthermore, recent studies [15, 16] indicated the possible application of novel supplements such as soy isoflavone and ursolic acid to prevent muscle atrophy in rodents. More recently, pharmacological treatment with fibroblast growth factor 19 markedly ameliorated two different types of muscle atrophy after aging and glucocorticoid treatment, probably via an obligate co-receptor for fibroblast growth factor 15/19, β-Klotho [17]. This chapter outlines several recent pharmacological approaches to inhibit muscle wasting.

21.2 Myostatin Inhibition

Myostatin, a potent negative regulator of muscle growth [18], was a novel member of the transforming growth factor-β superfamily. Mutations of myostatin can lead to marked hypertrophy and/or hyperplasia in developing animals. Severe muscle wasting in HIV patients and muscle unloading in mice and humans increase the amount of myostatin [19]. Muscle wasting also exhibits the increased level of myostatin [4]. Studies on sarcopenic muscles have yielded conflicting results [19–21], although many researchers consider myostatin levels to increase with age. Intriguingly, sarcopenic muscles of mice exhibit abundant Smad3 (possible myostatin-downstream regulator) protein but not myostatin [20]. More recently, muscle loading has been shown to elicit more abundant existence of myostatin in satellite cells of type II fibers in older than in younger males in spite of no difference in myostatin in satellite cells at the baseline [22]. Therefore, myostatin-dependent signaling may be activated in sarcopenic mammalian muscles. Although the adaptive changes in myostatin have yet to be clearly elucidated in the sarcopenic muscle, pharmacological myostatin inhibition is an intriguing strategy to attenuate sarcopenia. Treatment with a myostatin inhibitor (PF-354) seems to positively affect aged mice [23]. PF-354-treated mice for 4 weeks exhibited a significantly greater muscle mass and increased performance, such as habitual activity, distance to exhaustion, and treadmill time. Intriguingly, the PF-354-treated aged mice exhibited the decreased

amount of muscle ring finger 1 (MuRF1) and phosphorylated Smad3 in the muscle. In addition, their group [24] showed that a lower dose of PF-354 increased fiber size and force of the hind limb muscle. More recently, a randomized, phase two trial of a myostatin antibody (LY2495655: LY) was conducted using multinational individuals (e.g., Australia, Germany, the USA) aged 75 years or older [11]. This study investigated whether the subcutaneous injection of LY (315 mg) improves physical performance and increases the appendicular lean body mass (LBM). Becker et al. [11] demonstrated that treatment with LY for 24 weeks significantly improved several parameters of muscle power (fast gait speed, chair rise with arms, and stair climbing time) from the baseline in frail elderly subjects. Therefore, there is therapeutic potential of the antibody-directed inhibition of myostatin for treating sarcopenia.

Myostatin-inhibiting approaches have conducted in a variety models of muscle disorders such as cancer cachexia, amyotrophic lateral sclerosis, and Duchenne muscular dystrophy [25–27]. The approach of pharmacological myostatin inhibition was earnestly applied to attenuate muscle atrophy associated with DMD. Three months of weekly injections increased the muscle mass (~35%) and decreased serum creatine kinases to near normal levels [28]. Propeptide-mediated myostatin inhibition also significantly improved tetanic force production [29]. The success of myostatin inhibition in the mdx mouse model led to multiple clinical trials. Initial therapeutic strategies were aimed at systematically abrogating myostatin/ActRIIB signaling to ensure a widespread effect on the musculature. However, there are a lot of problems such as the efficacy, potential adverse side effects, and interference to non-muscle tissues of ActRIIB signaling. Clinical studies of Becker muscular dystrophy, limb-girdle muscular dystrophy, and facioscapulohumeral muscular dystrophy patients treated with a high-affinity myostatin binding antibody (MYO-029), intravenously every 2 weeks for 6 months, were discontinued after they did not improve the function or strength despite being well-tolerated [30]. Efforts to develop ACE-031, a recombinant pseudo ActRIIB receptor that improved muscle mass and whole-body strength in mdx mice [31], were finished because of dilated blood vessels, nosebleeds, and gum bleeding in boys with DMD. Subcutaneous ACE-031 dose trial every 2–4 weeks to ambulatory boys with DMD was not associated with serious or severe adverse events and demonstrated trends in pharmacodynamic effects on the body mass density and LBM [32]. However, this study was also discontinued due to safety concerns involving telangiectasis and epistaxis [32]. To minimize negative side effects, the uses of more highly specific antibodies to myostatin and a more direct approach (intramuscular injection) have recently been employed, with some positive effects [33, 34]. However, a therapeutic approach based on pharmacological myostatin inhibition may be very difficult for DMD patients particularly young boys, having a more active metabolism and being prone to the influences of the drug that are different to those in elderly people.

21.3 Testosterone

Testosterone increases muscle protein synthesis, and its effects on muscle are modulated by exercise and nutrition [35]. Application with testosterone improves sarcopenic characteristics such as decreases in the grip strength [37] and muscle mass [36]. A study of long-term treatment with supraphysiological amount of testosterone showed increased leg and arm strength and leg LBM [38]. Although testosterone supplementation has been shown to consistently increase whole-body and appendicular LBM [36, 38–40], the effects on physical function and muscle performance were contradictory in previous trials [39, 41, 42]. Storer et al. [43] hypothesized that such contradictory data from many previous trials are attributable to their relatively short duration, small sample size, and the heterogeneity of testosterone doses, regimens, and on-treatment testosterone levels. They recently demonstrated that testosterone replacement (7.5 g of 1% testosterone) in older men (> 60 years old) for 3 years significantly improved these parameters of stair-climbing power, muscle strength, power, and fatigability on conducting leg press and chest press exercise. Testosterone has been shown to positively regulate insulin-like growth factor I (IGF-I) [44], Wnt [45], and myostatin [46]. In addition, a 600-mg testosterone treatment of the elderly leads to an increase in the number of proliferating satellite cells possessing proliferating cell nuclear antigen and active Notch-1. The potential risks may outweigh the benefits, although high doses of testosterone significantly increase the strength among elderly males. Risks associated with testosterone therapy in older men include thrombotic complications, sleep apnea, an increased risk of prostate cancer, and increased hematocrit [47]. Novel, nonsteroidal compounds, called selective androgen receptor modulator (SARM), bind to the androgen receptor with differing levels of sensitivity compared with testosterone [48]. SARM has shown tissue-selective activity and improved pharmacokinetic properties and may be, theoretically, markedly safer than testosterone. The potential clinical utility of enobosarm (GTx-024), an orally bioavailable nonsteroidal SARM, was demonstrated at low doses in preclinical trials [49]. In a 12-week study, a 3-mg enobosarm dose-group showed increased total LBM and stair climb power in 120 healthy elderly men and postmenopausal women [50], with a similar frequency of adverse events such as headache, diarrhea, and pharyngolaryngeal pain between placebo- and enobosarm-treated patients. Dobs et al. [51] conducted a randomized, double-blind, phase II trial to assess the safety and efficacy of enobosarm using more than 150 male and postmenopausal female patients with cancer. After study termination (up to 113 days), significant increases in total LBM were noted from the baseline in both enobosarm-treated groups (1 or 3 mg once daily) [51]. However, in female patients with cancer, enobosarm did lead to a similar gain in LBM compared with a placebo [51]. Phase I trials using another SARM, LGD-4033, led to an increase in the muscle mass, but there was no effect on the fat mass in a 21-day short-term trial [52]. The POWER phase III trials of enobosarm (multicenter and multination) are ongoing involving subjects receiving first-line chemotherapy for non-small cell

lung cancer [53]. These full results will soon be published and will provide the clue of future anabolic trials.

21.4 Ghrelin

Ghrelin is mainly produced by cells in the stomach, hypothalamus, and intestines [54]. Ghrelin is a natural ligand for the growth hormone (GH)-secretagogue receptor that possesses a unique fatty acid modification. Ghrelin enables to enhance food intake and promote adiposity and to stimulate GH secretion. In contrast, ghrelin makes T lymphocytes and monocytes to suppress their production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β [55]. Attractive candidates for the treatment of cachexia are ghrelin and the agonists of the ghrelin receptor [56] because of their combined anabolic effects on skeletal muscle and the appetite. Three weeks of intravenous ghrelin (2 μg/Kg) to patients with chronic obstructive pulmonary disease elicits to significant increases in the handgrip strength, LBM, and Karnofsky performance score [57]. In addition, treatment with ghrelin (2 μg/Kg, twice daily) significantly improved LBM and the left ventricular ejection fraction in patients with chronic heart failure [58]. In a long-term (1 year) study participated by older healthy adults, an oral ghrelin mimetic (MK-677) significantly increased in appetite [57]. However, the study failed to increase the function or strength in the ghrelin-mimetic treatment group than those of the placebo group [58]. More recently, Pietra et al. [60] demonstrated that the oral treatment of rats with anamorelin HCL (ONO-7643), a potent and selective novel ghrelin receptor agonist [59], significantly increased body weight and food intake at all dose levels (3–30 mg/Kg) compared with the control. In addition, patients with non-small cell lung cancer and cachexia were conducted to phase 3 stages using two types of anamorelin [13]. Twelve weeks of treatment with both anamorelin for cachectic patients induced significant increases in LBM but not handgrip strength with negligible adverse effects (hyperglycemia <1%). However, the heterogeneity in the clinical effects of anamorelin is recently pointed out [61]. Therefore, further validation of this trial is necessary by varying the range of doses during treatment, increasing the sample size, and observing other outcomes.

21.5 Soy Isoflavone

Isoflavone is a flavonoid abundantly including in soybeans. Since the structure of isoflavone and estrogen is considerably similar, isoflavone exerts a physiological function similar to estrogen [62]. For example, muscle mass in ovariectomized mice increased by the supplementation of a high-fat diet with isoflavone [63]. Since long-term (120 days) supplementation with isoflavone for male mice inhibited fat accumulation in the skeletal muscle [64], isoflavone may also affect the skeletal muscle

in male mice. Unfortunately, many researchers have investigated the effect of isoflavone supplementation solely by evaluating the muscle mass and not myofiber size [65, 66]. Such a method would include some shortcomings because of a greater accumulation of fat and/or connective tissue in the atrophied tissue [67, 68]. It is possible that isoflavone's positive effect for maintaining muscle mass does not reflect maintenance of the myofiber size. In contrast, Abe et al. [69] investigated the effect of isoflavone treatment on muscular atrophy by evaluating the size of muscle fibers. In their study, higher amount (20% of diet) of supplementation with isoflavone has been shown markedly to inhibit fiber size of the tibialis anterior muscle but not the other fast-twitch lower limb muscles after denervation at 4 days. In addition, they indicated a significant increase of p-Akt and insulin receptor substrate 1 protein in the denervated muscle of mice after isoflavone supplementation. However, it is standard for in vivo supplementation with isoflavone to utilize amounts of >1% of the diet [63, 64]. The data of Abe et al. [69] is not of practical, since humans can't eat such high levels of isoflavone with each meal. More recently, our group [15] demonstrated that treatment with AglyMax (isoflavone aglycones) at lower amount (0.6%) attenuates the denervation-induced muscle fiber atrophy in mice. AglyMax seems to be absorbed faster and in larger amounts than those of glucoside in humans. This influence would be due to the decrease in apoptotic-dependent signaling.

21.6 Trichostatin A (TSA)

TSA is a popular inhibitor of class I and II histone deacetylase (HDAC). Acetylation/deacetylation of cellular proteins such as histone acetyltransferases and/or HDACs regulates muscle mass. Under atrophic conditions, this process becomes perturbed and causes the degradation of muscle-specific proteins [70, 71]. At first, Lezzi et al. [72] investigated TSA's functional role in in vitro myogenesis and the in vivo regeneration process. Analysis of the gene expression of myoblasts with exposed TSA indicated the marked elevation of myogenesis-linked molecules such as pRb, myosin heavy chain, follistatin, and muscle glycogen synthase. Intriguingly, such a TSA-dependent induction of follistatin is limited in C2C12 muscle cells but not C3H10T1/2 and NIH3T3 mouse myoblasts, osteogenic MC3T3-E1, and adipogenic 3 T3-L1 cell lines. Furthermore, muscles from animals treated with TSA show the increased production of follistatin and enhanced mRNA expression of regenerating markers (embryonic and neonatal myosin heavy chain) following muscle injury. In the denervated muscle, abundant HDAC4 proteins increase the atrogin1 and MuRF1 mRNA by downregulating the Dach2 level. Under this condition, HDAC4 protein accelerates the expression of myogenin, which creates a positive feedback loop and regulates HDAC4 expression [73, 74]. Bricceno et al. [75] demonstrated that under a denervation condition, TSA decreases atrogene's expression and controls the muscle mass by reducing the myogenin level and HDAC4 activity and promoting the Dach2 expression level [75]. TSA treatment improves the body weight and number and size of muscle fibers [76]. Forkhead box O (FOXO) is directly regulated by

acetylation and deacetylation processes. TSA may inhibit HDAC activity and inactivate FOXO, which attenuates contractile dysfunction and skeletal muscle atrophy [71]. In addition, TSA treatments of C2C12 myotubes under nutrient-deprived condition repress the FOXO target genes [microtubule-associated protein light chain 3 (LC3), MuRF1, and atrogin1] [71]. Similarly, TSA application of dexamethasone-induced atrophic mice significantly attenuates muscle atrophy [75]. More recently, treatment with TSA resulted in the downregulation of MuRF1 but not atrogin1 protein and markedly reduced the fiber size in the unloaded soleus muscle [77]. This atrophy-attenuating effect on muscle of TSA was not attributable to the changes of FOXO3a, although the data of FOXO3a protein was obtained using only crude homogenates of whole muscle. TSA's attenuating effect on muscle atrophy would be via MuRF1 irrespective of the upstream modulator (FOXO). Treatment with TSA increases the morphological and physiological potential in normal and dystrophic mice by induction of the follistatin. Indeed, the TSA-induced promotion of myoblast recruitment and fusion is blocked by treatment with recombinant myostatin [72]. However, unloading or TSA treatment for the soleus muscle seems not to induce myostatin gene expression or follistatin protein irrespective of markedly attenuating unloading-induced atrophy by TSA treatment [77]. In addition, treatment with TSA for tumor-bearing mice increased the follistatin expression without modulating the skeletal muscle mass [78]. These studies show that alteration of the myostatin/follistatin axis has no association or is not sufficient to protect the muscle mass specifically under unloading conditions or cancer-induced cachexia, respectively. Therefore, TSA treatment is not the same under diverse clinical settings.

21.7 Ursolic Acid

Ursolic acid is the major waxy component in apple peel. Since ursolic acid exerts beneficial effects in animal models of diabetes and hyperlipidemia, it is the active component of antidiabetic herbal medicines [79]. Kunkel et al. [80] demonstrated that ursolic acid reduced two different skeletal muscle atrophy-inducing stresses (muscle denervation and fasting). Intriguingly, the acute treatment of fasted mice with ursolic acid seems to reduce two atrogene mRNAs [80]. Chronic treatment of ursolic acid to unstressed normal mice induced muscle hypertrophy by reducing atrogin1 and MuRF1 mRNA. Supplementation with ursolic acid further activates the phosphorylating status of Akt in skeletal muscle in vivo [80, 81], but it is not still elucidated whether it directly influences skeletal muscle or not. Using serum-starved skeletal myotube model, Kunkel et al. [80] found that ursolic acid rapidly stimulated IGF-I receptor and insulin receptor activity. Importantly, ursolic acid alone was not sufficient to increase activation of the insulin or IGF-I receptor. Intriguingly, the augmented phosphorylation of p70S6 kinase by acute resistance training was maintained even after 6 h only when ursolic acid was injected immediately after exercise and not with placebo treatment [81]. Therefore, ursolic acid may enhance another pathway regulating muscle mass and not directly act on muscle fibers. On

administering a high-fat diet for 6 weeks, the continuous intake of ursolic acid (0.14% of total food) increased the skeletal muscle mass, muscle fiber size, distance run, and grip strength in mice [82]. More recent study conducted 3 weeks of administration with ursolic acid (100 mg/Kg) for a mouse model of chronic kidney disease (CKD) [16]. Ursolic acid markedly attenuated muscle atrophy induced by CKD by decreasing the expression of inflammatory cytokines and myostatin. Intriguingly, ursolic acid for CKD-induced atrophic muscle significantly suppressed the levels of phosphorylation of nuclear factor-kappaB (NF-κB, p65) and p38. These results clearly indicate anti-inflammatory property of ursolic acid. Since some researchers only investigated the possibility of supplementation with ursolic acid, further research is needed to more descriptively elucidate the effect of supplementation with ursolic acid on skeletal muscle and the attenuation of muscle wasting. Figure 21.1 summarizes the therapeutic action of both TSA and ursolic acid in muscle wasting.

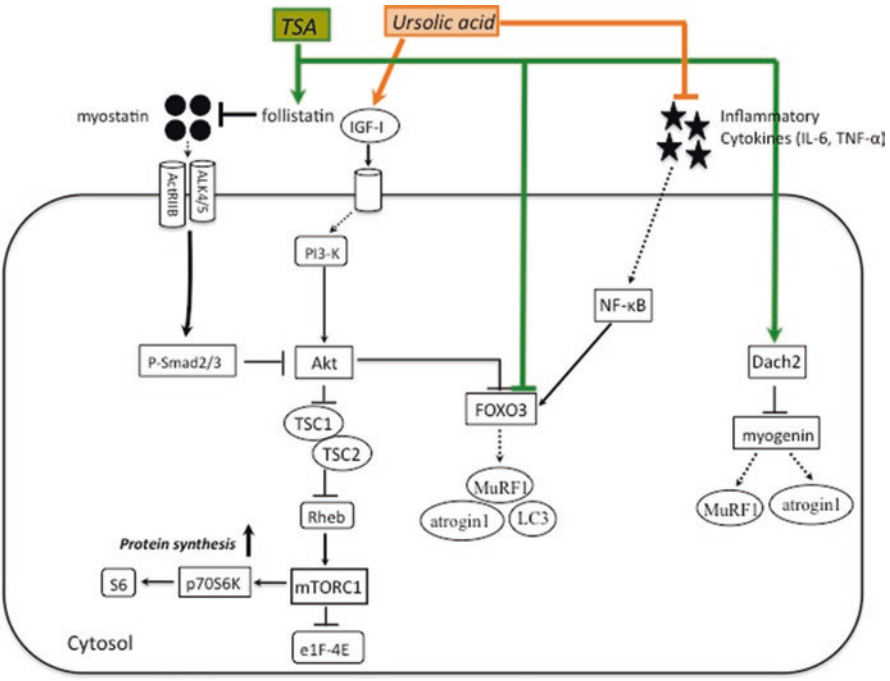

Fig. 21.1 Schematic representation of TSA and ursolic acid therapeutic action in muscle wasting. *ALK activin receptor-like kinase, ActRIIB activin receptor IIB, IGF-I* insulin-like growth factor I, *TSC* tuberous sclerosis complex, *TORC1* component of TOR signaling complex 1, *Rheb* Ras homolog enriched in brain, *mTORC1* mammalian target of rapamycin complex 1, *eIF4E* eukaryotic initiation factor 4E, *FOXO* Forkhead box O, *LC3* microtubule-associated protein light chain 3, *atrogin1* atrophy gene-1, *MuRF1* muscle ring finger 1, *TNF-α* tumor necrosis factor-α, *NF-κB* nuclear factor-kappaB

21.8 Angiotensin-Converting Enzyme (ACE) Inhibitor

Angiotensin II (Ang II) was firstly demonstrated in rats which caused a significant loss of body weight through increased proteolysis in the skeletal muscle and a reduction of food intake [83]. Ang II infusion decreases in IGF-I signaling and increases the rate of protein breakdown [84]. In Ang II-induced muscle wasting, levels of ubiquitin-conjugated proteins, expression of atrogenes, and 20S proteasome activity are robustly increased [85, 86]. ACE inhibitors have been used as a treatment for cardiovascular disease as well as secondary stroke prevention. ACE inhibitors would improve the muscle function through modulations in the metabolic and endothelial function, angiogenesis, and anti-inflammatory effects [87]. ACE inhibitors can increase IGF-I levels and mitochondrial numbers, thereby helping to counter many forms of muscle wasting [88]. Mechanisms of sarcopenia and cachexia are undoubtedly complex, and these processes are regulated by similar molecules but involve markedly different systems (TNF-α-NF-κB- and autophagy-dependent signaling are clearly different) [4, 5]. ACE inhibitors reduce the risk of weight loss in patients with cardiac heart failure [89]. The patients with CHF and CKD exhibit a two- to fivefold increase in plasma Ang II levels, in many cases, even in the presence of ACE inhibitory therapy [90, 91]. Circulating aldosterone and Ang II levels were elevated in despite clinically satisfactory ACE inhibition [91]. Ang II may act to reduce muscle mass in the elderly [92, 93]. The long-term utilization of ACE inhibitors may attenuate the decline in walking speed and muscle strength in older hypertensive individuals. This enlarges significantly lower limb muscle mass than users of other antihypertensive agents [92]. In both younger and older people with heart failure, ACE inhibitors improve the exercise capacity [92, 94], but they usually fail to improve the grip strength [95]. In functionally impaired older people, treatment with ACE inhibitors has been shown to improve some muscle performance test (6-min walking distance). However, nifedipine with ACE inhibitors in older people found no difference between treatments in terms of the muscle strength, functional performance, or walking distance [96]. Further evidence would be required before recommending ACE inhibitors to attenuate further atrophy in sarcopenia by using directly sarcopenic patients, not simple older people. Now, the effect of leucine and ACE inhibitors in sarcopenia (defined by European Working Group on Sarcopenia) is being investigated in a multicenter, masked, placebo-controlled, 2*2 factorial randomized trial [97]. The trial has recruited 440 patients from primary and secondary care services across the UK. Therefore, it is not clear whether ACE inhibitors improve sarcopenic symptoms. In general, frail subjects exhibit a tendency to have more cardiovascular problems and slower walking speeds. These agents are already commonly prescribed [98, 99], since ACE inhibitors are associated with cardiovascular benefits and, as older people frequently have underlying cardiovascular problems.

21.9 Cox2 Inhibitors

Cyclooxygenase (Cox) exists Cox1, Cox2, and Cox3. Cox2 exhibits pro-inflammatory actions and is induced by mitogens and cytokines in the skeletal muscle as well as in immune cells. Cox2 has both cyclooxygenase and peroxidase activities. Cox1 and Cox2 proteins would be affected differentially in the skeletal muscle after exercise. After acute resistance exercise (3 sets of 10 repetitions at 70% of maximum), the homogenates of young men (25 ± 1 year old) indicated that Cox1 protein levels were not altered at 4 and 24 h postexercise [100]. In contrast, this study showed that Cox2 protein levels were nearly threefold higher at 4 h and five-fold higher at 24 h postexercise, compared with pre-exercise. PGE2 and Cox2 are downstream effectors of cytokine activity [101, 102]. Preclinical and clinical trials strongly support the effective role of Cox2 inhibitors for the cancer cachexia [103]. Although many researchers use a variety of Cox2 inhibitors to inhibit PGE2 in diverse tumor-bearing mouse models, meloxicam and celecoxib have been widely used to study of Cox-2 induced muscle loss [101, 102]. Interestingly, celecoxib-treated cachectic patients with either neck and head or gastrointestinal cancer showed a marked improvement in the body mass index and quality of life [104]. To understand the safety and efficacy of celecoxib, a nonrandomized phase II study on cancer cachectic patients has been performed [105]. The treatment group showed a decrease in TNF-α and a significant increase in LBM along with improvement of the grip strength. The treatment with celecoxib for rheumatoid arthritis in cachectic rabbits showed reductions in the weight loss and levels of inflammatory IL-6 and NFκB [105]. Celecoxib may positively affect other types of cachexia, such as chronic obstructive pulmonary disease (COPD). In a cigarette-smoking rat model, celecoxib reduced the pulmonary inflammation and interalveolar wall distance by inhibiting serum nitric oxide production and inducible nitric oxide synthase in lung tissues [106]. A more recent study showed that significantly increased expressions of Cox2 existed in the lungs of patients with COPD and smoking controls compared with nonsmoking controls [107]. Interestingly, celecoxib (50.0 μmol/L) completely blocked Cox2 expression and apoptosis in vascular endothelial cells in vitro induced by cigarette smoke extracts [107]. Celecoxib has been utilized for other types of muscle wasting, with contradictory results. For example, celecoxib fails to slow the decline in the muscle strength, vital capacity, or ALS Functional Rating Scale-Revised, or motor unit number estimates, although it was well-tolerated and exhibited no apparent adverse effects [108]. Participants who received celecoxib-creatine twice daily for 6 months exhibited a more mild decline in ALS Functional Rating Scale-Revised than historical controls [109].

Meloxicam also inhibits the growth of murine adenocarcinoma tumors (MAC13, MAC16). Treatment with meloxicam has shown to inhibit the lipopolysaccharide-induced expression of Cox2 and atrogenes and markedly reduces the loss in muscle mass of rats [100, 111]. Cox2 pathway also regulates muscle wasting of chronic arthritis. This pathway has been shown to increase the TNF-α mRNA expression as well as inhibit the GH-IGF-I axis contributing to protein degradation. The application

with meloxicam attenuated muscle loss by preventing arthritis-induced atrogene upregulation in arthritic rats [113]. Figure 21.2 summarizes the therapeutic action of Cox2 inhibitors in cachectic muscle wasting.

21.10 Epigalocatechin-3-Gallate (EGCG)

Green tea is a popular beverage which can have benefits in endothelial cell lines [114] and cancer [115], as well as the skeletal muscle [115, 116]. The compound EGCG is occupied about 41% of the total catechins (flavonoid polyphenol) soluble in hot water [117]. EGCG has strong anti-inflammatory and antioxidant potentials, and it seems responsible for most of the health benefits linked to green tea. Using senescent rats (34 months old), Alway et al. [115] investigated to the effect of EGCG administration on atrophy and recovery processes of skeletal muscle after hind limb suspension. Although EGCG administration did not inhibit the fiber atrophy of muscles, this treatment selectively enhanced recovery of plantaris muscle fibers (EGCG, 2715.2 ± 113.8 μm^2 vs. placebo, 1953.0 ± 41.9 μm^2) but not soleus fibers. This enhanced recovery of the plantaris muscle is in part ascribed to the lower rate of apoptosis in the myonucleus by treatment with EGCG. In addition, treatment with EGCG may also suppress autophagy signaling by downregulating Beclin1 and LC3-II/LC-I protein abundance and promoting recovery of the plantaris muscle after unloading [118]. In contrast, based on monitoring nucleocytoplasmic movement of FOXO1-green fluorescent protein (GFP) in live skeletal muscle fibers, Wimmer et al. [119] demonstrated that the addition of EGCG causes a more moderate loss of nuclear FOXO1-GFP than those of IGF-I or insulin. These data indicate the role of EGCGs in the anti-atrophy of skeletal muscle fibers by blocking the ubiquitin-proteasome system. Although treatment with EGCG may be applicable

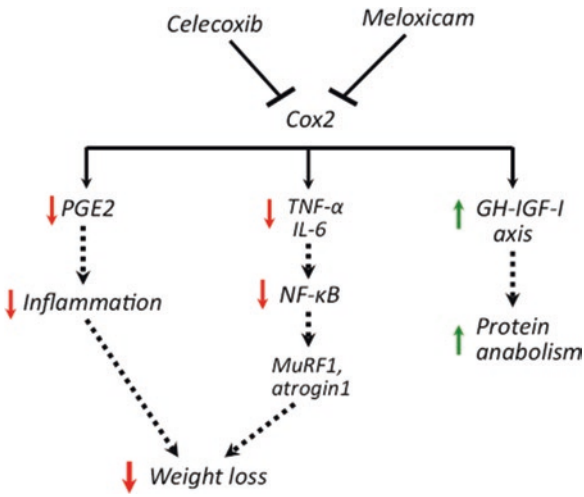

Fig. 21.2 Schematic representation of therapeutic action of Cox2 inhibitors in cachectic muscle wasting. *PGE2 prostaglandin 2, Cox2 cyclooxygenase 2, GH* growth hormone, *IGF-I* insulin-like growth factor I, *atrogin1* atrophy gene-1, *MuRF1* muscle ring finger 1, *TNF-α* tumor necrosis factor-α, *NF-κB* nuclear factor-kappaB

against muscle wasting in humans, almost all experiments using animals utilized gavage but not normal eating to evaluate of EGCG's effect. Since it is unusual for gavage to be applied to humans, an EGCG supplemental approach is needed. In fact, dietary EGCG and β-alanine in aged mice failed to show synergistic effects on several gene expressions (IL-6, superoxide dismutase 1, peroxisome proliferator-activated receptor gamma coactivator 1-alpha, sirtuin1, and IGF-I) on voluntary wheel running [120]. Therefore, the supplemental effect of EGCG should be further investigated based on normal ingestion and not gavage.

21.11 Conclusion

The recent advances in our understanding of muscle biology have led to new hopes for pharmacological, hormonal, and nutritional treatment of muscle wasting.

Supplementation with proteins (amino acids) only did not influence sarcopenic symptoms, although resistance training combined with amino acid-containing supplementation is usually recommended to prevent age-related muscle wasting and weakness [9, 10]. A myostatin-inhibiting approach is the most intriguing manner to prevent sarcopenia but not muscular dystrophy in humans.

Supplementation with ghrelin is also an intriguing candidate to combat sarcopenia as well as cachexia. Treatment with soy isoflavone, TSA, and Cox2 inhibitors seems to be effective modulators attenuating muscle wasting, although further systematic research is needed on this treatment in particular concerning side effects.

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Conflict of Interest Kunihiro Sakuma and Akihiko Yamaguchi declare that they have no conflict of interest.

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Chapter 22
Nutritional Support to Counteract Muscle Atrophy

Daniel John Owens

Abstract Malnutrition is an important factor contributing to muscle atrophy. Both underfeeding and obesity have negative consequences for the preservation of muscle mass and function. In addition, adequate nutrition on an exercise background is an efficacious strategy to counteract the severity of muscle loss associated with numerous clinical muscle wasting conditions. As such, significant research efforts have been dedicated to identifying optimal calorie control and the requirements of particular macro- and micronutrients in attenuating muscle atrophy. This chapter will explore current nutrition strategies with robust evidence to counteract muscle atrophy with a particular focus on protein, as well presenting evidence for other promising emergent strategies.

Keywords Protein · Amino acids · Food · Calories · Antioxidants · Vitamins

22.1 Background

In normal skeletal muscle, mass is maintained by a constant turnover of myofibrillar proteins through simultaneous synthesis and degradation. When synthesis rates decline or degradation increases such that muscle proteins are being degraded quicker than they are synthesized for a sustained period, muscle mass is lost. Many clinical strategies have aimed to alleviate the elevated degradation rates observed in pathological states; however, nutritional strategies are likely to be more effective in elevating muscle protein synthesis (MPS) than attenuating degradation rates.

Nutrition plays a crucial role in stimulating MPS, highlighted by the fact that the master regulator of protein synthesis, the mammalian target of rapamycin (mTOR) complex, can sense amino acids to increase its activity. The mTOR complex is a key regulator of cell growth in eukaryotic cells, promoting cellular anabolic processes including protein, pyrimidine, and lipid biosynthesis and inhibiting catabolic

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processes such as autophagy. Numerous upstream signals including amino acids converge at mTOR to stimulate protein synthesis. Crucially, resistance exercise appears to sensitize the muscle to amino acid feeding, and thus unsurprisingly, protein nutrition on a background of resistance exercise offers a potent stimulus for muscle anabolism. Dietary protein provides the building blocks, i.e. the essential (EAAs) and non-essential amino acids (NEAAs), necessary to sustain such increases in the production of new proteins mediated through mTOR signalling.

Despite the crucial role of dietary protein in maintaining muscle mass and permitting muscle growth (hypertrophy), reports suggest that just 33% of women and 50% of men meet the RDA (0.8 g/kg body mass/day) for protein [1]. Moreover, appetite, digestion and absorption of food is impaired in certain disease states; up to 50% of cancer patients report changes in eating behaviour at the time of diagnosis, leading to weight loss [2, 3]. This raises concerns for individuals suffering from muscle wasting conditions, whom are already rapidly losing muscle mass. In addition to protein intake, maintenance of an overall calorie balance through energy intake matched to energy expenditure is also important in maintaining muscle mass, and as such, this also directly implicates nutrition. The following sections of this chapter will describe current strategies for dietary protein intake to counteract muscle atrophy and will highlight the importance of energy balance with a smaller focus on emerging nutritional strategies that support muscle mass.

22.2 Dietary Protein and Amino Acids

Studies conducted over the past 30 years have demonstrated that amino acids stimulate MPS in healthy humans [4–6]. Importantly, it is the EAAs that appear to be critical for the amino acid-induced stimulation of MPS [5, 7]. In particular, the branched chain amino acid leucine acts as a potent ‘anabolic trigger’ capable of activating the mTOR complex [8, 9], which coordinates downstream signals to initiate the translation machinery and inhibits catabolic process, such as autophagy. At present, it is thought that leucine is sensed by mTOR via its ability to dissociate the negative mTOR regulator Sestrin2 from the positive mTOR regulator, GATOR [10]. Sestrin2 binds leucine with an affinity of ~20 μM in vitro, and Sestrin mutants lacking leucine-binding affinity are incapable of altering the concentration of leucine sensed by mTOR [10, 11]. It has been demonstrated that in certain populations with muscle atrophy, a leucine-enriched diet is necessary to stimulate the amino acid-induced MPS response [8]. Taken together, leucine is a highly important amino acid for maintaining and building muscle mass.

However effective leucine may be for *stimulating* MPS, isolated amino acids are unable to *sustain* increased rates of MPS. To achieve this, all of the amino acids are required implying that high-quality whole protein intake is necessary [12]. Moreover, in certain muscle wasting states such as bed rest or joint immobilization, anabolic resistance has been observed, i.e. the MPS response to amino acid administration is reduced. Although uncharacterized for a range of muscle wasting

conditions, it is likely that increased high-quality protein intake is necessary to offset increased rates of proteolysis, decreased rates of MPS and anabolic resistance to feeding seen in different pathological conditions.

22.2.1 Sarcopenia

From an ageing perspective, additional dietary protein may be warranted. In a large-scale study (n = 2066), dietary protein intake was assessed by using an interviewer-administered food-frequency questionnaire in men and women aged 70–79 years old [13]. Changes in total lean mass (LM) and non-bone appendicular lean mass (aLM) over 3 years were measured by dual-energy X-ray absorptiometry. Participants in the highest quintile of protein intake (1.1 g/kg/day) lost ~40% less LM and aLM than did those in the lowest quintile of protein intake (0.7 g/kg/day). Similarly, in a study of a heterogeneous group of 20 housebound elderly people (70–85 years) with chronic diseases, nitrogen balance was only achieved with protein intakes of 0.97 g/kg/day, whereas individuals with lower intakes (0.67 g/kg/day) were in a negative nitrogen balance [14]. To complicate matters, when protein is consumed as part of a mixed meal containing carbohydrates, the stimulation of protein synthesis is reduced in elderly people [15, 16]. The precise mechanisms for this are not known; however, it may be that digestion and absorption of amino acids are impaired in elders due to the presence of carbohydrates. Interestingly, this is not observed when protein is consumed with fats [17, 18].

Taken together, it is apparent that the RDA of 0.8 g/kg/day may indeed be insufficient to support the maintenance of lean mass in sarcopenic elders; however, a higher reference value is yet to be established.

22.2.2 Immobilization and Bed Rest

In situations of joint immobilization and bed rest, muscle protein is lost due to decreased rates of MPS, whereas degradation rates remain unchanged [19]. As such, increased protein intake may also offer some protection against muscle wasting by rescuing MPS rates. Stuart et al. show that higher amounts of dietary protein (1.0 g/kg bod mass) were effective in preventing muscle loss due to bed rest, whereas lower doses (0.6 g/kg) were insufficient to prevent such atrophy [20]. In other scenarios, leucine has been investigated as a potential supplemental strategy to offset atrophy during bed rest. In healthy middle-aged adults, 3–4 g leucine per meal partly protected leg lean mass during the first week of 14 days of bed rest [21]. Further evidence to support increased protein intake during bed rest is provided by studies showing anabolic resistance to amino acid feeding during disuse and immobilization [22, 23]. Simply reducing physical activity for 2 weeks has been demonstrated to reduce MPS in response to amino acids in elderly individuals [24].

22.2.3 Severe Cachectic States

In more severe cachectic states such as cancer, increasing dietary protein alone is not likely to outweigh the marked increase in muscle protein breakdown and maintain protein balance. In the few studies performed, total parenteral nutrition (feeding of a person intravenously, bypassing the usual process of eating and digestion) has typically resulted in increases in fat mass with inconclusive effects on lean mass [25]. However, some studies do suggest whey protein supplementation enriched with leucine can stimulate MPS in cancer patients. In a randomized placebo controlled trial, whey protein (40 g) enriched with leucine was capable of stimulating MPS in cancer patients compared to a conventionally used medical food, which was ineffective [26]. Notwithstanding such evidence, a more potent stimulus such as combining both resistance training and optimal protein nutrition is most likely to offer the best benefits to maintaining muscle mass as well as benefitting multiple other organ systems. Resistance-type exercise (RE) can increase rates of MPS for up to 48 h in healthy humans [27]. A number of studies demonstrate that RE is a positive treatment to support muscle mass in severe wasting conditions (where exercise is still possible) such as HIV. For example, structured resistance training results in marked improvements in both muscle strength (60% improvement in 1 repetition maximum strength) and size (5.3% increase in lean body mass) in patients with muscle wasting AIDS [28]. In healthy individuals, combining RE with high-quality protein intake stimulates and sustains MPS to a greater extent than either alone [29, 30]. Such evidence for a combined protein and RE treatment is lacking in severe cachectic states; however in one study, the effects of 14 weeks of whey protein supplementation vs RE vs combined whey and RE were examined in HIV patients [31]. Similar to earlier studies in healthy individuals, RE had a positive effect, but surprisingly there was no added benefit of whey. This finding could be explained by the fact that the whey group were advised to consume the supplement ad libitum and thus compliance cannot be certain.

22.2.3.1 Protein Timing and Distribution

It has been suggested that simply targeting a specific daily intake of protein may not be the optimal strategy to ensure individuals are maximizing the benefits of protein nutrition. Paddon-Jones and Rasmussen argue that a strong emphasis should also be placed on protein timing and distribution [32]. For example, when 20 g of whey protein is consumed every 3 h, this appears to be superior than pulsed (10 g every hour) or bolus (40 g twice a day) feeding patterns for stimulating MPS throughout the day. This suggests that the optimal distribution of protein intake on anabolic responses in skeletal muscle has the potential to maximize peak muscle mass [33, 34].

Overnight MPS rates are also understood to be limited by the level of amino acid availability. In combination with a progressive resistance training programme, protein provision prior to sleep can enhance gains in muscle mass and strength.

Recent studies investigating the impact of presleep protein ingestion suggest that at least 30–40 g of protein is required to display a robust increase in muscle protein synthesis rates during overnight sleep [35]. When combined with resistance exercise, 27.5 g of protein prior to sleep has been demonstrated to significantly improve the overnight MPS response and subsequently lead to increased lean mass and strength [36]. Taken together, pre-sleep protein can be an effective dietary intervention to improve overnight MPS.

A schematic representation of a suggested 'optimal' protein feeding strategy is highlighted in Fig. 22.1.

It should be considered that protein and amino acid supplements to counteract muscle atrophy are only effective if they also preserve or improve muscle *function*, i.e. there is little rationale for maintaining non-functional muscle mass. To this end, the efficacy of amino acid and protein supplementation alone for preservation of muscle function as well as mass is lacking. A number of studies have shown no change in muscle function in response to protein supplementation despite improvements in lean mass [37–40]. However, on a background of contractile activity (i.e. muscle contractions such as those experienced during resistance training (RT)), there is substantially better evidence in support of the efficacy of amino acid and protein supplements suggesting RT sensitizes the muscle to protein and promotes positive changes in muscle performance [29, 30]. This is a crucially important message, because stronger individuals are at lower risk of all-cause and cancer-caused mortal-

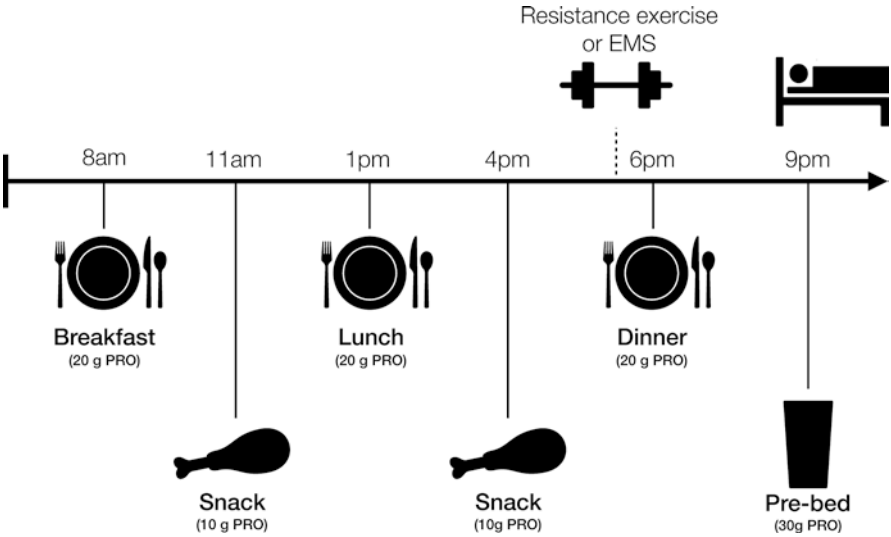

Fig. 22.1 An example meal plan for a 75 kg male aiming to meet a daily protein intake of approximately 1.2–1.4 g/kg. High-quality protein feeds that are rich in leucine should be evenly distributed throughout the day (approximately every 3 h) and in close proximity to resistance exercise (or electromyostimulation (EMS) where exercise is not feasible). A pre-bed feed of supplemental protein such as casein provides a source of slowly digested amino acids that may sustain amino acid uptake into circulation during sleep and enhance the MPS response to contractile activity

ity [41, 42]. Therefore, nutritionally strategies aimed to increase muscle mass should be considered in the context of exercise to yield the greatest health benefits.

22.3 Calorie Control

Both underfeeding and overfeeding are important considerations in muscle wasting conditions. During short-term immobilization due to injury, energy intake typically exceeds expenditure; however during longer periods of immobilization, there is an apparent energy balance [43]. Individuals who are calorie restricted during bed rest have an exacerbated muscle loss, highlighting the importance of energy availability in the maintenance of muscle mass. Unfortunately, there are less data characterizing metabolic rate and free-living energy balance in other muscle wasting conditions. Intuitively, it could be suggested that like bed rest conditions, other clinical conditions causing muscle atrophy would also be exacerbated in a prolonged calorie-restricted state.

On the other hand, by advising increased protein intake to support lean mass without reductions in other macronutrients, such as carbohydrates, overall calorie intake may exceed expenditure. Over time, this will lead to gains in fat mass, particularly if physical activity is reduced [44]. Therefore, careful consideration of the macronutrient composition in persons with muscle wasting conditions is crucial. It may be postulated that if minimal exercise can be performed and physical activity energy expenditure is low, the need for carbohydrates is largely reduced. This approach is yet to be investigated in clinical settings but may offer multiple benefits in more complex diseases such as cancer [45] and certainly in obese individuals as carbohydrate restriction can improve insulin sensitivity [46]. Similarly, oversupply of dietary fats leads to insulin resistance and impairs the MPS response to amino acid ingestion [47]. Energy balance should be the aim of macronutrient manipulation in individuals with accelerated muscle loss, with a larger portion of daily energy intake derived from proteins.

22.4 Dietary Antioxidants

Oxidative stress is thought to be a contributor to muscle loss with age and the production reactive oxygen species is well known to be elevated during prolonged immobilization and bed rest [48]. High levels of reactive oxygen species may inhibit protein synthesis and increase proteolysis [49, 50]. Consequently, researchers have aimed to establish whether targeted antioxidant treatments can scavenge the increased ROS produced in aged and immobilized muscle. There is both evidence in support and against the use of antioxidants as an effective treatment for disuse atrophy in humans.

Both vitamin E and vitamin E analogues have been widely investigated as antioxidant interventions to protect against disuse muscle atrophy. Vitamin E is a highly abundant, naturally occurring antioxidant. Vitamin E actually refers to eight structural isomers of tocopherols and tocotrienols, of which α-tocopherol is the best known and possesses the highest antioxidant capacity [51]. The majority of evidence that suggests vitamin E can reduce the severity of disuse atrophy has been derived from animal models. Several studies report that vitamin E either completely or partially protects immobilized rodent hind limb muscle from atrophy [52–56]. Despite the aforementioned findings that imply vitamin E can attenuate disuse muscle atrophy, the precise mechanisms underlying this are poorly understood. Many of the studies that have aimed to identify how vitamin E exerts these effects have shown changes in proteolytic gene expression and selected muscle proteins [56, 57]. In addition, it is not known whether vitamin E accumulates in appreciable amounts at the key sites of ROS production to be able to scavenge ROS to an appreciable degree. Therefore, it could be postulated that vitamin E exerts its effect through modulation of gene expression as opposed to through its scavenging capacity, although this is speculative at present.

The purpose of this chapter is to explore nutritional interventions, and therefore pharmaceuticals and nutraceuticals are not discussed. However, it is worth mentioning that numerous vitamin E analogues have been explored for their potential to ameliorate elevated ROS and exert beneficial effect on skeletal muscle. One such analogue that has received considerable research attention is Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a water soluble vitamin E analogue with direct ROS scavenging activity. Trolox appears to offer favourable effects in different models of atrophy and recently in models of sarcopenia [58–62].

To summarize, several studies have suggested that some naturally occurring antioxidants and their analogues have the potential to decrease inactivity-induced muscle atrophy of both limb and respiratory muscles. The use of antioxidants as a therapeutic intervention to protect against disuse muscle atrophy is still a preliminary idea. It is accepted that more research is required to uncover whether antioxidant treatments are safe and efficacious to help prevent inactivity-induced muscle atrophy. At the very least, individuals with muscle atrophy conditions should aim to increase their intake of antioxidant rich foods.

22.5 n3–PUFA

Omega-3 polyunsaturated fatty acids (n-3 PUFA), specifically n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are a group of nutrients that are known for their anti-inflammatory properties. The n-3 PUFA may also possess anabolic properties. Intake of 4 g/day of long-chain n-3 PUFA augments the MPS response to amino acids and insulin via mTOR-mediated mechanisms [63]. Moreover, in animal models of cancer, n-3 PUFA supplementation amounting to approximately 1–2% of total daily caloric intake has been shown to support whole-body protein

synthesis, whole-body protein net balance and muscle mass [64, 65]. These fatty acids occur naturally in nuts and oily fish like salmon, mackerel and tuna. However, the most comprehensive study to data suggests a minimum of 2-weeks supplementation with 5 g/day of fish oil capsules (providing 3500 mg EPA and 900 mg DHA) is necessary to permit detectable increases in muscle n-3 PUFA lipid composition. Taken collectively, these studies support the efficacy of n-3 PUFA as a promising adjunct to help support muscle mass in situations of muscle wasting.

22.6 Vitamin D

Vitamin D is a secosteroid hormone predominantly obtained in humans by exposure to ultraviolet B radiation (UVB; sunlight). Lack of sunlight exposure and predominantly indoor lifestyles have led to a large number of vitamin D deficiency cases worldwide (defined as <30 nmol/L 25-hydroxyvitamin D or 25[OH]D) [reviewed recently in 66]. The classical function of vitamin D is its role in Ca^{2+} homeostasis and thus bone mineralization [67]. It is now understood that the biological effects of vitamin D are much wider than Ca^{2+} homeostasis. As skeletal muscle expresses the vitamin D receptor [68], and following generation of a vitamin D receptor knockout mouse that harbours muscle abnormalities [69], great attention has been drawn to the potential for vitamin D to influence muscle health. Research has shown that vitamin D deficiency is associated with sarcopenia in some populations [70] and associates with increased fall risk in frail elders [71]. Meta-analyses suggest that individuals with vitamin D concentrations <25 nmol/L may show improved proximal strength when supplemented with vitamin D_3 to correct their vitamin D status. In young healthy populations, improving vitamin D status with a supplemental form of vitamin D_3 also augments resistance training adaptations [72].

Given that vitamin D plays numerous roles in tissues other than muscle and that deficiency is highly prevalent, it is important that individuals with muscle wasting conditions are screened for their 25[OH]D status, which can be easily corrected with moderate daily doses of vitamin D_3 (2000 IU/day) [reviewed in 73]. Current guidelines set by the US Institute of Medicine suggest that 25[OH]D concentrations <30 nmol/L are considered deficient and concentrations <50 nmol/L are inadequate [74]. Therefore, best practice should currently be considered to maintain serum 25[OH]D concentrations >50 nmol/L.

22.7 Summary

In summary, nutrition plays a pivotal role in the preservation of muscle mass in normal and pathological conditions. In the simplest sense, total caloric intake will largely determine weight loss or gain. More specifically, the protein contribution to overall caloric intake appears to be a key factor affecting muscle protein balance. When coupled with exercise, protein intake is a potent stimulus for muscle growth. It is likely

that a resistance exercise and nutrition strategy will yield the greatest benefits since dietary interventions alone may preserve muscle mass but not function, whereas a combination of the two may confer benefits to both. Where possible (i.e. depending on the severity and the cause of the muscle atrophy), a regime of evenly distributed high-quality protein intake (of approximately 20–30 g servings of protein) that is rich in leucine and separated by approximately 3–4 h throughout the day is a good starting point. Consuming protein close to RT or stimulated contractile activity and ingested also before sleep will stimulate the greatest MPS response. Additionally, a diet rich in antioxidants (particularly vitamin E) and oily fish will at worst confer benefits to global health and at best may also contribute to attenuating muscle atrophy.

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Chapter 23
Nutritional Considerations in Preventing Muscle Atrophy

Sanda Maria Cretoiu and Corina Aurelia Zugravu

Abstract Muscle atrophy may occur under different circumstances throughout a person's life. These conditions include periods of immobilization of a limb or of the whole body and aging accompanied by the onset of sarcopenia. Muscle mass is reduced as a result of decreased protein synthesis or increased protein degradation. Most studies aim to prevent the degradation of muscle proteins, but the way in which protein synthesis can be stimulated is often neglected. This study will provide an up-to-date review regarding nutritional considerations and resistance exercise countermeasures in the prevention of muscle mass loss and recovery of muscle mass in muscle atrophy secondary to immobilization or in sarcopenic obesity. We do not address muscle atrophy in disease states associated with inflammation (rheumatoid arthritis, COPD, cancer cachexia, AIDS, burns, sepsis, and uremia) which are governed by particular mechanisms of muscle loss.

Keywords Muscular atrophy · Muscle disuse · Sarcopenic obesity · Nutrition · Protein turnover

23.1 Short Overview

There is more and more talk about the concept of quality of life. However, nutrition is an overseen factor because diet quality and dietary strategies for health promotion could greatly influence the quality of life. Skeletal muscle mass is of great importance for health status and its critical for a healthy life.

Muscle atrophy is defined as a weakening, shrinking, decrease, and loss of muscle mass. The most used synonyms to describe muscular atrophy are muscle waste,

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muscle loss, muscle catabolism, and muscle withering. There are several causes of muscle atrophy, from short periods of muscle disuse to neurogenic atrophy.

In general, muscle atrophy has negative health consequences such as low strength [1]; compromised metabolic health, due to a decline in basal metabolic rate [2]; the development of insulin resistance [3]; and accumulation of body fat [4]. Around the age of 40, human muscles undergo continuous transformation, the most relevant being muscular atrophy [5]. Accentuated muscle mass and strength/performance loss is known as geriatric sarcopenia which unfortunately remains frequently overlooked and undertreated, contributing to a poor quality of life [6].

This chapter focuses on the role of nutrition in promoting a healthy recovery of the impaired functional capacity of skeletal muscle atrophy in young and older adults. It also highlights the necessary tools for nutritional screening and nutritional assessment which underpin recommendations for improving this condition.

23.2 Disuse Muscle Atrophy

23.2.1 Introduction

Changes in muscle mass and quality, besides altering the muscle strength and its functional capacity, have repercussions in the metabolism of the macronutrients carbohydrate, fat, and protein [7]. During lifetime, one experiences situations which require short or long periods of physical inactivity (e.g., rehabilitation after injury, mobility limitations of limbs, recovery from illness) even in previously healthy and young individuals [8]. It was demonstrated that even short periods of muscle disuse (5–14 days) could cause substantial loss of skeletal muscle mass and strength [9]. Muscle atrophy in young, mature, and aging individuals derives from a loss in muscle protein resulting from increased protein degradation and decreased protein synthesis when patients are immobilized for a certain period, due to reduced respiratory muscle activity during mechanical ventilation, or is found in the absence of gravitational load during space missions (unloading) [10, 11]. Also, the loss of skeletal muscle mass and the fiber cross-sectional area is frequently seen in patients with limb and joint immobilization due to fractures or arthritis, in patients with spinal cord injury, or in patients requiring prolonged bed rest or admitted in ICU (*i*ntensive *c*are *u*nit) [12]. To study the causes that can lead to muscle atrophy in humans, animal models are used as depicted in Fig. 23.1. Usually, the volume and cross-sectional area changes during disuse atrophy are determined in humans by magnetic resonance imaging (MRI) [13].

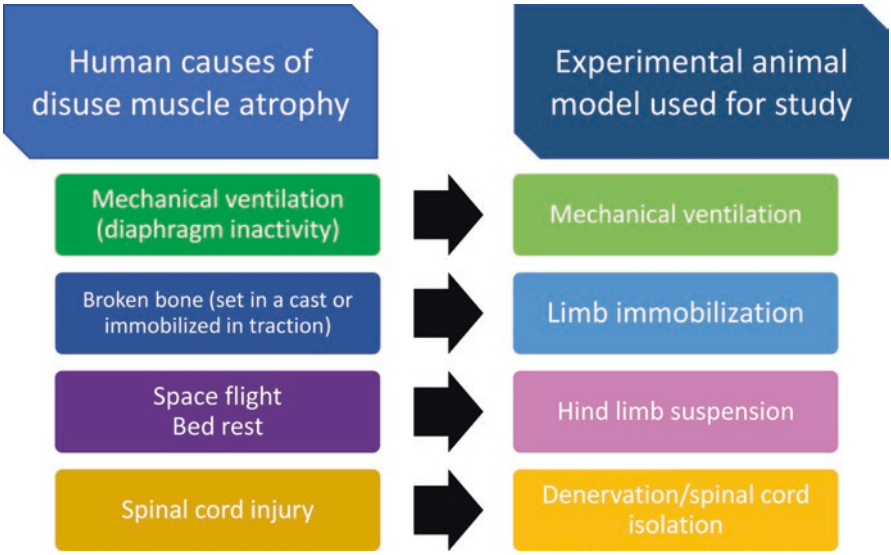

Fig. 23.1 Diagram depicting the most frequent causes of muscle atrophy in relation to the animal models used to study them

23.2.2 Nutritional Strategies During Muscle Disuse

Skeletal muscles represent a source of amino acids that are mobilized in stressful situations, and if muscle protein breakdown exceeds muscle protein synthesis, muscle wasting occurs. Hence, muscle protein turnover is essential for the maintenance of muscle mass during prolonged inactivity or unloading. It is believed that proper protein synthesis could prevent loss of muscle mass. Maintaining the muscle mass during immobilization and restoring it after disuse involve processes dependent upon protein and cellular turnover. To ensure ideal protein synthesis, a proper caloric intake may be a useful strategy for mitigating muscle loss during muscle disuse [14]. A deficient diet which does not preserve energy balance has been shown to decrease protein synthesis by ~20% [15], and therefore an adequate protein intake must be provided during muscle immobilization. Muscle waste cannot be completely abolished, but a protein intake must be maintained at a high level (1.0–1.2 g per kilogram per day) [16] to attenuate as much as possible the disuse atrophy [17]. Moreover, it was shown that protein or essential amino acid ingestion stimulates rates of muscle protein synthesis in a dose-dependent manner [18]. However, a study by Dirks et al. concluded that "dietary protein supplementation (~20 g twice daily) does not attenuate muscle loss during short-term muscle disuse in healthy older men" [19].

23.2.2.1 Amino Acid/Protein Supplementation

Protein and/or amino acid supplementation is considered to be effective in increasing muscle anabolism during extended periods of immobilization and preventing muscle atrophy [20]. Stuart et al. evaluated if a diet with high protein content might have a positive impact on bed-rest-related protein catabolism and concluded that "bed rest does not cause an increase in whole-body-protein breakdown, but decreased whole-body-protein synthesis is demonstrable when dietary protein is low." They suggested that increasing dietary protein will prevent a decrease in muscle atrophy during disuse [21].

23.2.2.2 Essential Amino Acid Supplementations

Availability of essential amino acids (EAA) has been shown to stimulate muscle protein anabolic response [22], partly through activation of mTORC1 (rapamycin complex 1) signaling [23]. Downstream of the mTORC1 signaling pathway, the expression of several transporters (LAT1, CD98, SNAT2, and PAT1) was shown to be rapid and transiently upregulated following EAA ingestion in humans [24]. Among EAA, extra leucine might regulate and stimulate specific intracellular pathways associated with muscle protein synthesis [25, 26]. Paddon-Jones et al. reported that a total dose of 49.5 g of EAA per day (divided into three intakes of 16.5 g of EAA each containing 3.1 g of leucine) might prevent a quantifying decline in muscle mass during 28 days of bed rest in healthy subjects [27].

A study comparing muscle protein metabolism in elderly and young individuals found that the elderly are less responsive than the young individuals to the ingestion of EAA [28]. In vivo, evidence that in elderly humans small boluses of leucine improve muscle protein retention was brought by Katsanos et al., who showed that 26% Leu in a mixture of EAA could reverse an attenuated response of muscle protein synthesis [29]. Their study also demonstrated that young individuals' muscle protein synthesis was improved following the EAA ingestion independent of the leucine concentration in their blood [29]. Brooks et al. demonstrated the efficacy of combined resistance training with EAA supplementation, which attenuated the losses in muscle mass, and strength as a countermeasure against muscle wasting during 28 days of bed rest and energy deficit [4]. Two years later, a study by the same team, regarding dietary manipulation alone (EAA) or in combination with resistance exercise, showed that muscle atrophy was less influenced among participants who received only EAA, compared with those who received EAA and exercises [30]. Furthermore, Dreyer at al. found that resistance exercise and ingestion of EAA with carbohydrates enhance muscle protein synthesis to a greater degree than either stimulus alone, by enhanced activation of the mTOR signaling pathway [31]. Supplementary attention must be given in the future to unravel the molecular mechanisms responsible for how EAA enhances muscle protein synthesis and their importance in muscle protein anabolism.

23.2.2.3 Branched-Chain Amino Acids (BCAAs)

Leucine, valine, and isoleucine are known to have a unique capacity to stimulate muscle protein synthesis, and they are frequently used as nutritional supplements. Louard et al. showed that BCAA intravenous infusion not only fails to increase the rate of muscle protein synthesis in human subjects but actually reduces the rate of muscle protein synthesis and muscle protein turnover [32], while a very recent review of the literature concluded that dietary BCAA supplements alone do not promote muscle anabolism [33]. However, it seems that amino acids from the diet are more effective in preventing disuse atrophy than those in food supplements [34]. Sundström recently demonstrated the improvement in whole-body net protein balance from a supplemental intravenous amino acid infusion to ICU patients [35]. Martin et al. have shown that whey diet promoted a faster recovery of muscle functional properties as compared to the casein diet during immobilization [36]. In rat animal models, it is accepted that BCAA stimulates muscle protein synthesis rate [37]. Oral BCAA administration (600 mg/kg/day, 22.9% L-isoleucine, 45.8% L-leucine, and 27.6% L-valine) in Sprague-Dawley rats protects against microgravity- and immobilization-induced muscle atrophy via the inhibition of the Ub-proteasome pathway responsible for the expression of atrophy-related genes [38].

23.2.2.4 Other Amino Acids

Taurine, a natural amino acid, is a known potent antioxidant due to its contents of sulfonic acid and for its claimed effects as an energizer. Frequently used as supplement cocktails for athletes, taurine has the ability to control muscle metabolism and gene expression, and it was proposed to improve resistance and recovery by an effect which increases the amino acid levels in skeletal muscle [39]. Ghandforoush-Sattari et al. studied the pharmacokinetics and effects of oral administration of taurine in healthy volunteers, using a daily dose of taurine of 4 g [40]. Although there are few studies on humans, the findings about the importance of taurine in animal models of skeletal muscle atrophy cannot be overlooked. Khalil et al. concluded in their study that "taurine may be helpful to counteract apoptosis and up-regulated MuRF1 gene expression related to muscle atrophy" [41].

In the literature, there is a limited number of studies that relate to the beneficial effects of cysteine supplementation on muscle atrophy. An in vitro study on cultured myotubes was recently performed by Dutt et al. and suggested the positive effects of S-allyl cysteine (SAC), an active component of garlic (*Allium sativum*), on alterations which appear in protein metabolism during muscle atrophy [42]. Another study showed the beneficial effects of a cocktail of amino acids (cysteine, threonine, serine, aspartate, asparagine, and arginine), which spared muscle protein catabolism and muscle wasting during infection in rats [43].

23.2.2.5 Oral Creatine Supplementation

Therapeutical applications of creatine as a popular "ergogenic" supplement were analyzed by Derave et al., who concluded that a short-term (less than 2 months) and discontinuous creatine supplementation might have a positive effect on muscle function [44]. Although there are numerous speculations that creatine supplementation could lead to an increase of lean body mass in active individuals, during short muscle disuse (7 days leg immobilization), type I and type II muscle fiber showed no net changes during and after creatine loading, as demonstrated by Backx et al. [45]. However, creatine supplementation during resistance training of older adults enhances energy stores, including phosphorylcreatine and glycogen. This allows a better buffering of ATP during high-intensity exercise as showed by Chilibeck et al. in their meta-analysis [46]. A study by Hespel et al. investigated the effect of oral creatine supplementation (20 g down to 5 g daily) on muscle volume and function during leg immobilization and rehabilitation and concluded that it stimulates muscle hypertrophy during rehabilitative strength training. The effect seemed to be mediated by a creatine-induced change in MRF4 and myogenin expression [47]. However, despite some promising results, there is a long way until we can assert with certainty that oral creatine supplementation represents a good nutritional intervention strategy to prevent muscle atrophy during disuse.

23.2.2.6 Antioxidant and Anti-inflammatory Supplementation

Muscle protein synthesis is influenced, among other factors, by oxidative stress and inflammation, which are associated with immobilization [48, 49]. Since both factors are leading to increased proteolysis and muscle atrophy during periods of prolonged disuse, it was considered that antioxidant supplementation might represent an effective countermeasure for this condition [48, 50]. More than 25 years ago, muscle inactivity was correlated with increased muscle lipid peroxidation [51], and particular attention has been given to its prevention with the antioxidant vitamin E [52, 53]. The first study using vitamin E, selenium, ascorbic acid, β-carotene, coenzyme Q10, N-acetyl-L-cysteine, and catechin as antioxidants concluded that antioxidant supplementation did not attenuate the disuse atrophy [54].

There are findings which indicate that the protective effect of vitamin E is due to a non-antioxidant mechanism, which involves the modulation of muscle proteolysis-related genes such as μ-calpain; caspase-3, caspase-9, caspase-12; and two atrophy-related ubiquitin ligases (MuRF1 and MAFbx) found to be upregulated by vitamin E. The same study of Servais et al. showed that vitamin E failed to modify markers of oxidative stress (GSH/GSSG, SOD, GPx, CAT, UCPs) and partly prevented the decrease in type I and IIa fiber size, thus relatively preventing muscle atrophy during unloading [53].

Reactive oxygen species (ROS) are major signals involved in muscle homeostasis and play an important role in muscle atrophy associated with decreased levels of neuromuscular activity [49]. Astaxanthin is an antioxidant belonging to a group of

chemicals called carotenoids, which might ameliorate muscle atrophy in combination with intermittent loading, by preventing the overexpression of ROS [55]. From the same group of antioxidants, orally administered micelle with β-carotene, a dietary source of vitamin A (0.5 mg once daily), for 2 weeks, to mice, were reported to have chemopreventive effects in an early stage of muscle atrophy by repressing the expressions of Atrogin-1, MuRF1, USP14, and USP19 [56].

These results evidently exemplify the antagonistic findings related to the role of antioxidant treatments in preventing disuse muscle atrophy.

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenolic phytoalexin which has been shown to reduce oxidative stress, restore mitochondrial function, and promote myogenesis and hypertrophy in vitro [57]. A study on experimental rat models demonstrated that although resveratrol appears to have modest therapeutic benefits, it increased the fiber cross-sectional area of type IIA and IIB fibers in response to reloading after hind limb suspension [58]. In another study, rats affected by mechanical unloading were treated with resveratrol supplements, in a dose equivalent to 400 mg/kg, for 6 weeks (4 weeks before unloading and during the 2 weeks of unloading). Resveratrol was shown to maintain a net protein balance and preserve muscle mass and muscle maximal force contraction by acting as an exercise mimetic [59].

Green tea polyphenols have been regarded as substances with antioxidants, antimutagenics, antidiabetics, anti-inflammatory, and anti-obesity properties [60, 61]. Under this generic name, several active substances, extracted from the leaves of the *Camellia sinensis* plant, are pooled: epigallocatechin gallate, epicatechin gallate, gallocatechin, and epigallocatechin [62]. It was shown that tea catechins prevent contractile dysfunction in skeletal muscle and muscle atrophy in unloaded muscle due to the lower oxidative modification of myofibrillar protein through the antioxidant activity [63]. Alway et al. recently suggested that green tea extract attenuates muscle loss and improves muscle function during disuse and appears to be most effective for muscles that have a high percentage of fast (type II) fibers. This study, performed on rats, provides a rationale for conducting a clinical study on the effects of green tea extract on muscle atrophy [64].

23.3 Sarcopenic Obesity

23.3.1 Introduction

The global population, although having a higher life expectancy, is struggling with obesity more and more. At the confluence of these two global trends, one can find the serious problem of sarcopenic obesity. It has been assessed that its prevalence is of 20% in seniors, though difficulties in defining the disease and incorrectly evaluating it might distort the figures [65]. Though sarcopenic obesity is usually a problem of old age, it might also affect younger obese adults. However, its identification is

limited due to the availability of low but affordable accurate body composition evaluation techniques but also due to heterogeneity in diagnostic criteria [66]. A high number of body composition indices and cutoffs were used to define sarcopenia and obesity. This leads to conflicting results regarding its prevalence and risk prediction [66]. The majority of studies have focused on sarcopenic obesity in older adults, and the prevalence in younger obese adults is yet to be defined. However, we might expect more studies in the future in this area, since the prevalence of class 3 obesity and of sarcopenia are on the rise [67].

23.3.2 Definition and Evaluation of Sarcopenic Obesity

The term "sarcopenia" originates from two Greek words, "sarco" which means flesh and "penia" which means loss [68, 69]. In the beginning, "sarcopenia" was the term used to explain for the loss of muscle mass accompanying aging and being regarded as a physiological process [70], but older adults tend also to gain fat, sometimes developing obesity as they age. National survey data from the USA, published in 2014, showed that more than one-third (35%) of American older adults are obese [71]. In the meantime, we witness a steady growth of the lifespan, hence a rapid augmentation of the elderly population, resulting in a sum of potential health hazards related closely to the simultaneous rise of the fat tissue and the loss of muscle mass. Natural body composition consists in both fat mass and muscle mass, which are combined in different percentages, varying from one individual to another. But high fat will generally signify obesity and low muscle mass, sarcopenia. If the two are combined, we have sarcopenic obesity, frequently encountered in elderly, due to changes in the body composition linked to the natural process of senescence. The need for an accurate definition is as important as the need to properly evaluate the presence and the extent of the problem. When the concept of sarcopenic obesity started to be used, it was believed that age-associated decline of muscle strength was caused mainly by the simultaneous decline of muscle mass [72, 73]. Thus, the study of muscle mass could somehow be the succedaneum to the study of muscle function. In consequence, several scientists proposed better ways to define and measure "sarcopenia." In 1998, Baumgartner et al. defined sarcopenia being the lower muscle mass index with two or more standard deviation than the reference values measured in young healthy individuals by the DXA (X-ray absorption) method [74]. Also, in 2002, Janssen et al. proposed the definition for sarcopenia in the form of a calculated percentage of muscle mass/body mass × 100, measured by the bioelectrical impedance, considering the occurrence of sarcopenia by recording a standard deviation below the reference values [75]. The criteria by which the occurrence of sarcopenia is most recently defined refer to a calculation made between muscle mass and fat when using residues from linear regression models [76].

However, studies have shown that the decline in muscle function cannot be explained mainly by the parallel decline in muscle mass. It is true that decreased muscle mass and contractile force is accentuated with the advent of aging, but the expectations are always overtaken by the drop in mass [77–79].

The occurrence of a discrepancy between mass and strength is due to progressive deterioration of fiber counts and size, increased collagen volume, reduced contractility of intact fibers, motor unit modification through neurological disorders and micro-infiltration of fat [80–82], etc.

We have evidence that muscle strength is more important than muscle mass when it comes to determining the poor health and the functional limitation in old age individuals [77, 83]. Thus, scientists arrived at a complex choice between muscle mass and muscle strength as valid markers of age-related muscle impairment. In this context, for the apparition of osteoporosis, bone mineral density measurement was originally used as a diagnostic marker because it reflects morphometric bone changes that occurred over the lifetime and was accelerated by menopause [84]. Further studies have shown that not only bone structure but also other factors such as bone quality, weight loss, and fragility may contribute to the risk of fracture.

The authors suggest that age-related changes in muscle tissue should be the central point of interest, due to their functional consequences. Research has confirmed that muscle macro-architecture is a poor witness of the amount of actively contracting proteins. In cross-sectional and longitudinal studies, muscle mass correlated purely with physical function [77, 85]. More recent definitions are based on strength. Usually, a normalization of strength by body size or by fat mass is done, sending to the discrepancy between the "engine" and the "mass to be moved," which is the crucial aspect in sarcopenic obesity. Older adults are particularly susceptible to the deleterious effects of excess body fat on physical function because of the lowered muscle mass and strength that occurs with aging (sarcopenia) and of the need to carry greater body mass (obesity) [84]. Thus, definitions based on non-"normalized" strength have been proposed [86]. There are no generally accepted criteria to define low muscle strength; however in practice, it is easier and cheaper to try to measure it than to evaluate muscle mass. More sophisticated and expansive methods (DXA, CT) should be kept for situations where there is a need for thorough clinical examination and for the evaluation of the efficacy of an intervention. The European Working Group on Sarcopenia in Older People (EWGSOP) defines now sarcopenia as being the presence of both low muscle mass and low muscle strength or performance [87]. Recently, the Foundation for the National Institutes of Health (FNIH) sarcopenia project did suggest that a practical way to define sarcopenia is by using ALM (appendicular lean mass) with adjustment for BMI to define low muscle mass [88]. In this project, large datasets from 9 large observational studies with over 25,000 participants have been used, and the resultant ALM/BMI ratio cutoff values were of < 0.789 for men and < 0.512 for women [88, 89]. Kim et al. compared indices of somatic muscle mass and described their clinical implications [90]. In the future, studies comprising the definitions of sarcopenia in relation with its consequences on disability, cardio-metabolic risk profiles, and mortality will be needed [91].

23.3.3 Causes and Consequences

The increasingly prevalent phenotype of high fat and low muscle functioning has led to an entire population of older adults which is at an increased risk for disability [92], hence institutionalization [93] and mortality [94]. The combination of sarcopenia and obesity poses even more significant risks for ill health-related outcomes and disability than either one of the two alone [95, 96]. The current trend is to identify the main promoters of healthy aging leading to increased healthy active years of life by influencing the factors that positively and negatively impact nutritional health (Fig. 23.2).

But understanding the pathways leading to these discouraging outcomes might result, in future, in finding practical solutions even after the wrong has been already done [97]. Some other factors playing a part in sarcopenic obesity are the following:

23.3.3.1 The Role Played by Age and Body Composition

As people age, essential changes of main body compartments are noticed. Fat body mass increases especially in the late decades of life and peaks at about age 60–75 years [98, 99]. Muscle strength and mass, on the other side, decline progressively starting around 30 and accelerating after the age of 60 [100, 101]. Subcutaneous fat declines also, but this change is accompanied by the tendency of growth of visceral and intramuscular fat [102, 103]. Fat muscle infiltration is a driver to lower

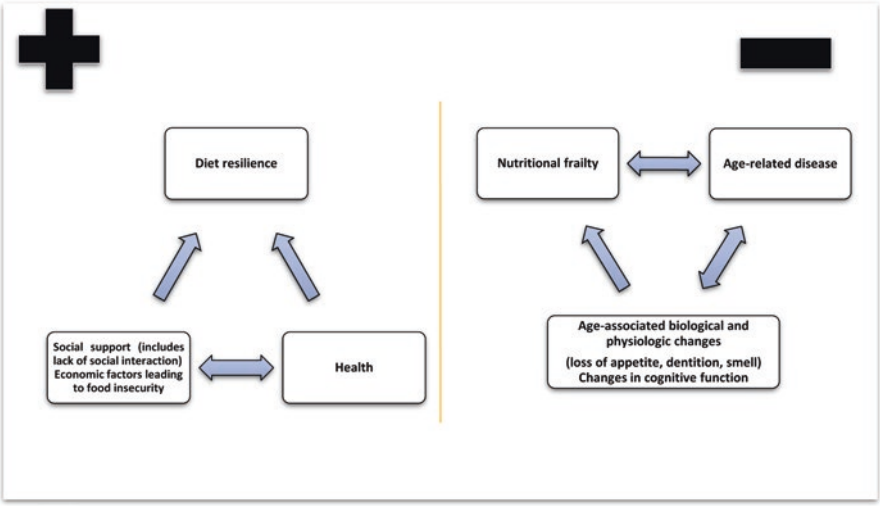

Fig. 23.2 Healthy aging is impaired by several factors which have a positive or negative impact on nutritional behavior

functioning and performance. These changes are due to the progressive decline in energy expenses, both because, with age, the basal metabolic rate is slowing and because the level of physical activity is also decreasing, while food intake remains stable or sometimes increases. Stenholm et al. noticed that "Aging is also associated with a decline in a variety of neural, hormonal and environmental trophic signals to muscle" [84]. Physical inactivity, hormonal changes, pro-inflammatory state, malnutrition, loss of alpha-motor units in the central nervous system, and altered gene expression accelerate the loss of muscle mass and mass-specific strength [68, 104].

23.3.3.2 The Deficit of Physical Activity

It is well known that changes in our level of physical activity are significant risk factors for obesity. Once a person becomes obese, vicious cycles are set, where physical activity becomes even less accessible, because of their weight. This may contribute to decreased muscle strength [105]. Sarcopenia reduces metabolic rates both during rest and active periods, which leads to further weight gain, accompanied by an even stricter sedentary lifestyle, etc. Several studies show that if resistance exercise is combined with diet in weight loss intervention, an improvement of muscle strength and muscle quality is noticed, thus confirming the hypothesis about the link between adiposity and impaired muscle functioning [106–108].

23.3.3.3 Involvement of Insulin Resistance and Inflammation

Older concepts regarding fat tissue, as being a slow metabolic compartment of the human body, have proven to be wrong. Now we know that adipose tissues are very active, synthesizing proteins and hormones that interfere at a large scale with the human metabolism. Their action is obvious on muscles, where, by means of cytokines (interleukin-6 and tumor necrosis factor -α) [109] and/or by means of adipokines, they produce upregulating inflammation responses (leptin and adiponectin) [110, 111] and they contribute to strength and mass decline [112–114].

Sarcopenic obesity seems to be also modulated by an age-related upregulation of myostatin. Sakuma (2013) found that the inhibition of myostatin induced by gene manipulation or neutralizing antibody ameliorates sarcopenic obesity via increased skeletal muscle mass and improved glucose homeostasis [115]. In the Taichung Community Health Study-Elderly, it is shown that obesity and sarcopenic obesity are associated with increased levels of serum hs-CRP (high sensitivity CRP) among males [116]. Results from the Trial of Angiotensin Converting Enzyme Inhibition and Novel Cardiovascular Risk Factors study [112] show that "C-reactive protein and IL-6 are positively associated with fat mass, but negatively related to lean mass," thus suggesting "that obesity-related inflammation may lead to sarcopenia and sarcopenic obesity." Another interesting study [86] found that older obese persons with low muscle strength had higher levels of IL-6 and CRP than their peers. One of the doubtless obesity consequences is insulin resistance, which is mediated by inflam-

matory molecules that interfere insulin receptor signaling pathways [117]. Muscle fat infiltration might be one of the causes of insulin resistance in obese persons [118, 119]. Insulin resistance might also promote muscle catabolism, and studies have proven that it correlates with reduced muscle strength [120, 121]. Old diabetics lose both muscle strength and quality swiftly [122]. However, it has been shown that resistance training improves insulin sensibility and glycemic control [123, 124].

23.3.3.4 The Influence of Hormones

Testosterone is a hormone that increases muscle protein synthesis. In men, the levels of testosterone decrease by approximately 1% per year of age [125]. In women, testosterone levels also lower rapidly, from 20 to 45 [126]. Another anabolic hormone is the growth hormone (GH), a peptide of 191 amino acids produced mainly by the anterior pituitary gland. It controls the postnatal growth of multiple tissues, including skeletal muscle [127]. The secretion of GH is maximal at puberty when it is accompanied by high levels of insulin-like growth factor-I (IGF-I) [128] followed by a gradual decline during the next years of life. Circulating GH levels decline progressively after 30 [129]. In senior men, daily GH secretion is 5- to 20-fold lower than that in young ones [130]. Many types of research have indicated an age-related decrease in anabolic hormones. Hormonal supplementation has been conducted on a large scale, but it was not highly effective against sarcopenia [130–132].

The other factor in sarcopenic obesity, obesity, is associated with high levels of free fatty acids in circulation [133] which lower GH synthesis and the plasmatic level of IGF-I [134]. Several hypotheses can link sarcopenia to muscle impairment, like depressed growth hormone secretions [135] or a lower testosterone level in obesity [136]. It is well known that a low level of anabolic hormones is associated with low muscle strength [137, 138].

23.3.3.5 Malnutrition/Weight Loss

It is well known that, for different reasons, from economic ones, to lower appetite or edentation, older adults have the tendency to eat meals lower in protein [139]. This impairs the protein muscle turnover. Even more, obese elderly might try to lose weight, lowering even more of their protein intake. It has been observed that periods of weight loss [140, 141] often coincide with accelerated sarcopenia. Even though acute stable-isotope-based methodologies have demonstrated that the anabolic muscle response to a given amount of protein may decline with age (anabolic resistance), protein supplementation or a higher level of intake of protein-rich food might be an effective approach to delay the age-related loss of muscle [142].

23.3.3.6 Association Between Obesity and Muscle Impairment

Stenholm et al. (2008) examined the hypothesis that obesity (BMI $\geq$ 30 kg/m^2) and low muscle strength (lowest sex-specific hand grip strength tertile) are connected, in four epidemiological studies that included persons aged 65 years and older: BLSA (Baltimore Longitudinal Study of Aging, USA; 1959–2007; Shock 1984); Health 2000 Survey, Finland (2000–2001; Aromaa 2004); InCHIANTI, Italy (1998–2000; Ferrucci 2000); and LASA (Longitudinal Aging Study Amsterdam, Netherlands; 2001–2002; Sonnenberg 2008; Deeg 2002) [84]. Following the four studies, it was concluded that, depending on sex, age, and body weight, individuals with reduced muscle strength were more likely to develop obesity twice as much as those with normal resistance, but obesity decreasing muscle strength are not necessarily correlated.

23.3.4 Consequences

As the name states, sarcopenic obesity combines sarcopenia and obesity, both being associated with different metabolic disorders, hence being able to raise morbidity and mortality [143]. Sarcopenic obesity might have a greater impact on metabolic diseases and cardiovascular morbidity and mortality than any of its two components alone [144, 145]. Several cross-sectional studies in senior Koreans have shown that persons with sarcopenic obesity have the worst cardiovascular risk profiles, with hyperglycemia, hypertension, dyslipidemia, insulin resistance, and lower cardiorespiratory fitness [146–148]. A similar Taiwanese study showed the association between sarcopenic obesity and the highest risk of metabolic syndrome [149]. In a cross-sectional study from the National Health and Nutrition Examination Survey III (NHANES III), sarcopenia enhanced dysglycemia and insulin resistance associated with obesity [150].

There are studies that have investigated the consequences of sarcopenic obesity on cardiovascular disease (CVD) and mortality. Stephen and Janssen (2009) found that sarcopenic obesity is associated with increased CVD risk [151]. As expected, in the British Regional Heart Study, patients with sarcopenic obesity had a higher risk of mortality compared to normally weighted subjects without sarcopenia [152]. A meta-analysis that took into consideration several prospective cohort studies showed that sarcopenic obesity is associated with a 24% increase in the risk of all-cause mortality, particularly in men [153]. On the other hand, sarcopenic participants with obesity from the New Mexico Elder Health Survey were more likely to be disabled than participants who were either obese or sarcopenic [154]. In the 8-year follow-up of the New Mexico Aging Process Study, it has been demonstrated (Baumgartner 1998) that older participants with sarcopenic obesity at baseline had over twofold higher risk of developing IADL (instrumental activities of daily living) disability

than those without initial sarcopenic obesity. However, two other cross-sectional studies based on NHANES III [155] and a sample of older women in Verona [156] did not find an association between sarcopenic obesity and poor physical functioning. Muscle mass was used as an indication for sarcopenia, a fact that might explain the lack of an association with physical functioning [155, 156].

An interesting question for research remains the link between sarcopenia and gender. Women have a higher fat mass, as well as lower, absolute, and relative muscle strength than men [157, 158] due to hormonal characteristics. It is foreseeable that they are more prone to develop obesity and lower strength. Some recent studies show that obesity consequences may be more severe in women than in men [159, 160], because even a small decline in muscle strength can lead to high problems in locomotion and efficiently bearing the excess weight. A cross-sectional study from Brazil [161] showed that sarcopenic obesity was present in 7% of this population of middle-aged women, and it was associated with poor physical performance, limitations being beyond those driven by pure sarcopenia or obesity alone.

23.3.5 Treatment

The pathogenesis of sarcopenic obesity is multifactorial, so choosing the best treatment might be a challenge. Aging, with a decrease in all compartments of energy expenditure and a reduction of physical activity, can lead to excess adiposity. Meanwhile, through the same pathways, chances for sarcopenia rise, being further exacerbated by other changes linked to aging: lower protein intake, increased skeletal muscle fatty infiltration, altered skeletal muscle substrate metabolism, increased expression of myostatin, impaired sensitivity to the anabolic effects of insulin with associated mitochondrial dysfunction, and age-related reductions in growth hormone and testosterone secretion [162]. In consequence, optimal management has to address the different facets that determine the onset of the disease. Lifestyle interventions have to combine weight loss, exercise, and nutritional changes. Recent research shows that a combination of exercise, nutritional intervention, and pharmaceutical treatment (hormones) might offer the best results [163, 164].

23.3.5.1 Weight Loss

Even though weight loss seems to address the main pathways that have led to sarcopenic obesity, for older adults, it remains rather problematic, due to the associated loss in lean body mass and the consequent worsening of sarcopenia [165]. However, weight loss is feasible in frail, obese elderly [166], subjects being sometimes more compliant than younger individuals [167]. Bouchonville and Villareal investigated the effects of diet in lowering body weight (~10%) in obese older adults and found that, apart from some minor loss in lean body mass, a greater reduction in fat was noticed [162]. In the end, researchers found an improvement in relative sarcopenia

(percent body weight as lean body mass) and an improvement of frailty [168]. An adequate protein intake combined with a proper exercise program can have reparatory effects on muscular protein synthesis that resulted from previous hypo-energetic diets [169]. Muscariello et al. showed that a diet moderately rich in proteins was able to preserve muscle mass in sarcopenic women [170]. Thus, adequate protein intake could contribute to the prevention of lean-mass loss associated with weight reduction in obese older people.

23.3.5.2 Exercise

Sarcopenic obesity has been attributed in part to the decline in physical activity, noticed as people get old [171]. Studies have shown that exercise has excellent effects in sarcopenic obesity, by means of the increase of synthesis of protein in somatic muscles [168], the reduction of the expression of myostatin [172], the increase in IGF-1 in muscle [173], the recovering of skeletal muscle sensitivity to insulin, a hormone with anabolic effect [174], the improvement of the nutrient delivery to muscle [175], the enhancement of mitochondrial function [176], and the activation of skeletal muscle satellite cells [177]. Even more, Lambert et al. demonstrated that exercise-induced weight loss lowered skeletal muscle inflammatory gene expression in frail, obese older adults, an effect that has not been seen in diet-induced weight loss [178]. Complex programs have to be designed, combining progressive resistance training (PRT), flexibility, aerobic exercise, and balance training [179]. Resistance training seems to be crucial for sarcopenic obesity prevention and treatment. PRT was associated with improvements in muscle strength, waist circumference, and multiple metabolic outcomes. Its effects were positive also for senior women with sarcopenic obesity [180, 181], and planning specially designed programs has proven to have even greater effects [182].

23.3.5.3 Combined Weight Loss and Exercise

Naturally, the combination of diet and exercise are presumed to give best effects. These interventions act synergistically to improve sarcopenia and ameliorate frailty more than either diet or exercise alone [107].

23.3.5.4 Nutritional Modifications

Many efficient modifications have been suggested, and usually, they target the quantity and the timing of protein/amino acid ingestion. As stated before, aging is associated with a reduction in protein consumption and in the use of the amino acids in muscle protein synthesis [183]. Recent recommended dietary allowance for protein intake underline higher necessities for the elderly [184, 185], since the previous was judged as not adequate in older adults [169, 186]. It has been demonstrated that a

higher intake of essential amino acids restores the synthesis of muscle protein similarly to what has been noticed in younger adults, suggesting that there might be a threshold effect that can be overcome with a higher protein intake [187]. Research advises that in order to prevent sarcopenia in older adults, an intake of 25–30 g of high-quality protein should be ingested at each main meal [188]. Lower ingestion has been associated with suboptimal muscle protein synthesis in seniors [189]. If the intake is higher than 30 g of protein per meal, no positive effect has been reported in muscle synthesis and repair [190]. Some researchers proposed supplementation with leucine since it is a branched-chain amino acid with high potency in stimulation of protein synthesis [191–193]. In a recent study of Sammarco et al., sarcopenic obese patients with high-protein diet showed an improvement in muscle strength [194]. Furthermore, dietary protein enrichment might represent a protection from the risk of sarcopenia enhancement following a hypocaloric diet.

23.3.5.5 Pharmacologic Therapy

Lifestyle interventions remain the corner key for the sarcopenic obesity treatment. However, due to practical reasons, pharmacologic therapies might be useful. Some alternatives, though limitative, are the use of myostatin inhibitors and the use of some anabolic agents, like testosterone and mediators of the IGF-1 system.

23.3.5.6 Inhibitors of Myostatin

There is a growing body of evidence that inhibition of myostatin in sarcopenic obesity can lead to positive modifications of adiposity and lean body mass. Myostatin is a member of the TGF-β superfamily of secreted growth factors, being synthesized both by skeletal muscle and adipose tissue, and it plays a role of negative regulator of muscle mass [195]. Research suggests that skeletal muscle may be considered an endocrine organ that contributes to the regulation of body composition. Myostatin seems to be a biomarker of sarcopenia in the elderly. There is an inverse correlation between the myostatin level and muscle mass, the highest levels being observed in frail older adults [196]. On the contrary, animal models show that myostatin deficiency is associated with excessive muscularity and a low level of fat tissue in myostatin-deficient cattle [197]. A similar fact has been observed in children with a mutation in the myostatin gene [198]. As a consequence, one might raise the idea of myostatin inhibition, as a suitable strategy for the treatment of sarcopenic obesity. Data on animal models are promising: in mice, it led to the lowering of adipose tissue [199], reduced the markers of inflammation [200], increased muscle mass [201], and protected against age-related sarcopenia [202]. It was proved on animal models that muscle mass and function can be improved through therapy of inhibitory propeptides or by myostatin antibodies and also was observed that the inhibition of

myostatin induced an upregulated intramuscular satellite cell function and IGF-1 signaling increased thermogenesis and endurance to obesity [162]. However, trials in humans had disappointing results. One study found that myostatin inhibition in patients with muscular disorders, respectively, muscular dystrophy was correlated only with ameliorations in muscle function, but not in muscle strength [203].

Further uncertainties are linked with observations in individuals with the K153R polymorphism in the myostatin gene (this is a variant that reduces the capacity of myostatin to influence muscular strength and mass) [204].

The variant may contribute to exceptional longevity [205], but there were reports that it is also associated with a diminished muscle force in some but not all [206] affected individuals. Other questions regarding myostatin are linked to the safety of long-term administration, especially in relation with the cardiovascular system, since there is proof that myostatin expression is correlated with heart disease [207].

We can conclude that for now, more long-term studies are needed before using myostatin inhibitors in protocols of treatment of sarcopenic obesity.

23.3.5.7 Testosterone

Aging is accompanied by a decline in testosterone, paralleling the loss in lean body mass and the gain in fat, which are the paramount components of sarcopenic obesity. The testosterone therapy might be an answer in sarcopenic obesity prevention and treatment. Most studies carried out on healthy subjects reported positive changes in fat mass and lean body mass but were mixed regarding muscle strength. One research work studied the effects of twelve months of testosterone administration in a double-blind trial in healthy older individuals unsystematized to progressive resistance training versus no exercise [208]. The results, for the exercising subjects, were positive for the improvement in fat mass and fat-free mass, but the physical function and muscular strength were not modified. Some positive changes in upper body strength were noticed in the non-exercise subjects treated with testosterone but none in physical function. Researches on healthy older male persons reported beneficial effects of testosterone administration on human body composition [209].

However, higher concentrations of testosterone therapy are associated with adverse events.

The conclusion for the moment is that testosterone treatment in healthy older male individuals has favorable results on human body composition, which provides protection against sarcopenic obesity, but is necessary to supervise the potential adverse effects (growth of subclinical prostate cancer, erythrocytosis, aggravating of obstructive sleep apnea, fluid retention, etc.).

The 2010 Endocrine Society Guidelines submit that therapy in older persons has to be limited to cases where there is proof of hypogonadism and the patients should know the benefits and risks of treatment [210].

23.3.5.8 Other Therapies

Aging is correlated with other hormonal and mediator changes, like the progressive decline in growth hormone (GH) secretion and IGF-1 production [211], which are connected with the lowering of lean body mass and increase in fat mass [212]. GH substitution was studied already for a long time, as an ameliorator of the changes in body composition [213]. However adverse effects were important: arthralgias, edema, and glucose intolerance. A research paper published in 2007 suggested that GH should not be used as antiaging therapy [214].

More recently, advanced techniques have been employed, like the augmentation of endogenous pulsatile GH, aiming to shunt the adverse effects connected with exogenous GH.

Capromorelin is a growth hormone secretagogue which has positive effects for physical condition and body composition in healthy older persons but, unfortunately, has negative properties for glucose homeostasis [215].

Makimura et al. observed that GHRH (growth hormone-releasing hormone) analog is associated with enhanced lean body mass and decreased fat mass. They suggested that there is no correlation between GHRH analog and disturbances in glucose metabolism or other adverse events [216].

The results might be promising, and future studies are needed to determine whether tesamorelin, a synthetic form of GHRH, may be helpful for the cure of sarcopenic obesity in older individuals.

Some other androgenic therapies have been tested. There are conflicting data regarding the use of dehydroepiandrosterone (DHEA) on muscle mass and strength. DHEA administration amplifies the anabolic events of heavy resistance exercise in aged persons [217].

A recent meta-analysis of studies in senior men revealed that DHEA administration can be associated with a minor but important positive effect on human body composition [218].

Another interesting topic refers to treatment with anabolic steroids and their effects in older human body. In this category is included oxandrolone, a synthetic anabolic androgen. Treatment with this compound had advantages like improvements in lean body mass and fat mass and also in muscle strength [219] but had significant disadvantageous consequences on plasmatic lipid profiles.

Another study, carried on patients with cancer cachexia, suggested that a nonsteroidal selective androgen receptor modulator, enobosarm, might ameliorate the lean body mass without the toxic effects associated with androgens [220]. There are also other modern treatments developed and tested, like using inhibitors of transcription factor nuclear factor kappa B (NF-κB) for protection against cancer-related cachexia, with promising results that might be transferred in elderly with sarcopenic cachexia [221].

23.3.6 Conclusions

Taking into consideration the worldwide rise in the incidence of obesity especially at older ages, in relation with the age decline of muscle mass, sarcopenic obesity will gain momentum, with negative consequences in maximizing disability, morbidity, and mortality. These will lead to a lowering of the quality of life of seniors and will also negatively impact the public health systems. Weight loss and exercise can bring their own and separate contribution. However, strategies combining especially tailored resistance training and bespoke high-quality protein intake in older adults show the strongest effects. While promising, pharmacological therapies are yet riddled with numerous adverse effects, so for the moment, the impact of their use in long-term interventions has yet to be evaluated.

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Chapter 24
Physical Exercise for Muscle Atrophy

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Abstract The most direct characteristic of muscle atrophy is reduction in muscle mass, which is due to increased protein degradation or reduced protein synthesis in skeletal muscle. The loss of muscle mass can directly affect the quality of daily life, prolong the recovery period, and become the main risk factor for chronic diseases. However, there is currently no effective way to prevent and treat this disease, and therefore it is imperative to explore effective therapeutic approaches for muscle atrophy. It is well known that physical exercise is important for maintaining good health and long-term adherence to exercise can reduce the risk of cardiovascular diseases, obesity, and diabetes. It is also well established that exercise training can promote the synthesis of muscle protein and activate signaling pathways that regulate the metabolism and function of muscle fibers. Therefore, exercise can be used as a method to treat muscle atrophy in many of these conditions. Mitochondria play an important role in skeletal muscle homeostasis and bioenergy metabolism. Mitochondria are sensitive to contractile signals, and hence exercise can improve mitochondrial function and promote biosynthesis, which ultimately maintains the healthy state of cells and the whole body. On the other hand, frequent unaccustomed exercise will change the structure and function of skeletal muscle fibers, which is called exercise-induced muscle damage. When the exercise-induced muscle damage happens, it can cause temporary muscle damage and soreness, giving a negative effect on the muscle function.

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Keywords Muscle atrophy · Physical exercise · Mitochondria · Excessive exercise

24.1 Introduction

The skeletal muscle mass is about 40% of body weight, and it is important for exercise and metabolic balance [1]. Skeletal muscle is the largest reservoir of protein in the body [2]. Skeletal muscle is not only the foundation of physical exercise but also the major glucose metabolism organ in the human body. It is also the energy storage tissue of the body in pathological state of energy deficiency [3]. Muscle atrophy is mainly manifested as a significant reduction in muscle mass, which is due to the increased protein degradation or reduced protein synthesis in skeletal muscle [4]. According to different pathogeneses, muscle atrophy can be classified into three types: the primary disorders of skeletal muscle, the secondary disorders of skeletal muscle, and the aging-induced sarcopenia [5]. The occurrence of various kinds of muscle diseases can directly cause the primary muscle atrophy, and the muscle atrophy often appears concomitant with muscle diseases, such as Duchenne muscular dystrophy (DMD). On the other hand, secondary muscle atrophy is caused by external factors including diseases and weightlessness. The increased protein degradation or the reduced protein synthesis in skeletal muscle always happen to be associated with many terrible diseases like cancers [6], heart failure [7], muscle genetic diseases [8], and neurodegenerative disorders [9]. The last one is age-related decline in skeletal muscle mass and function. The loss of muscle mass and motor ability that accompanies aging always occurs in old people; it is usually manifested as muscle weakness and muscle atrophy. Moreover, muscle atrophy is also found in healthy people: during leg fractures [10], immobilization, bed rest, and age-associated [11] spinal injury [12]; for those who need prolonged bed rest because of injury, stay in a weightless environment or simply live a sedentary lifestyle. Finally, muscle wasting signaling starts due to lack of muscle contraction and stimuli, subsequently increasing the protein degradation and cell apoptosis. Muscle atrophy occurs when protein degradation exceeds protein synthesis [13].

24.2 Exercise: Skeletal Muscle Protection

The loss of muscle mass can affect people's daily life, reduce their ability of daily living activity, prolong the recovery period of illness, and become the most important risk factor for chronic diseases. Therefore, it is critical to develop novel approaches for quick and effective treatment of muscle atrophy. When muscle atrophy occurs, the decrease in muscle protein synthesis and the increase in protein degradation happen simultaneously, which results in a rapid decrease in muscle size [14]. It is well known that proper physical exercise is beneficial to the health of the human body; meanwhile physical exercise can also improve cardiopulmonary function, reduce the risk of cardiovascular disease, and prevent obesity and diabetes

[15]. Exercise can activate the signaling pathway that stimulates the metabolism of skeletal muscle fibers and enhances contraction and physiological function of the muscle. Many health guidelines recommend that adults should do at least 30 min aerobic exercises five times a week to keep in good health [16]. Regular and appropriate physical activity is beneficial for the health of the body and can improve the body's resistance to diseases [17]. It is well known that the exercise training can increase muscle protein synthesis and muscle weight, so in many cases, exercise training can be used as an important method for the treatment and prevention of muscle atrophy [18].

On the other hand, exercise training can improve muscle metabolism and ameliorate the abnormalities in muscle function without changing the functional performance of the heart [19]. It has been reported that exercise training can increase the volume of mitochondria by up to 40% [20]. During the physical exercise, the factors regulating mitochondrial biogenesis are elevated, which directly enhance the synthesis of mitochondrial protein. In aging skeletal muscle, the mitochondria are found smaller, with slow metabolism, and reduced biosynthesis, resulting in a rapid decline in muscle mass and muscle performance parameters. Moderate exercise training can protect the mitochondria from volume and biogenesis reduction caused by aging and hence relieve the age-related skeletal muscle mass decrease [21]. Although the aging-driven skeletal muscle atrophy is only one type of muscle atrophy, the results still indicate that exercise can resist the adverse consequences caused by muscle atrophy through the induction of mitochondrial biogenesis.

24.2.1 Physical Exercise Types

Physical exercise can be roughly divided into endurance training and resistance training. Endurance training is based on aerobic exercise that improves muscular endurance, while resistance training is based on strength exercise. Marathon, swimming, and cycling are common endurance trainings, which are characterized by high-frequency, longtime, and low-power consumption. On the other hand, resistance training such as fitness and throwing is characterized by low frequency, high resistance, high strength, and short duration. For different kinds of exercise, the parameters such as duration, frequency, intensity of exercise, and the effects on the muscles will be different [22]. Specific functional adaptability of skeletal muscle will be developed according to distinct exercise patterns [23]. Skeletal muscle mass and strength will increase response to resistance exercise [24], while endurance exercise can stimulate the mitochondrial biogenesis and improve the respiratory function of mitochondria for adaption to higher intensity of metabolic activity [25]. In general, exercise has many beneficial effects on skeletal muscle, which is good for the health.

Though both endurance training and resistance training are good for human health, the endurance exercise is considered more effective in preventing cardiovascular disease, whereas resistance training is more effective in maintaining muscle mass and

protecting age-related muscle atrophy [26]. Benefits of endurance exercise in cardiovascular diseases such as hypertension and coronary heart disease are because of increased angiogenesis and promoted capillarity and, more importantly, due to enhanced resistant to inflammation. Combination of these two types of exercise can increase the bone density and insulin sensitivity as well, thus preventing the occurrence of type 2 diabetes. In addition, exercise training is also the main preventive method against obesity, glucose intolerance, and many metabolic diseases [27–30].

24.2.2 Endurance Exercise Preconditioning Prevents Disuse Muscle Atrophy

Muscle atrophy is caused by reduced protein synthesis and increased protein degradation. The loss of contractile proteins, cytoplasm, organelles, and nuclei in muscle cells will eventually lead to a decrease in the size of muscle fibers [31]. Previous results based on animal studies have shown that increased protein degradation and reduced protein synthesis can cause disuse atrophy [32]. The relative stability of total skeletal muscle mass is achieved by balance of protein synthesis and degradation. Nutrients and nutrient-derived hormones play a key role in keeping muscle mass stabilization by regulation of the synthesis and degradation of muscle protein. Proteins are made up of essential amino acids, and the protein intake through diet is essential for the synthesis of muscle proteins [33].

24.2.3 Muscle Protein Breakdown

As for protein degradation, skeletal muscle can remove misinterpreted, damaged, misfolded, or unnecessary proteins through four special complementary pathways, including calpain, caspase-3, autophagy, and ubiquitin proteasome pathway [34].

1. Calpain

   The calpain protein family is calcium-dependent proteases, which play an important role in the breakdown of myosin, actin, and other structural proteins. In fact, targeted inhibition of calpain can effectively prevent muscle wasting in various disease states [35–39].

2. Caspase–3

   Caspase-3 is a member of the cysteine-aspartic acid protease family, which is generally believed as the most important terminal shear enzyme in the process of apoptosis. Recent studies have shown that caspase-3 can be combined with calpain to participate in the hydrolysis of myofibrillar proteins [40, 41]. Moreover, when caspase-3 protein is suppressed or the gene is knocked out, the occurrence of disuse

muscle atrophy can be effectively suppressed [38, 39, 42, 43]. Therefore, it is believed that calpain and caspase-3 together play a crucial role in inhibiting muscle atrophy as they begin the initial breakdown of the muscle contractions [34].

3. Autophagy

Early studies suggested that there is not much direct correlation between cell autophagy and muscle atrophy [35]. However, recent studies have shown that autophagy may play a crucial role in the disuse atrophy, by selective degradation of organelles such as mitochondria and removal of apoptotic cells [44].

4. Ubiquitin Proteasome Pathway

Previous report has showed that these protein degradations are also accomplished through the ubiquitin proteasome pathway. Small peptides, misfolded proteins, and unnecessary proteins can be degraded via ubiquitin proteasome pathway [45].

24.2.4 Muscle Protein Synthesis

Protein synthesis in cells is a complex process regulated by a complex network composed of multiple regulatory factors. Amino acids are combined to various proteins according to the genetic information on messenger RNA (mRNA). Within hours of disuse, muscle protein synthesis is reduced by about 25–50% and will remain inactive throughout the period [46–49]. The Akt/mTOR signaling pathway plays an important regulatory role in controlling the change of muscle mass [50]. In disuse atrophy, the Akt/mTOR pathway is suppressed by reducing the phosphorylation of Akt and subsequently inhibiting the expression of downstream target gene mTOR. When the signaling pathway is attenuated, the formation of the translation initiation complex will be greatly reduced, resulting in declined muscle protein synthesis. To sum up, it is believed that increase of protein degradation and decrease of protein synthesis can induce the occurrence of disuse atrophy [14].

24.2.5 Reactive Oxygen Species (ROS)

It has been reported that the expression of reactive oxygen species (ROS) in skeletal muscle is increased in disuse atrophy [51–53]. The amount of mitochondrial protein in skeletal muscle and respiration of mitochondria will significantly decrease along with the increase of ROS. ROS regulate the redox signaling pathway in muscle fibers, and hence increase of ROS can reduce the synthesis of skeletal muscle protein and enhance protein hydrolysis [54]. Combined with above conclusions, we believe that the disuse atrophy is closely related to the decrease of the antioxidant capacity of skeletal muscle [53, 55–58]. It is found that the root cause of decreased antioxidant scavenging ability of skeletal muscle is the reduction of antioxidant

clearance ability, which is not necessarily concomitant with reduced antioxidant enzyme content [55, 58]. It has been proved that ROS are vital in the upstream events that lead to disuse atrophy, and increased synthesis of ROS can effectively activate the associated signaling pathways. Furthermore, increased ROS can activate the activity of transcription factor, which further elevate the expression of endogenous antioxidant proteins [59].

24.2.6 Heat Shock Protein 70

The heat shock proteins are a group of highly conserved protein known as molecular chaperone proteins. Heat shock protein plays a role in cell protection by combining with the denatured proteins to assist the recovery or transport of the proteins for lysosomal degradation. It has been found that the expression of heat shock protein in the body is increased after exercise. Generally, the heat shock protein has three functions: (1) to promote the folding of newly synthesized proteins, (2) to help fold back the denatured protein, and (3) to transfer the synthesized protein to the specific organelle [60]. Heat shock protein 70 (HSP70), as a member of the family, is the most popular research object at present. HSP70 (also known as HSP72) has a highly conservative peptide structure, which facilitates its repair and functional restoration of the denatured protein in cell. Temperature, oxidative stress response, mechanical action, metabolic reaction, and cytokine stimulation all have influence on the expression of HSP70. More importantly, physical exercise can cause a series of stress reactions in the body that can directly promote the expression of HSP70 protein.

24.2.7 PGC-1α

PGC-1 is an important regulatory factor for mitochondrial proliferation and therefore mainly expresses in tissues that require a large amount of energy, such as the heart, skeletal muscle, and liver. PGC-1α is a transcription co-activator, which involved in many physiological functions, such as mitochondrial biosynthesis, promoting blood vessel formation, glucose metabolism, and fatty acid oxidation [61, 62]. It has been reported that after 18 h of endurance exercise, the expression level of PGC-1α is markedly increased in rat soleus muscle [63]. Kang's study found that the expression of PGC-1α in female Sprague-Dawley rats subjected to anaerobic sprinting exercise is increased by 5.6 times compared to the control group [64]. Another group of rats were given 20 min of aerobic treadmill running for 6 weeks, and the level of PGC-1α mRNA was found to increase by 25% in rat soleus muscle [65]. Other studies have also verified that exercise training and prolonged physical activity can effectively promote the expression of PGC-1α in skeletal muscle [66–70]. Although very little is known about the molecular mechanisms involved in the

exercise-induced adaptive response, PGC-1α is currently accepted as the main regulatory factor. In brown fat cells, PGC-1α is found to be a transcriptional activator of peroxisome proliferator-activated receptor γ (PPAR γ) [71]. Studies have also shown that PGC-1α plays a key role in mitochondrial development. The expression level of PGC-1α is the rate-limiting factor of mitochondrial gene expression in skeletal muscle, and overexpression of PGC-1α can promote the synthesis of mitochondria. An acute exercise or prolonged endurance exercise can both stimulate the deacetylation of PGC-1α in skeletal muscle; the exercise activates the signaling pathway associated with energy metabolism, thus inducing the expression of PGC-1α, whereas PGC-1α is expressed higher in slow muscle fibers which are more suitable for endurance exercise [72, 73]. Phosphorylation and deacetylation of PGC-1 can induce the expression of ag group of mitochondrial genes [74, 75]. On the other hand, prolonged disuse muscle atrophy is accompanied with damage of cellular oxidative metabolism and increase of glycolysis. This process involves the disruption of electron transport chains in mitochondria and the reduction of mitochondrial content [64, 76]. When the mitochondrial function is manifested, it causes the increase of ROS and glycolysis, elevation of metabolic stress, reduction of fat oxidation, and accumulation of substrate, which eventually leads to low efficiency of ATP production [77]. In summary, physical exercise can induce the upregulation of PGC-1α expression by activating multiple metabolic process and eventually prevent metabolic defects and protect against the disuse atrophy.

24.3 Mitochondria

Mitochondria are the cell organelles responsible for aerobic respiration. It plays an important role in metabolism and maintenance of homeostasis [78]. There are a lot of mitochondria in muscle tissue, which provides enough energy for muscle contraction. Therefore, the number and function of mitochondria are the key factors affecting the health of skeletal muscle [79–81]. Exercise training can improve the functional activity of mitochondria and promote the biosynthesis of mitochondria, which help maintain the stability of muscle cells. Some chronic diseases, such as obesity and diabetes, can reduce the number or function of skeletal muscle mitochondria [82–84]. Mitochondria play an active role in maintaining environmental balance and bioenergetics in skeletal muscle [85]. In skeletal muscle, the content of mitochondria is dynamically balanced, and muscle cells can regulate the number of mitochondria according to the energy required by tissue metabolism [86] whereas long periods of inactivity, chronic disease, and aging can reduce the number and function of mitochondria [87–89]. Although some diseases do not directly harm mitochondria, mitochondrial function abnormalities are often noticed being involved in the development of diseases; and the change of mitochondrial genome usually leads to change of physiological functions as well [13]. In skeletal muscle, the conversion from type I to type II is documented. In humans with mitochondrial myopathy, oxidative muscle

fibers can be transformed to glycolysis ones. Mitochondrial dysfunction changes the form and reduces the function of skeletal muscle, and the changes in the energy source further reduce muscle strength, ultimately affecting the health of the muscle [90]. It has been reported that increased mitochondrial DNA mutation and decreased mitochondrial DNA total content are observed in the aging in the skeletal muscles, which is related to the decreases of muscle mass and function in elders [91]. Additionally, when the synthesis of mitochondrial and oxidative phosphorylation (OXPHOS) proteins is interrupted, ATP synthesis and production of ROS will decrease [92]. ROS is associated with many diseases, including muscle diseases [93]. Excessive ROS can activate cell apoptosis and protein degradation through caspase and ubiquitin proteasome pathways [94]. A growing body of research has been focusing on improvement of the mitochondrial function, and at present many treatments for mitochondrial dysfunction, such as exercise therapy, nutritional therapy, and drug therapy, have been developed. The principles of these therapies are to counteract the effects of mitochondrial dysfunction by regulating some signaling pathways involved in mitochondrial biosynthesis [93, 95].

24.4 The Damage of Excessive Exercise

For people who do not exercise regularly, the body will have discomforts, such as muscle pain and muscle stiffness, after an acute strenuous exercise. This kind of phenomenon is the most common cause of muscle incommensurate reaction, which is called exercise-induced muscle damage [96]. Once the muscle is subjected to a long unaccustomed exercise, the structure and function of myofibrils will be changed [97, 98]. Destruction of the muscle fiber structure, inflammation, and muscle protein degradation will directly lead to the reduced muscle strength, decreased athletic ability, edema, and delayed the pain of exercise [99, 100]. Exercise-induced muscle damage can be divided into two stages, the initial injury stage and the secondary injury stage; the former is the injury during the movement; the latter is because of the delayed inflammatory response [101, 102]. The mechanism on muscle injury caused by training especially by strength training is relatively clear now [103], and more researches have been focused on the relationship between the degree of muscle microlesion and concentricity or eccentric contraction [104].

24.4.1 Muscle Damage Markers

Until now, there are only few definitive studies on muscle damage caused by strength training. Although muscle response and exercise intensity have been documented, there are still no clear results on other aspects. There are many definite markers for muscle injury; the most common are muscle strength, delayed onset muscle

soreness, blood creatine kinase activity, indirect markers of collagen breakdown, median frequency of EMG signal, and ultrastructural damage.

Muscle strength: the most common approach is measurement of post-training muscle strength, which has been used in many studies [105–107]. By comparison between the muscle strength before and after exercise, the results showed that the average level of muscle strength in the exercise group is lower than the value detected before a 2-day exercise. Decreased muscle strength is associated with excessive muscle contraction, and the intensity exercise can lead to a change in the process of overlaying and excitation contraction of the filaments.

Delayed onset muscle soreness: delayed onset muscle soreness (DOMS) is a muscle maladaptive response that occurs 24–48 h after strenuous exercise [108]. Muscle tendinous junctions are the most vulnerable part in the muscle structure and can be easily damaged in mechanical stress [109]. Multiple studies have found that damage of muscle tendinous junctions are the root cause of muscle soreness [108, 110].

Blood creatine kinase activity: as is known to all, proteins are generally not able to pass through the sarcoplasmic reticulum. Therefore, when the intramuscular proteins are detected in the blood, the muscle fibers and sarcolemma are determined as damaged [111]. Creatine kinase (CK) is found specific expressed in skeletal muscle and myocardial tissue, which is thought to be the most obvious marker for the breakdown of muscle cell structure [112, 113]. Some studies have found a rise in CK levels between 48 and 72 h after exercise [113].

Indirect markers of collagen breakdown, hydroxyprolin (HP), hydroxylysine (HL), and pyridinoline (PYD), are markers of collagen breakdown. Many articles have reported that the content of these markers is abundant when muscle damage does occur [112, 114].

Median frequency of electromyography (EMG) signal: the changing in EMG signal median frequency is one of the evidences to evaluate the muscle injury especially for the eccentric exercise [115, 116].

Ultrastructural damage: muscle ultrastructural damage is also a direct marker of muscle injury, and muscle fiber damage is usually caused by the disorder of muscle fiber structure [117–119]. At the same time, muscle fiber injury, T-tube injury, Z-line injury, and cytoskeleton injury can also be detected in muscle injury [120].

24.4.2 The Prevention and Treatment of Exercise-Induced Muscle Damage

When the exercise-induced muscle damage happens, it can cause temporary muscle damage and soreness, which has a negative effect on the muscle function of the later exercise. Nowadays many interventions can be adopted to treat exercise-induced muscle damage or to eliminate resulting adverse reactions, such as pharmacology [121], nutritional [122], electrotherapies [123, 124], exercise [125, 126], and artificial therapy [127]. Further studies are required to elucidate the underlying

mechanism for the treatment for muscle damage and to determine the most appropriate dosage, frequency, and intensity for optimum treatment efficiency.

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Part VI
Treatment Strategies of Muscle Atrophy

Chapter 25
To Contrast and Reverse Skeletal Muscle Atrophy by Full-Body In-Bed Gym, a Mandatory Lifestyle for Older Olds and Borderline Mobility-Impaired Persons

Ugo Carraro, Karma Gava, Alfonc Baba, Andrea Marcante, and Francesco Piccione

Abstract Older olds, that is octogenarians, spend small amounts of time for daily physical activity, contributing to aggravate their independence limitations up to force them to bed and to more and more frequent hospitalizations. All progressive muscle contractile impairments, including advanced age-related muscle power decline, need permanent management. Inspired by the proven capability to recover skeletal muscle contractility and strength by home-based functional electrical stimulation and guided by common sense, we suggested to older olds a 15–30 min daily routine of 12 easy and safe physical exercises. Since persons can do many of them in bed (full-body in-bed gym), hospitalized elderly can continue this kind of light training that is an extension of the well-established cardiovascular-ventilation rehabilitation before and after admission. Monitoring arterial blood pressure before and after the daily routine demonstrates that peripheral resistance decreases in a few minutes by the functional hyperemia of the trained body muscles. Continued regularly, full-body in-bed gym helps to maintain the independence of frail older people and may reduce the risks of serious consequences of accidental falls.

Keywords Skeletal muscle atrophy · Home-based full-body in-bed gym · Older olds · Borderline mobility-impaired persons

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25.1 Background

There are about 700 named skeletal muscles in the human body, including 400 that only specialists care. Better known are the roughly 300 skeletal muscles that are serious bone movers, plus another 100 little muscles of the hands, feet, and face. The aim of this short report is to convince older persons to counteract muscle atrophy-sarcopenia-cachexia to maintain at their best function and shape of the majority of their body muscles, though they will inexorably decay decade after decade [1].

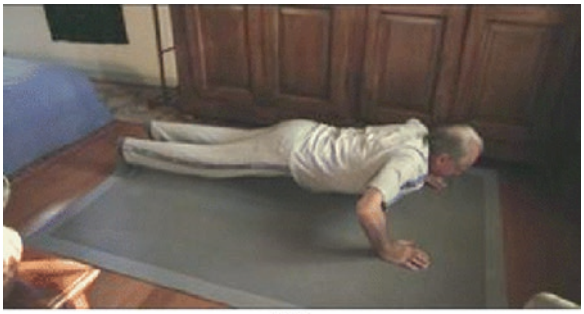
Older olds, due to advanced age or associated diseases, spend only a small amount of time for daily physical activity. The consequent muscle atrophy contributes to limit their independence up to force them to bed and to hospitalization for long periods. Immobility-related muscle atrophy is associated with neuromuscular weakness, functional limitations, thromboembolism, and high costs [2–4]. All progressive muscle contractile impairments, muscle atrophy included, need permanent managements. Besides eventual pharmacological treatment, a home-based physical exercise approach is helpful in counteracting muscle atrophy. Awaiting the development of implantable devices for muscle stimulation, as effective as pacemakers for cardiac arrhythmias or cochlear implants for hearing loss, education of sedentary patients to home physical exercises during and after hospitalization could be an effective, low-cost alternative.

Cardiovascular and ventilation rehabilitation of surgical patients are well established. A major component of them is to reverse muscle atrophy and weakness [5, 6]. Furthermore we demonstrated that a home-based functional electrical stimulation (h-bFES) strategy recovers skeletal muscle contractility and strength by even in the worse cases of muscle atrophy and degeneration after severe neuromuscular traumatic injuries [7–16]. Thus, we suggested to sedentary elderly a daily short (15–20 min) sequence of 12 easy and safe physical exercises that they could perform in bed (full-body in-bed gym) to improve their muscle function and mass and, thus, mobility [17, 18]. Full-body in-bed gym is, indeed, an extension of the in-bed approaches for cardiocirculatory and ventilation physiotherapy rehabilitation that improves mobility of octogenarians and of younger mobility-impaired persons counteracting decay of the neuromuscular and osteoarticular systems.

25.2 Suggested Exercises

Active persons, able to make 25 consecutive push-ups in 3 min (Fig. 25.1A), need the following exercises as a seasonal warm-up to be able to perform very demanding physical activities.

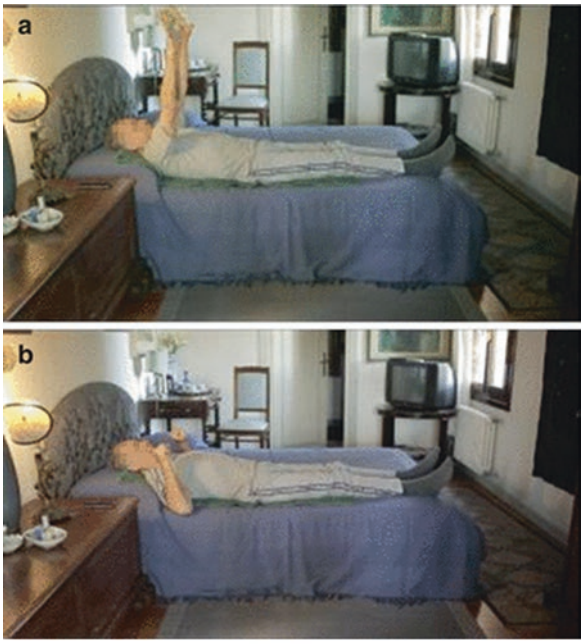
On the other hand, extreme sedentary people, after asking advice to their family physician, may gradually start with five repetitions of each of the following suggested exercises. After the first or second training week, they may add groups of 5 additional repetitions, up to 30, every 1 or 2 weeks. If compliant, older olds will



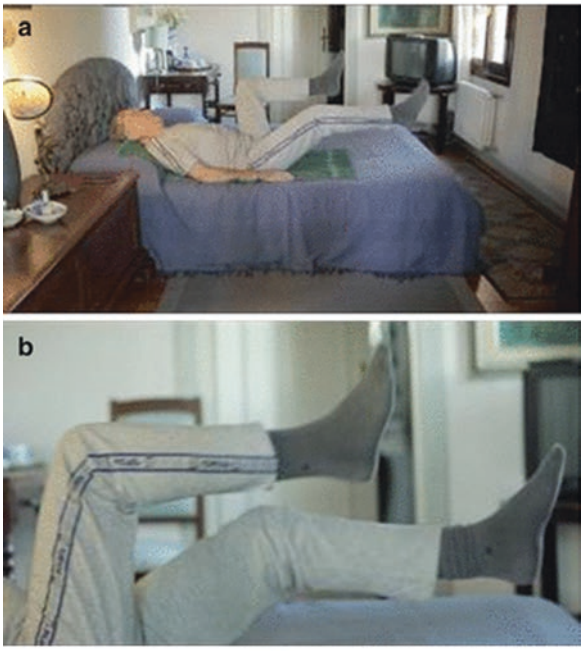
(A)

(B)

Fig. 25.1 Full-body in-bed gym, the 12 exercises. (Pictures are from the figure of Chap. 6 of the Springer – Nature Book: "Rehabilitation Medicine for Elderly Patients", Stefano Masiero, Ugo Carraro Editors). (**A**) Twelfth exercise: Push-up (for active person) usually performed as the last exercise of the routine. To increase its effectiveness, at the end of the series, maintain the flexion position breathing open mouth up to reach an evident face perspiration. (**B**) First exercise: (a, b) flexion and extension of the ankles. (**C**) Second exercise: (a, b) arms up and arms down. Notice the raised hands full open and then closed. (**D**) Third exercise: (a, b) cycling movements. (**E**) Fourth exercise: deep breathings, raising the open arms during inspiration. (**F**) Fifth exercise: to raise the pelvis, maintaining the up position for 2 s. (**G**) Sixth exercise: (a, b) forward bending. Notice the extended arms. (**H**) Seventh exercise: (a–d) neck torsions: a and b, up and down; c and d, right and left. Rotating the head (not shown). (**I**) Eighth exercise: sit and raise the body on your hands. (**J**) Ninth exercise: (a, b) lift the legs when in sitting position. (**K**) Tenth exercise: stand up. (**L**) Eleventh exercise: get up on toes



(C)

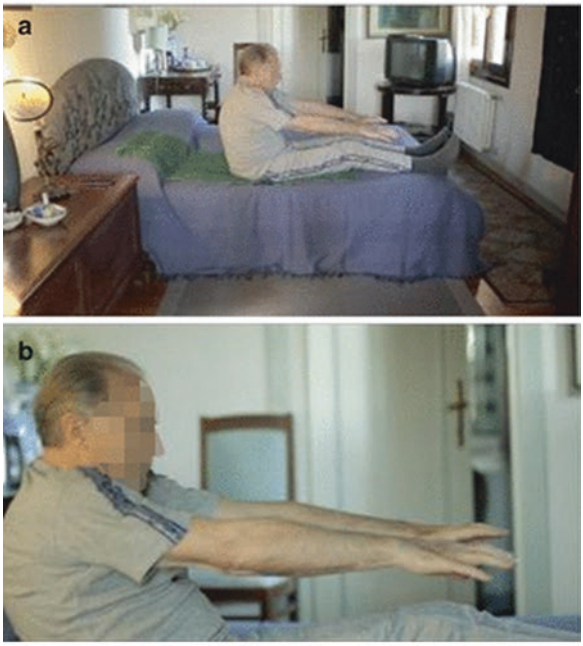


(D)

Fig. 25.1 (continued)

(E)

(F)



(E)

Fig. 25.1 (continued)

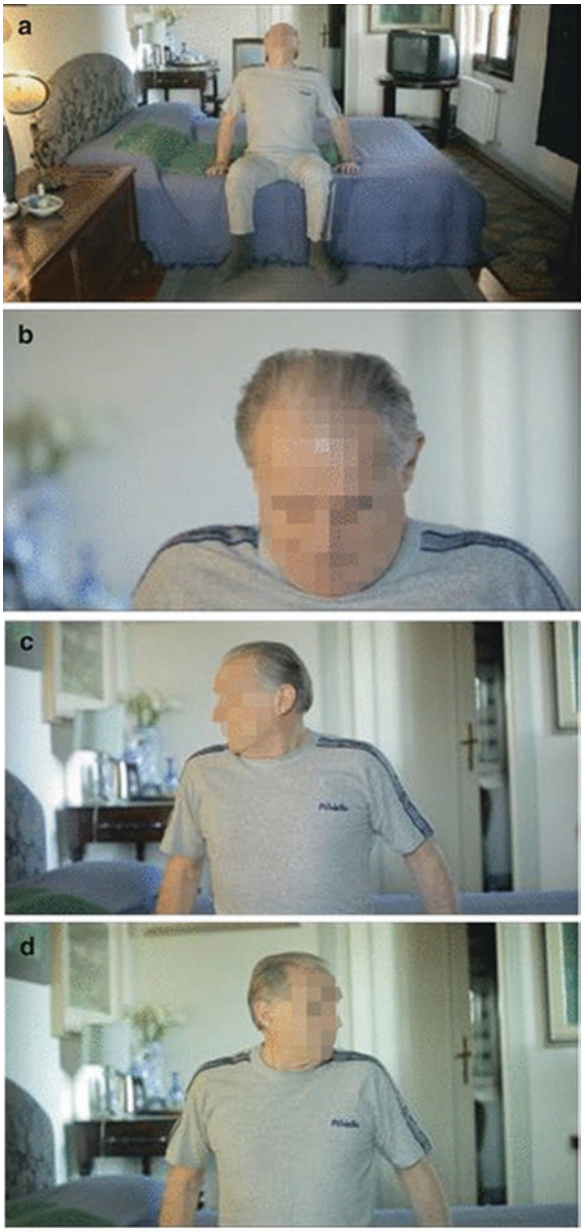


Fig. 25.1 (continued)

(I)

(J)

Fig. 25.1 (continued)

(K)

(L)

Fig. 25.1 (continued)

progressively increase their muscle mass and strength even reaching and maintaining only 15 or 20 daily repetitions. At the beginning, it will be safer to perform the routine at very slow speed. When their maximum number of each exercise is reached, improved effects will be obtained by speeding up the exercises. The daily routine may last from 10 (in the beginning) to 30 min (for complete session in accustomed persons).

Figure 25.1B–L show each exercise, but see also captions of figures for more details. For an educational video, see at the link: http://www.bio.unipd.it/bam/video/InterviewCarraro-tutorial.mp4.

If sedentary persons, without major comorbidities but with rest-related muscle weakness, challenge themselves and avoiding stress, in a few days of full-body in-bed gym, they may increase their muscle strength, fatigue resistance, and independence in daily life activities. Cautious in-bed gym may help patient's recovery after the acute phase of hospitalization, prevent the risk of thromboembolism after surgical interventions, and concur to reduce arterial hypertension [19].

Figure 25.2 shows that after a routine that ends with slight muscle fatigue, increased heart and ventilation frequencies, and sweat at the forehead, the maximal arterial pressure is increased immediately after the routine, but it decreases, together with the minimal arterial pressure, after a few minutes. This behavior is strong evidence that peripheral resistance decreased and thus functional hyperemia of the body skeletal muscles occurred.

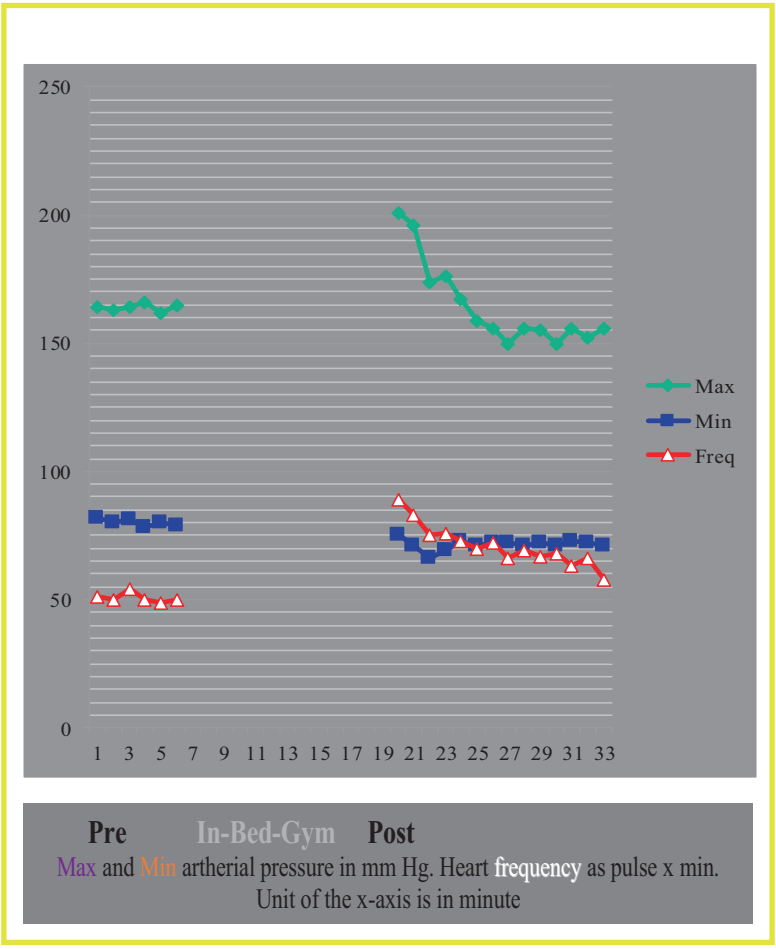


Fig. 25.2 Home-based full-body in-bed-gym. Short-term effects on cardiovascular system

Furthermore, full-body in-bed gym could mitigate the bad mood that is usually associated to mobility limitations, strengthening confidence of patient in recovering partial or total independence, thus reducing the risk of accidental falls [20].

However, if elderly persons cannot, or are reluctant to, perform volitional physical exercises, functional electrical stimulation (FES) may mimic them and be almost equally useful [7–16].

Stimulators for neuromuscular electrical stimulation (ES) that are especially suited for elderly people requirements were designed and implemented in Vienna, Austria [21]. As detailed in Kern et al., 2014 [13], older persons may be exposed to regular neuromuscular ES training. These constant voltage stimulation devices can be safely applied during home use. Starting 2 times a week, for a total amount of 24 training sessions (3 × 10 min for each session), ES is safe and effective. The subjects are ought

to be instructed to increase the stimulation intensity until their maximal tolerance is reached. Using this approach a full knee extension is achieved in all subjects. The outcome is a significant increase in muscle strength, associated with an increase of fast muscle fibers, which are the first to respond to ES and are well related to the power of skeletal muscle; ES significantly increased the size (diameter) of fast-type muscle fibers and the number of Pax7- and NCAM-positive satellite cells. Moreover, analyzed muscle biopsies did not present signs of muscle damage and/or inflammation [13, 22].

Altogether, these results demonstrate that physical exercise, either voluntary or induced by ES, improves the functional performance of aging muscles. Of course, physical training can't stop the aging process [1], but we showed that ES is a safe home-based method that can counteract atrophy of fast-twitch muscle fibers [12, 13]. Age-related muscle power strength is partially attributable to a loss of innervation followed by reinnervation and muscle type groupings [23]. Furthermore these events are delayed by a lifestyle of high-level amateur sport activities [24, 25]. Diseases involving permanent denervation show a premature functional aging process but much more severe muscle deterioration. Despite doubts and criticisms [26, 27], we have shown that h-bFES with appropriate protocols can inhibit degeneration of denervated muscle and even reverse it [7, 8, 28]. Furthermore with appropriate protocols, ES may also enhance reinnervation after nerve injury [29–32].

Therefore, FES should be extended from critical care units to rehabilitation centers, nursing facilities, and at home of the elderly population if volitional muscle activity is impaired or elderly are reluctant to perform volitional physical exercises.

In conclusion, it is never too early and it is never too late to increase daily levels of volitional or FES-induced muscle contractions!

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Chapter 26
Overview of FES-Assisted Cycling Approaches and Their Benefits on Functional Rehabilitation and Muscle Atrophy

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Abstract Central nervous system diseases include brain or spinal cord impairments and may result in movement disorders almost always manifested by paralyzed muscles with preserved innervations and therefore susceptible to be activated by electrical stimulation. Functional electrical stimulation (FES)-assisted cycling is an approach mainly used for rehabilitation purposes contributing, among other effects, to restore muscle trophism. FES-assisted cycling has also been adapted for mobile devices adding a leisure and recreational benefit to the physical training. In October 2016, our teams (Freewheels and EMA-trike) took part in FES-bike discipline at the Cybathlon competition, presenting technologies that allow pilots with

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spinal cord injury to use their paralyzed lower limb muscles to propel a tricycle. Among the many benefits observed and reported in our study cases for the pilots during preparation period, we achieved a muscle remodeling in response to FES-assisted cycling that is discussed in this chapter. Then, we have organized some sections to explore how FES-assisted cycling could contribute to functional rehabilitation by means of changes in the skeletal muscle disuse atrophy.

Keywords Cell plasticity · Electric stimulation · Managed competition · Central nervous system diseases

26.1 Background

As discussed in all chapters of this book, a great variety of *pathological states* lead people to muscle atrophy requiring different therapeutic strategies to restore adequate muscle trophism once several intra- and extracellular factors may influence cellular homeostasis [1, 2]. Among the more current pathological states, such as immobilization [3], denervated conditions [4, 5], neuromuscular joint disease [6–10], central nervous system diseases [11], aging [12], and others, which could conduct to muscle atrophy, in this chapter, we discuss muscle atrophy following the absence of voluntary muscular recruitment – specially determined by upper or lower motor neuron impairment – resulting from cerebrovascular events or spinal cord injury.

Muscle trophism[1] has remarkable adaptive properties in response to contractile activity (*muscular plasticity*); central nervous system (CNS) diseases lead to paralyzed muscles – resulting in skeletal muscle disuse atrophy – and trigger a reaction to atrophy by changes in the energy metabolism that interfere in the muscle fiber composition and in the balance between protein synthesis and degradation [2, 5, 7, 13].

Exercise represents an extrinsic stimulus that initiates many intracellular regulations that trigger pleiotropic[2] responses in skeletal muscle fibers, revealing that the physical activity-dependent muscle fiber plasticity is responsible for muscular remodeling coming from a large variety of *training programs* [14–16]. Several studies [12, 16–18] reported that muscular contraction is affected by neuronal, hormonal, mechanical, and metabolic parameters which can trigger adaptations by

[1] The fundamental nutrition involving the actual metabolic exchanges of the tissues.

[2] Pleiotropy is the phenomenon by which one gene influences two or more seemingly unrelated phenotypic traits, i.e., the capacity of a gene having multiple phenotypic expressions.

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means of events occurring before, during, and after physical activity. Therefore, a voluntary or forced physical inactivity has a great potential to promote atrophy compromising quality of life and life expectancy. Hypertrophy and restoration of atrophic states of the skeletal muscle fibers (*muscle mass*) are often associated with a *prerequisite* for strength that is a determinant of success in daily life activities and even in sport events [19].

The muscles of the body compose of a relatively large mass of alive tissue (around 50% of the human body weight) with high metabolic rate, so their adaptive properties by modifications in terms of number, size, and structural/functional properties in response to a large variety of stimuli make this tissue a great contributor to whole-body health and human functional movements [19, 20]. The two main kinds of training exercises able to promote muscle adaptations are called *endurance*[3] and resistance[4] training in which the training-induced adaptations in skeletal muscle follow specific sequences of cellular and molecular events based on the parameters of each training program [21].

For the purpose of this chapter, where we discuss benefits coming from FES-assisted cycling, the adaptations arising from endurance trainings are particularly relevant, since technological devices trigger active contraction in paralyzed muscles by electrical stimulation resulting in aerobic exercise artificially performed by cycling movements of the lower limbs. In this way, we can consider that FES-assisted cycling promotes a stress that can substantially modulate cellular signaling mechanisms inside paralyzed muscles resulting in adaptations to restore suitable muscle trophism, avoiding drastic and everlasting atrophy [21–23].

Although muscle hypertrophy is mainly related to resistance exercise training that results in increased muscle mass and muscle fiber cross-sectional area, endurance exercise trainings also promote muscular plasticity, mainly characterized by metabolic adaptations designed to enhance generation/utilization of metabolic energy and ultimately resist fatigue [13, 24].

For able-bodied people weighing around 70 kg, the protein turnover results in a rate of 300 g/day, and the metabolic changes influenced by endurance training modify this rate depending on mode, intensity, and duration of the exercises determined in the training program, possibly duplicating the protein synthesis compared with rested levels [22]. Apparently, the *appropriate levels of exercise* to promote the optimum muscular hypertrophy or restoration of atrophic states have the challenge to increase the basal level in values of work until reaching a reference point in the boundary of the maximum energy metabolism enabling to interfere in the muscle fiber composition and enhancing protein synthesis but a secure zone enable to avoid degradation and injuries coming from overtraining [1]. It is a particularly complex task to be accomplished, especially when preparing for competitions [25].

Despite the acute inflammatory response to exercise seeming to favor muscular hypertrophy and regeneration, a more persistent inflammatory response may damage

[3] Endurance training refers to aerobic exercise normally involving cyclic movements of a large number of muscles as observed in walking on a treadmill, swimming in a pool, or cycling a bike.

[4] Resistance training refers to exercises by which muscular strength is improved as observed during pumping iron gym.

the muscular tissue. Currently, little is known about compensatory and anti-inflammatory mechanisms by which a precise intensity of exercise could promote a safe reaction timely and enable to trigger hypertrophic mechanisms and restore tissue homeostasis, without risk of lesion. If it is complex to exercise precise safe intensity, duration, and frequency to able-bodied people, could you image how difficult it is to determine exercise parameters to *disabled people*?

Based on in vitro and in vivo animal models in the past, nowadays in vivo human studies have suggested that satellite cells play a crucial role in skeletal muscle fiber repair and remodeling in response to exercise [13, 21]. Satellite cells are skeletal muscle stem cells which provide the main source of new myonuclei in postnatal skeletal muscle tissue and are still present in adult muscles between the sarcolemma and basal lamina of their associated muscle fibers, even if in a quiescent state. Hypertrophy stimuli or damage conditions activate satellite cells to proliferate and differentiate in a remodeling or repair process, respectively.

The weakness exhibited by *poststroke hemiparesis* [11] and *postspinal cord injury people* [26, 27] – who have to perform tasks coordinating muscles that still respond to voluntary control in the midst of paralyzed muscles – is due to a multifactorial cause justified by both intracellular junction of the muscular fibers and the neuromuscular junction of an innervated paralyzed muscle. In these cases, the disturbed muscle activation generating muscle atrophy may be related to the reduction of descending inputs, which affect both the paretic and non-paretic limbs with different magnitudes.

Carraro and collaborators [12] described the atrophy found in paralyzed muscles by chronic diseases as a premature or accelerated aging of muscle health condition in which the chronic disease causes an irreversible and permanent damage, interfering with nervous system control. Although the cited researchers have addressed their comparison for extreme cases of irreversible *conus* and *cauda equina* syndrome and there are differences between atrophy coming from denervated and innervated muscles [4], the similarity with a premature or accelerated aging of muscle seems also suitable to the disuse of the **paralyzed muscles with preserved innervation** that we discuss in this chapter.

26.2 Health Conditions Dealing with Muscular Atrophy in Response to Disuse Unsolved by Voluntary Muscle Recruitment Approaches

Even in a body with intact nervous system, muscular atrophy could represent an impairment to be tackled with resistance or endurance exercise training if, for some reason, muscle groups were not constantly recruited in a basal activity level due to a lack of use according to the lifestyle habits of each person. Then, muscle disuse conditions induced by immobilization, aging, and/or hospitalization also represent health conditions in which the atrophy could be solved by strategies promoting

training, involving approaches, and triggering voluntary muscle recruitment to restore the basal activity level.

Nevertheless, health conditions determined by structural or functional impairments in the descending projection pathways of the central nervous system coming from the cortex are responsible to drive movements of the limbs and trunk by regulation of the skeletal muscles and may generate a kind of atrophy unsolved by voluntary muscle recruitment strategies, once the common final pathway in any voluntary muscle recruited in the body has to trigger the motor neurons on the ventral horns of the spinal cord.

Figure 26.1 illustrates three health conditions (stroke, Parkinson's disease, and spinal cord injury) in which the parallel and hierarchical organizations of the central nervous system are affected and may prevent the adequate triggering to the common final pathway responsible for promoting voluntary recruitment (natural coordination). In these cases, other non-volitional strategies must be employed in order to

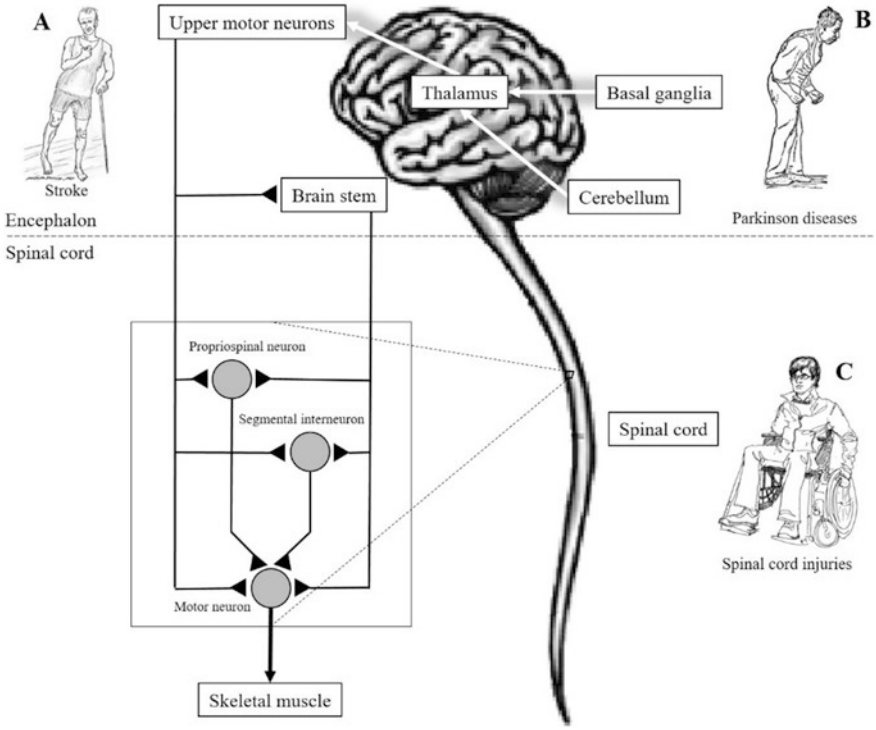

Fig. 26.1 General parallel and hierarchical organization of the descending projection pathways from the central nervous system responsible to drive movements through the common final pathway to the skeletal muscle. The drawings (available on the Internet) around the scheme. (Modified from the Martin's book [93]) represent health conditions determined by stroke (A), Parkinson's disease (B), and spinal cord injury (C) placed, respectively, nearby their affected nervous system components. In the illustrated cases (A, B, and C), the common final pathway (motor neuron) is intact, but not adequately triggering by the descending pathways (voluntary recruitment)

activate the final common pathway (lower motor neurons). One of the available strategies is to activate muscle fibers by the electrical stimulation: a kind of artificial coordination coming from external devices.

In this section, we discuss notably the health conditions dealing with muscular atrophy in response to disuse [28] unsolved by voluntary muscle recruitment approaches (natural coordination). They are usually caused by stroke [29–31] and spinal cord injury [32], and in both conditions, when the lower motor neuron (common final pathway) is not affected, a manifestation – historically described as pyramidal tract syndrome [33] – takes place and increases muscle tone (spasticity), with involuntary responses (spasms), hyperreflexia, and positive Babinski signal. Despite dysfunctional muscle activation remaining present in the muscles, it is insufficient to promote an adequate muscular trophism in order to prevent or attenuate atrophy.

Researchers seem to have reached a consensus around the hypothesis that the process responsible for initiating skeletal muscle atrophy are unique, despite similar upstream signals and downstream phenotypical adaptations [34]. If this hypothesis is validated then, countermeasures to attenuate atrophy may be more effective when designed to accommodate molecular process related to the atrophic stimulus, no matter the nature of the approaches being employed since they provide a basal muscular trophism.

Urso [34] has proposed a schematic explanation of how health conditions such as distraining, spinal cord injury, immobilization, and unloading may initiate a set of steps to install atrophy. All of them seem to interfere in the gene expression (exception to unloading pathway that seems also to interfere through a pathway initiated by increased collagen and metalloprotease levels), and no matter by which metabolic pathway each one follows, the final result is an imbalanced skeletal muscle protein turnover leading to atrophy. In spite of the not confirmed step in the Urso's scheme (Fig. 26.2), the schematic explanation can help us to envision FES-assisted cycling strategies.

Although in Urso's scheme [34], atrophy followed by poststroke hemiparesis conditions is not mentioned; it comes from the same pathway of the postspinal cord injury conditions, resulting in an innervated paralyzed muscle with partial voluntary recruitment. Ideally, if we could fix the muscle activation – by means of electrical stimulation, for instance – we would be preventing the cause of the atrophic process. However, as simple as it may seems, evidences have shown that a combination of unloading and reduced neural activity are jointly referred as "disuse" resulting in muscle atrophy [28]. So, in the approach discussed in this chapter, *the activation powered by electrical stimulation must be accompanied by a minimal continuous load applied in the trained limbs*, which hinders therapeutic strategies to the point of researchers stating that no good therapies are available to prevent or mitigate atrophy.

By means of animal models in rodents under disuse conditions with intact nerves, a rapid loss of muscular mass can be observed within 1 or 2 weeks, followed by a slowing of the rate of muscle loss until the muscle reaches a plateau represented by a new lower steady state, i.e., a new trophic state [28, 34–36]. Bodine [28] showed the effect of hind limb unloading and reloading on muscle mass on soleus (Sol),

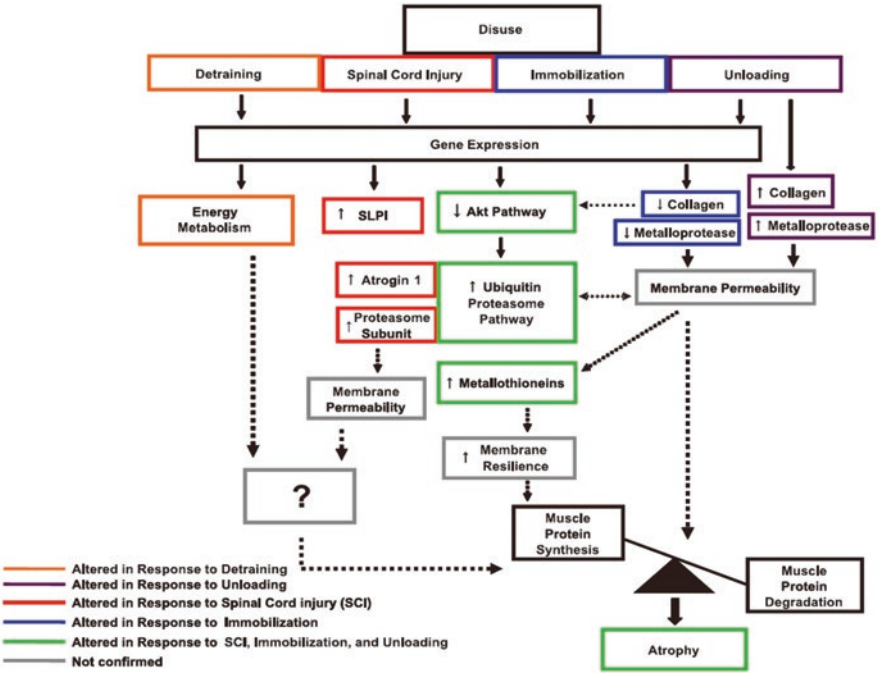

Fig. 26.2 Signaling pathways affected by various atrophy models [34]. Pathways influenced by detraining (orange), SCI (red), immobilization (blue), and unloading (purple). Common pathways affected by SCI, immobilization, and unloading are outlined in green. Pathways that are not well defined at this point are outlined in gray. Solid arrows indicate confirmed alterations. Hashed arrows indicate alterations that are less well characterized and in need of future research to ascertain their role in skeletal muscle atrophy

medial gastrocnemius (MG), tibialis anterior (TA), and extensor digitorum longus (EDL) of female Sprague Dawley rats during 21 days unloading time course followed by 14 days reloading (Fig. 26.3).

Even if we have to consider the differences between the rodents' and humans' metabolisms and the respective time courses to make conjectures, the pattern of muscle tissue loss may give us insights to analyze benefits on functional rehabilitation and muscle atrophy coming from stimuli triggered by FES-assisted strategies which are discussed in Sect. 26.4 of this chapter.

In humans, we can find evidences of a differential atrophy across muscle and fiber types in response to disuse, even if not all human studies have detected it [28, 34]. The lack of success to detect differential atrophy in human studies could be due to the small biopsy samples taken from single site in periphery of the muscle belly, usually from the vastus lateralis: a knee extensor. Seemly, if the ankle extensors (soleus and gastrocnemius muscles) were assessed, the soleus could be found more vulnerable to atrophy than gastrocnemius muscle and type I fibers and more promptly susceptible to the loss than type II, as reported by studies that utilized

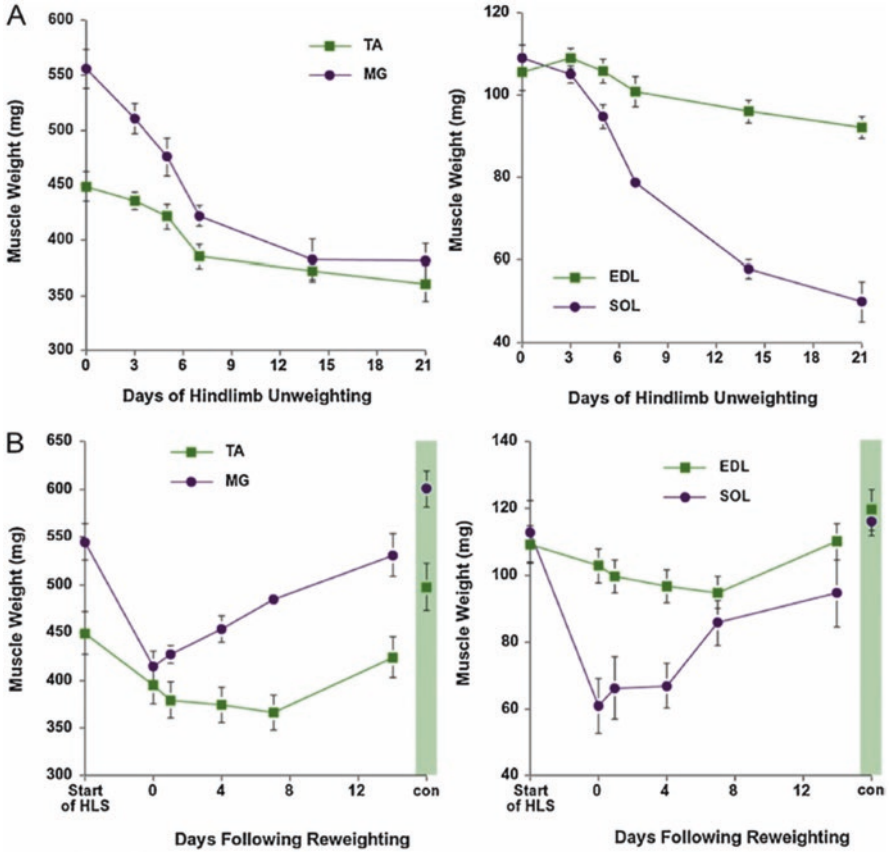

Fig. 26.3 Bodine's figure [28] presenting a 21 days' unloading time course followed by 14 days reloading in animal model of atrophy. Muscle wet weight of the tibialis anterior (*TA*), medial gastrocnemius (*MG*), extensor digitorum longus (*EDL*), and soleus (Sol) of female Sprague Dawley rats (n = 10/time point) following hind limb unloading (A – unweighting) and reloading (B – reweighting). Data points are mean ± standard deviation (SD). A separate cohort of controls was taken at the start (y axis) and the end (green-shaded area) of the experiment to assess normal growth over the experiment

magnetic resonance imaging (MRI) to examine volume and cross-sectional area changes during disuse atrophy [37].

If not all human studies have detected a differential atrophy due to methodological difficulties, to identify it in paralyzed muscles is an additional challenge. Motor weakness due to partial paralysis (paresis) or total paralysis (plegia) is commonly, but differentially, manifested in poststroke hemiparesis/hemiplegia [38] and post-SCI paraplegia/tetraplegia [39] conditions. Even under available therapy, patients with these health conditions present an increased tendency to reach muscle atrophic states leading to a permanent disability and requiring institutional care even after discharged from the rehabilitation programs.

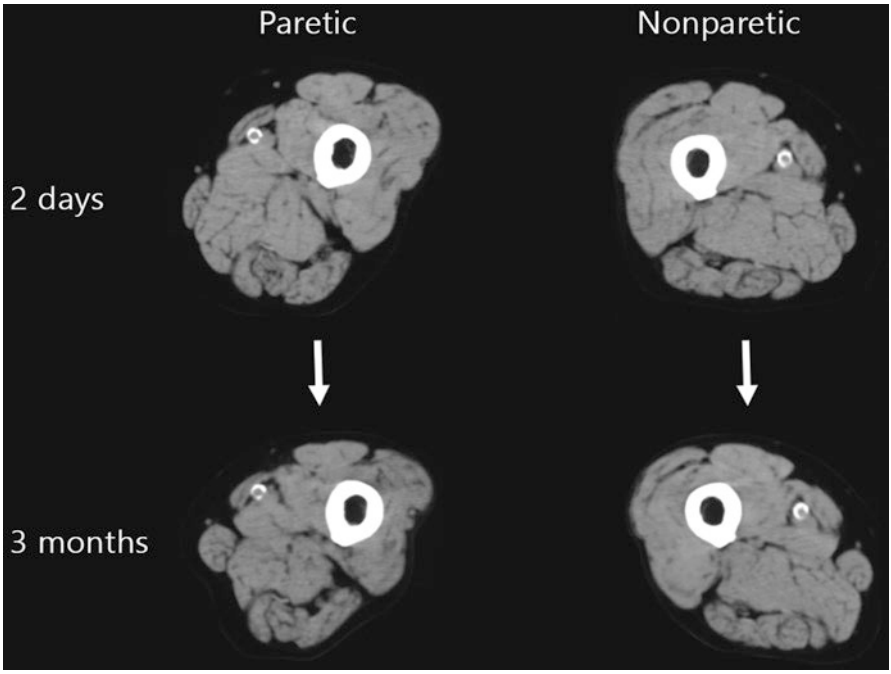

Fig. 26.4 Femoral muscle volume changes observed from a computed tomography image took from a 64-year-old elderly person. Images were made after 2 days and 3 months of immobilization due to the bedridden state from paretic (affected) and non-paretic legs [35]

For example, to realize how fast atrophic states take place in paralyzed muscles, Fig. 26.4 shows the results obtained from a computer tomography after 2 days and 3 months' immobilization due to the bedridden state which caused disuse muscle atrophy in the paretic leg as well as the non-paretic leg of a poststroke elderly subject (64-year-old). Even in healthy young subjects, the disuse atrophy of lower limb muscles was confirmed to occur following 35 days of bed rest [35].

In spite of the acquired health condition, in most of cases, the atrophic state of the muscles may be partially reversible if the activation through the motor unit[5] has been restored. Presumably, what determines atrophic states is a close relationship between oxidative stress[6] and disuse muscle atrophy that is better explored in Sect. 26.1 when we discuss about training protocols.

Additionally, evidences have presented that, in neurologic diseases, the paresis and the altered mobility due to central nervous system impairments are conducted to different and specific patterns of muscle loss (not suitably named by the term,

[5] The lower motor neuron and the skeletal muscle fibers innervated by that motor neuron's axonal terminals.

[6] Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage.

sarcopenia) described as muscle changes coming from disuse atrophy, spasticity, and myosteatosis. Since sarcopenia of the elderly and muscle atrophy and modifications coming from central nervous system diseases have different mechanisms, they do not probably respond equally to the same treatments [36]. The different responses to treatments are a strong reason for why we discuss alternative approaches to treat atrophic states for poststroke hemiparesis and postspinal cord injury conditions in this chapter.

26.3 Choices to Face Muscle Atrophy Resulting from Paralyzed Muscle with Preserved Innervation

The health conditions discussed in the present chapter (poststroke hemiparesis and postspinal cord injuries) – in which the common final pathway responsible to promote voluntary recruitment is not impaired but without corticospinal modulation arriving from descending pathways – are potentially responsible for the atrophy resulting from the paresis. However, muscle atrophy never is the primary debilitating loss followed by the event which generated such health conditions.

Systematic reviews published in the final of the last century [40, 41] had already described paresis, paralysis, spasticity, and sensory-perceptual dysfunction as primary effects leading to contractures and disuse muscle atrophy in association with metabolic and endocrine changes that result in a cyclic process that if not interrupted quickly will enhance the activity limitations and participation restrictions [42]. So *disuse muscle atrophy is a secondary effect to be avoided*.

This simple conclusion is crucial to define intervention choices, once according to the disease attributes – specially defined by the worsening of the health condition over time – we have to propose an intervention to avoid atrophy or restore the muscular trophism. The recent review [7] seems to agree that initiating exercise during a critical early period may optimize clinical outcomes, specially to prevent atrophy of sublesional skeletal muscles taking place within the first 6–12 weeks post-injury.

In spite of the apparent advantages of acute interventions, the clinical usage varies largely, especially because early exercise may trigger autonomic dysreflexia, unregulated hyperthermia or effects of neurogenic shock, and other side effects that are not precisely known to favor recommendations regarding exercising early after central nervous injuries [7]. For this reason, disuse muscle atrophy is an expected outcome to be fought in chronic health conditions followed by poststroke hemiparesis or postspinal cord injuries.

A common residual defect after upper motor neuron impairments coming from stroke or spinal cord injuries is the reduced number of recruitable motor units during activities which thereby limit endurance capacity. In both cases, as parts of their bodies are partially ready to exercise, training programs of aerobic exercise propelled by the non-paralyzed limbs are the first option to be considered by therapists

around the world. For poststroke hemiparesis condition, a kind of asymmetrical cycling propelled by the non-paretic hemibody is performed [43], whereas for post-spinal cord injury condition, upper limb aerobic exercises combined or not with passive lower limb exercises are commonly recommended [44]. Despite training programs focused on the residual non-paralyzed muscles which had reached significant results to break the cyclic process mentioned above, they do not avoid muscle atrophy in the paralyzed part of the body.

The scientific evidences had already revealed [41] that the paretic muscles of poststroke hemiparesis people performing asymmetrical exercises presented reduced muscle blood flow, a greater lactate production, a higher utilization of muscle glycogen, and a diminished capacity to oxidize free fatty acids when compared to the non-paretic muscles, not avoiding muscle atrophy in the paralyzed muscles.

Fortunately, a great variety of interventions have been investigated to improve the muscles and bones of paralyzed limbs, with the aim of reducing secondary conditions such as fragility fractures and endocrine/metabolic diseases. Some of these methods include early exercises [7, 45], functional electrical stimulation (FES) [7, 45–47], body weight-supported treadmill training [7, 45], cycling ergometry [48–54], and robot-assisted ambulation [55–57] which are available to be explored.

In individuals with paralyzed muscles and with preserved innervation, functional electrical stimulation (FES) can be used to produce isometric contractions, to facilitate gait, or to produce contractions against resistance during cycling or leg extensions [7, 58]. The results from a recent review appear to indicate that paralyzed muscle tissue can promote hypertrophy with FES within a 3-month time frame [7]. The magnitude of muscle hypertrophy may be related to either the amount of resistance or the length of intervention, but given the diversity of outcome measures, such comparisons remain speculative.

The most common therapy for both health conditions discussed in this chapter has been primarily directed at activities focused on compensatory strategies to promote independence in preparing for discharge, teaching new ways to move in bed, get dressed, transfer in and out of a wheelchair, as well as provision of assistive devices [59–61].

26.4 Neuromuscular Electrical Stimulation (NMES), Functional Electrical Stimulation (FES), and FES-Assisted Cycling: Devices, Protocols, Thresholds, and Cautions to Be Taken

The most familiar terms to define the clinical use of electrical stimulation are neuromuscular electrical stimulation (NMES) and functional electrical stimulation (FES). As precisely defined by Sheffler and Chae [62], NMES refers to the electrical stimulation of an intact lower motor neuron – described as the final common pathway – to activate paralyzed or paretic muscles. In turn, the clinical applications of

NMES provide either a functional or therapeutic benefit; then, Moe and Post [63] introduced, for the first time, the term FES to describe the use of NMES to activate paralyzed muscles in precise sequence and magnitude to accomplish functional tasks. In their study, they used electrical stimulation to assist the ambulation in the poststroke hemiplegic condition.

FES can induce the synchronized contraction of paralyzed yet innervated muscles of the corresponding intact alpha motoneurons. A stimulating current applied to electrodes placed over sensory-motor structures creates a potential field along the axons. This gradient induces a transmembrane current through an ionic flow, which may generate an action potential. The action potential then propagates along the nerve causing the contraction of the muscle.

Classically, a series of rectangular biphasic (symmetrical or asymmetrical) electrical pulses are delivered by the electrical stimulators. The stimulation pattern can be described by its global envelope and the pulse parameters: amplitude or intensity of pulses (current or voltage), frequency or pulse repetition rate, and duration of single pulses [64]. Controlling the injected charge and stimulation frequency allows modulation of the muscle force.

Nowadays, there are several applications based on FES, which include control of respiration [65] and bladder function [66], upper limb performance of activities of daily living [67, 68], and tasks involving upper [69, 70] and lower limbs, such as standing and ambulation, associated or not with another assistive technology [25, 47, 49, 51, 71–75]. In a simplified manner, when FES is used within an assistive device or system, the ensemble becomes a neuroprosthesis that is able to enhance functional activity as a result of the interface between the machine and the nervous system [62, 76].

FES-assisted cycling consists in a neuroprosthesis applying sequential stimulation, typically to the quadriceps, hamstrings, and glutei, to induce bilateral flexion and extension of the legs to generate a cycling motion.

Ergometers using FES-assisted cycling has been utilized during the last decades for rehabilitation purposes in order to improve the general condition and to prevent deterioration in subjects with central neurological impairments [77]. The benefits range from cardiopulmonary fitness to tissue changes due to adaptations in the trophic states influenced by the demanding effort to propel a system for stationary exercise or mobile cycling.

Some studies support findings for the potential clinical efficacy of FES cycling for reducing the risk of secondary medical complications in subjects with paralysis. The potential therapeutic benefits include conditioning the cardiopulmonary, muscular, and skeletal systems and improving other physiological and psychological performances [78]. Among the many benefits reported, the muscle remodeling in response to FES-assisted cycling seems to promote adaptations to restore suitable trophism, avoiding drastic and everlasting atrophy.

For decades, FES has been used to elicit rhythmic cycling exercise in order to promote central and peripheral hemodynamic responses [79]. Previous studies have shown that activating lower limb skeletal muscle pump augments venous return, improves ventricular filling, and increases oxygen uptake. FES leg exercise has

been shown to promote central and peripheral hemodynamic. However, FES leg exercise alone has often resulted in significantly lower submaximal oxygen uptakes compared with arm crank ergometry.

FES-assisted cycling has been proposed as an option to provide active lower limb involvement in alternative therapies and locomotion solutions, once that active muscle contraction of paralyzed muscles can be evocated to develop locomotion devices in a combination of artificial (FES-device system) and natural (nervous system) controls. Not only FES-assisted cycling is an example of applying the concept of propelling devices by paralyzed muscles and by electrical stimulation, but a range of FES-assisted devices could also be thought of based on the same concept [80]. As an example, in our group we have a special interest in investigating FES-assisted cycling [25, 51, 53, 54] and FES-assisted transfer [50, 81].

Several FES-assisted cycling ergometers are commercialized, such as the MOTOmed (Reck, Betzenweiler, Germany) or the BerkelBike (BerkelBike BV, AV's-Hertogenbosch, Netherlands). We developed an FES-assisted tricycle system over the structure of a commercial recumbent trike with adjustable crankset position and 24-speed system with adapted chain tensioner (Fig. 26.5). All mechanical components were instrumented with a wireless inertial sensor that enables to estimate the crank position and angular speed for each crank spin. Crank position and angular speed were the inputs to the artificial control system activating cyclically in specific sequences of quadriceps, hamstring, and gluteus muscular groups. In addition to the sensor readings, the artificial controller is modulated directly by the user

Fig. 26.5 FES-assisted cycling system developed by our group to compete in the Cybathlon 2016 whose development detail was published in the IEEE Robotics and Automation Magazine [94]

through an interface based on buttons and display. While the display features information such as speed and stimulation intensity, the buttons may be used to update FES parameters and trigger alternative stimulation sequences.

The first reported FES-cycling event was organized in Cardiff (United Kingdom) in 2006. Organizers aimed at advertising FES as a recreational activity and not only a hospital-based therapy. According to them, muscle training should be an enjoyable activity in order to be attractive. In 2004, two FES-rowing athletes participated into the British Indoor Rowing Championships (BIRC) and completed the Olympic 2000 meters' distance in open competition with able-bodied athletes. In 2016, an international competition, Cybathlon, was held in Zurich (Switzerland) to promote assistive technologies, including FES cycling. Twelve international teams participated [82–85].

Although significant advantages have been reported about FES-cycling devices, little or no attention has been paid to cushioning systems for tricycles – an issue already discussed for wheelchairs and demonstrably important to avoid pressure ulcer and lesions. Trike cushions must promote safe impact and do not enhance risk coming from the tissue changes including weight and fat mass gain, skeletal muscle atrophy, and fat infiltration into the muscles, bone loss, and bone shape adaptations at the pelvis, vascular perfusion changes, and microstructural changes in the skin and muscle that are associated with disuse and affect the biomechanical behavior of these tissues [39]. In the wheelchair, cushioning systems represent support surfaces designed to accommodate on one side; the microchanges that occur for a seated person throughout the day, expecting compressive strength generated by posture and position or muscle tone modified by spasticity; and on the other, the macrochanges in the anatomy, tissue composition, and long-term tissue (patho)physiological changes.

Among the methods to minimize the structural and functional body impairments secondarily caused by poststroke hemiparesis and postspinal cord injury, including restoration of muscle trophism avoiding disuse atrophy, FES-assisted cycling seems to be an adequate recommendation, especially after 2 years of injury, when few attractive options are available to motivate disabled people who had already completed the rehabilitation process. Despite the benefits of remodeling muscular trophism of the paralyzed muscles in the midst of a lack of choices, FES-assisted cycling allows to change the handicap condition to a locomotion condition improved by technologies, in which people remain engaged in a social structure, paving the way for activity-based therapies to promote physical, mental, and social recovery [25].

Regarding preparation to FES-assisted cycling, no matter what the purpose (locomotion, leisure, or sports), before training, we have to investigate the responsiveness of paralyzed muscles to electrical stimulation. By anatomical reasons, the most responsive to FES are the people facing poststroke hemiparesis, once their lesions are addressed in the brain, preserving totally the common final pathway to muscle recruitment (lower motor neuron). For people with postspinal cord injuries, the nature (infectious) and the level (at the medullary cone and bellow) of the injury represent an obstacle to FES-assisted cycling, once the final pathway to muscle recruitment was impaired by the primary lesion. Figure 26.6 shows an image that

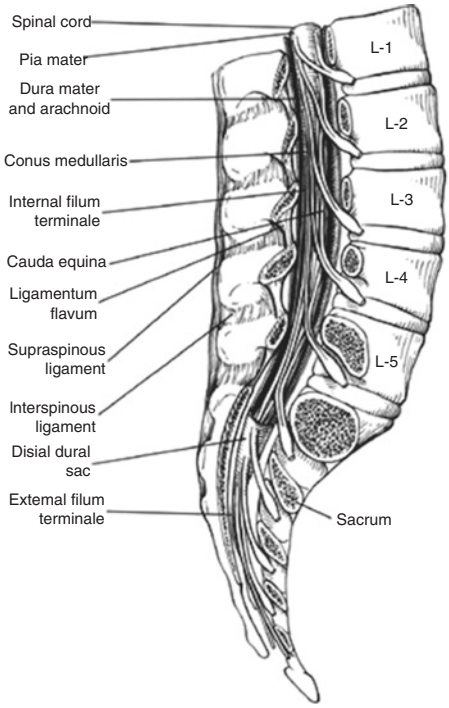

Fig. 26.6 An illustration of a section in the sagittal plane showing the vertebral-medullary relations of the spinal cord bellow the T12 (twelfth thoracic vertebral level). At this level, the medullary cone is impaired compromising the common final pathway to activate the muscles

allows identifying the anatomical relations mentioned above. Among 14 Brazilian participants who attended a public call to be prepared for FES-assisted cycling, 8 volunteers (57%) responded to the NMES. All of them had traumatic injury above the T12 level [25].

In our experience, being responsive to NMES do not ensure ability to perform FES, mainly if the task involves generating force by lower limb paralyzed muscles to overcome the gravitational action or to push stationary objects (as a bike pedal). Only one of the eight responsive participants had sufficient bone quality, tolerance to efforts, and minimum muscular response to achieve all the steps to initiate the FES-assisted cycling protocol [25].

Although low-energy fractures have been reported as common for individuals with spinal cord injury occurring during events that would not normally cause fracture, such as a transferring from bed to wheelchair or being turned in bed, fractures for this population who partake in training programs including FES, standing frames, and treadmill walking have not been studied extensively [46].

Our protocol was divided in two phases separated by the minimum performance thought by us as recommended to start FES-assisted cycling: 30 min of cycling at a cadence of 35 rpm (details of the protocol can be found in our previous publications already cited along the chapter). Although we did not record any measure directly related to changes in muscular trophism, muscle strength generated in the first assessment by NMES and rated by the Medical Research Council increased from

2/5 contractions performed by trials lasting less than 10 min and only involving quadriceps muscular group to 5/5 contractions performed by trials lasting 30 min involving quadriceps, hamstring, and gluteus muscular groups repeated three times per week at the end of 18 weeks. Certainly, this first phase of the protocol modified the atrophic state of the paralyzed muscles.

During the second phase of the protocol, all electrical stimulation was performed in the FES-assisted tricycle system developed by us. Surface electrical stimulation was conducted on the quadriceps (two channels), hamstrings (one channel), and glutei (one channel) muscles, starting with stationary training provided by a resistance roll to prevent free spin of the wheel. Following the first week performing the stationary mode, the participant was able to pedal during 20 min (outdoor training) by means of a closed-loop stimulation at a frequency of 20–30 Hz, maximum pulse width of 300 μs, and current intensity varying from 20 mA to 96 mA.

Inspired by the Cybathlon experience and motivated to provide benefits to other health conditions, we decided to explore the effects of the FES-assisted cycling to improve health-related states for poststroke hemiparesis people. According to Ferrante and colleagues [86], rehabilitation programs including FES-assisted cycling demonstrated a significant increase of the power output (the product between the torque and the speed) over each semi-revolution in which the paretic and non-paretic legs were pushing at the end of the 20 days of treatment, analyzing a 20-patient sample. The protocol was performed every day for 4 weeks by trials lasting 35 min, merging passive (5 min) and FES-assisted cycling (10 min) phases in a total of five phases beginning and finishing by passive cycling.

As highlighted in Sect. 26.2 of this chapter, the mentioned protocol combines activation powered by electrical stimulation and accompanied by a minimal continuous load applied in the pedal, generating a power output that triggers molecular events to prevent or mitigate atrophy. Only the electrical stimulation could not enable to restore effectively the muscle trophism.

Also in the investigation of FES-assisted cycling for poststroke hemiparesis people (Fig. 26.7), however, compared to a control group by a randomized clinical trial, Bauer et al. [87] evidenced that the potential changes in the muscular trophism were accompanied by improved ambulation and mobility.

To better explore the effects of the FES-assisted cycling for hemiparesis condition, we are developing a cycling system (Fig. 26.8) equipped with a multichannel stimulator able to trigger paralyzed muscles in the paretic leg coordinated by an artificial control work together with the voluntary control of the cadence generated by the non-paretic leg. The system will be developed to generate biphasic stimulus until 140 mA, with pulse width setting until 1000 μs, reaching 100 Hz of frequency. All the control will be performed by means of a graphic interface for mobile devices, allowing to explore a great variety of protocols.

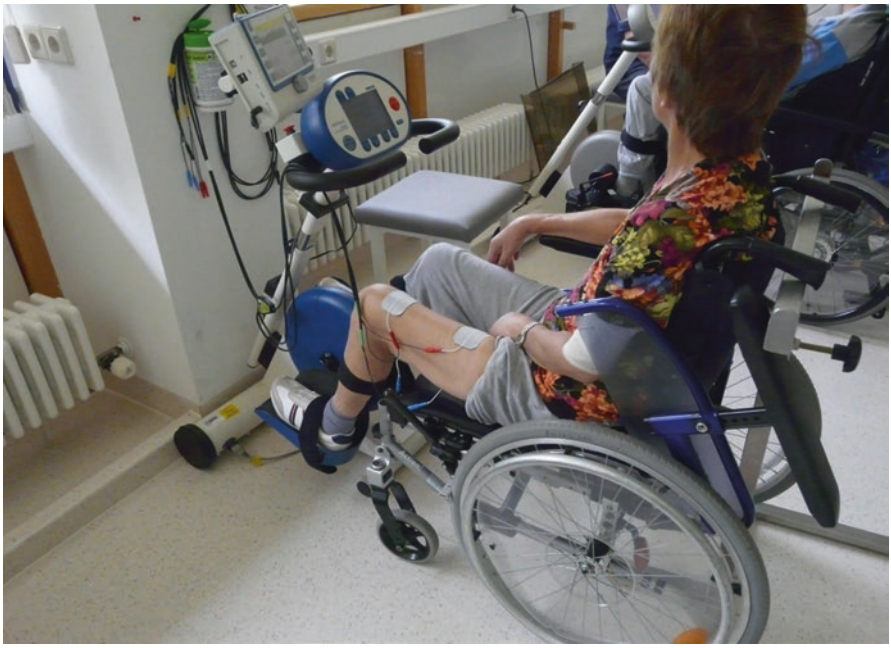

Fig. 26.7 FES-assisted cycling used by Bauer and colleagues [87] to investigate the effects in the ambulation and mobility of patients with poststroke hemiparesis from 7 days to 6 months after the cerebrovascular event in a randomized controlled pilot study

Fig. 26.8 Stationary cycle ergometer system in process of developing by our research team to explore protocols of FES-assisted cycling for poststroke hemiparesis people

26.5 Conclusion

Skeletal muscle atrophy as observed after a spinal cord injury is associated with cardiometabolic health consequences with increased risks of developing chronic secondary conditions and impacts the quality of life. Functional electrical stimulation-assisted cycling allows to activate several muscle groups in one exercise and has been seen as an interesting training strategy [88]. Some studies have already shown some interesting results in chronic SCI and poststroke individuals as a solution able to provide physical integrity benefits, increased muscle mass, and reduced spasticity accompanied with an improved quality of life [89–91].

FES can be used to propel tricycle and ergometer cycles, adding a recreational facet to the activity with interesting outcomes as observed in some rehabilitation centers. Motivation is indeed a central aspect in training programs. We have mainly discussed about surface FES in this chapter, but implanted neuroprosthetics can be considered as well with enhanced performances [85, 92].

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Chapter 27
To Reverse Atrophy of Human Muscles in Complete SCI Lower Motor Neuron Denervation by Home-Based Functional Electrical Stimulation

Helmut Kern, Paolo Gargiulo, Amber Pond, Giovanna Albertin, Andrea Marcante, and Ugo Carraro

Abstract After spinal cord injury (SCI), patients spend daily several hours in wheelchairs, sitting on their hamstring muscles. SCI causes muscle atrophy and wasting, which is especially severe after complete and permanent damage to lower motor neurons. A European Union (EU)-supported work demonstrates that electrical fields produced by large electrodes and purpose-developed electrical stimulators recover both quadriceps and hamstring muscles, producing a cushioning effect capable of benefitting SCI patients, even in the worst case of complete and long-term lower motor neuron denervation of leg muscles. We reported that 20 out of 25 patients completed a 2-year h-bFES program, which resulted in (1) a 35% increase in cross-sectional area of the quadriceps muscles ($P < 0.001$), (2) a 75% increase in

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mean diameter of quadriceps muscle fibers ($P < 0.001$), and (3) improvement of the ultrastructural organization of contractile machinery and of the Ca2+-handling system. Though not expected, after 2 years during which the 20 subjects performed 5 days per week h-bFES of the atrophic quadriceps muscles, the CT cross-sectional area of the hamstring muscles also augmented, increasing from 26.9+/−8.4 (cm^2) to 30.7+/−9.8 (cm^2), representing a significant ($p \leq 0.05$) 15% increase. Here we show by quantitative muscle color computed tomography (QMC-CT) that h-bFES-induced tissue improvements are present also in the hamstring muscles: a once supposed drawback (lack of specificity of muscle activation by large surface electrodes) is responsible for a major positive clinical effect. Interestingly, 2 years of home-based FES by large surface electrodes reversed also the denervation-induced skin atrophy, increasing epidermis thickness. Finally, we would like to attract attention of the readers to quantitative muscle color computed tomography (QMC-CT), a sensitive quantitative imaging analysis of anatomically defined skeletal muscles introduced by our group to monitor atrophy/degeneration of skeletal muscle tissue. Worldwide acceptance of QMC-CT will provide physicians an improved tool to quantitate skeletal muscle atrophy/degeneration before and during rehabilitation strategies so that therapy for mobility-impaired persons can be better prescribed, evaluated, and altered where needed.

Keywords Muscle atrophy · Home-based functional electrical stimulation · Quantitative muscle color computed tomography

27.1 Background

All skeletal muscle atrophy is the loss of muscle size and strength, which occurs with neural and skeletal muscle injuries, prolonged bed rest, space flight, normal aging, and diseases such as sepsis cachexia, diabetes, etc. If unabated, skeletal muscle atrophy can be extremely debilitating, increasing morbidity and mortality in affected people [1, 2]. After spinal cord injury (SCI), patients spend daily several hours in wheelchairs, sitting on their hamstring muscles. Spinal cord injury causes muscle wasting, which is especially severe after complete and permanent damage to lower motor neurons [3–6]. In previous studies, we have shown that denervated, atrophying muscles were rescued by 2 years of home-based functional electrical stimulation (h-bFES) when a purpose-developed electrical stimulator (now commercially available, “Stimulette den2x” of the Schuhfried Medizintechnik GmbH, Vienna, Austria) provided the needed high currents to large surface electrodes covering the quadriceps muscles [7–13]. Interestingly, we recently demonstrated that the skin exposed to 2 years of electrical stimulation (to induce contractions of the atrophic Quadriceps muscles) shows an improvement in epidermis thickness [14]. Here we report that the electrical fields also produce clinically relevant recovery in atrophic hamstrings muscles not in direct contact with the very large electrodes of the Vienna protocol of h-bFES for denervated, atrophying muscles. A once supposed drawback is, indeed, responsible for a major positive clinical result.

27.2 Methods

Patients of the EU Program: RISE [Use of electrical stimulation to restore standing in paraplegics with long-term denervated degenerated muscles (QLG5-CT-2001-02191)] with complete conus and cauda equina lesions were enrolled and gave appropriate informed consent. Using a custom-designed stimulator and large surface electrodes designed and implemented in Vienna (Austria), we stimulated denervated atrophic leg muscles according to the h-bFES strategy. Muscle mass, force, and structure of the stimulated quadriceps muscle were determined before and after 2 years of h-bFES, using the quantitative muscle color computed tomography (QMC-CT) [14–17], measurements of knee torque during stimulation, and muscle biopsies analyzed by light and electron microscopy [6, 10–12]. QMC-CT is a highly sensitive quantitative imaging analysis of one muscle or group of anatomically defined skeletal muscles introduced by ourselves to monitor skeletal muscle tissue. QMC-CT is based on acquisition of high-resolution CT scans and the use of special image processing tools allowing evaluation of soft tissues and skeletal muscle segmentation [15–18]. We developed QMC-CT as a by-product of the EU RISE project to complement follow-up in extreme cases of muscle degeneration, i.e., complete *conus* and *cauda equina* syndrome, a SCI sequelae in which leg muscles are completely disconnected from the nervous system. QMC-CT uses CT numbers, i.e., Hounsfield units (HU), for tissue characterization. In the process of assessing muscle quality, soft tissues were discriminated as follows: subcutaneous fat, intramuscular fat, low-density muscle, normal muscle, and fibrous-dense connective tissue (Fig. 27.1). To further evaluate the data, pixels within the defined interval of HU values (or, more generally, gray values when these data are not from CT scans) are selected and highlighted in colors (red for normal muscle tissue, yellow for intramuscular adipose tissue, green and blue for fibrous

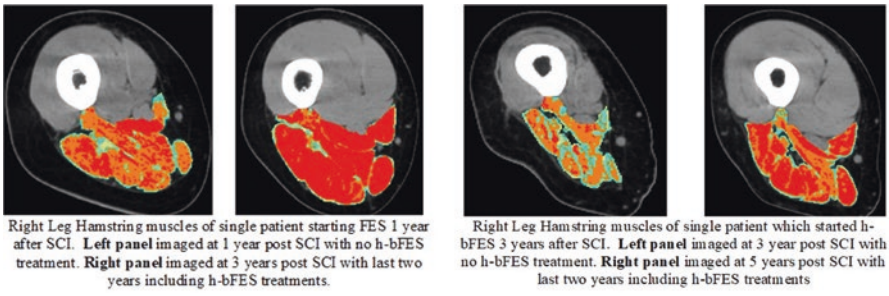

Fig. 27.1 Muscle color computed tomography of thigh muscles at 20 cm from femur head. Both the quadriceps and the hamstring muscles increased in size and tissue density (improved content of the red healthy muscle fibers) after 2 years of training using the Vienna protocol for h-bFES of permanently denervated human muscles. Comparison of the left and right left panels provides strong evidence of the deterioration that occurred in the long-term denervated muscles between the first and the third year post SCI. However, it is worth noting that, even starting 3 years post SCI, h-bFES is able to recover substantially the hamstring muscles

connective tissue), while other tissues with HU values outside the threshold ranges remain black, including the extra-muscle adipose tissue [15–18].

27.3 Results

We reported that 20 out of 25 patients of the EU Program: RISE [Use of electrical stimulation to restore standing in paraplegics with long-term denervated degenerated muscles (QLG5-CT-2001-02191)] completed a 2-year h-bFES program, which resulted in (1) a 35% increase in cross-sectional area of the quadriceps muscles ($P < 0.001$), (2) a 75% increase in mean diameter of quadriceps muscle fibers ($P < 0.001$), and (3) improvement of the ultrastructural organization of contractile material and of the Ca^{2+}-handling system [12]. Furthermore, a truly impressive 1187% increase in force output during electrical stimulation occurred ($P < 0.001$) which was sufficient to allow 25% of the end-point subjects to perform FES-assisted stand-up exercises [12]. Though not expected, after the 2 years during which the 20 SCI subjects performed h-bFES 5 days per week by large electrodes covering the quadriceps muscles, the CT cross-sectional area of the hamstring muscles also augmented, increasing from 26.9+/−8.4 (cm^2) to 30.7+/−9.8 (cm^2), representing a significant 15% increase ($p \leq 0.05$) [12].

QMC-CT analyses confirm that h-bFES-induced muscle improvements (noted in CT of quadriceps muscle) are present also in hamstring muscles [15–21]. Figure 27.1 shows, by computed tomography of thigh muscles at 20 cm from femur head, that the quadriceps and the hamstring muscles increased in size and tissue density (improved content of healthy muscle fibers) after 2 years of training using the Vienna protocol for h-bFES for permanently denervated human muscles. Comparison of the right panels provides strong evidence of the deterioration of the long-term denervated, atrophying muscles among 1 and 3 years post SCI. However, it is worth noting that, even starting 3 years post SCI, h-bFES is able to recover substantially the hamstring muscles.

27.4 Discussion and Perspective

Persons suffering with SCI must use wheelchairs to gain some mobility independence, this resulting in them sitting several hours each day on their hamstring and gluteal muscles. The prolonged seating contributes to severe atrophy of the muscles and edema of the legs, with increased risks of decubitus ulcers and deep thrombophlebitis. Of particular importance in SCI is whether the connection between the muscle and the nerve is preserved or the muscle is denervated due to complete peripheral nerve lesion. In the latter cases, the denervated muscle becomes unexcitable with commercial electrical stimulators and undergoes ultrastructural disorganization within a few months, while severe atrophy with nuclear clumping and

fibro-fatty degeneration appears later on within 3 and 6 years [4, 6–9]. Our work with h-bFES is important because it leads to muscle recovery, specifically in the worst case of complete, permanent lower motor neuron muscle denervation. Indeed, we have documented the recovery of the quadriceps muscles when directly stimulated. Interestingly, here we show that our h-bFES of quadriceps muscles by large electrodes is not selective but that co-contractions of the hamstring muscles occurred and resulted in increased size and quality of this muscle group. This was not expected. Indeed, biomedical engineers may be unhappy with this result because it shows that the stimulation is not precisely focused on one muscle and, indeed, co-contraction of the antagonistic muscle group (hamstring) interfered with the analysis of the quadriceps muscle strength during FES-activated contraction [12]. Nonetheless, the improved hamstring muscles contribute to the cushioning provided by the recovered muscle tissue, and this is a major clinical benefit of the Vienna protocol, validated by the EU Project RISE (Use of electrical stimulation to restore standing in paraplegics with long-term denervated degenerated muscles (QLG5-CT-2001-02191)) [12]. The h-bFES sustained increase in muscle mass is also important because of the increase in leg perfusion that preliminary analyses are demonstrating that they are extended to the skin [14]. The improvement will be even more if patients add h-bFES of gluteal muscles to their training workout.

We suspect that the concept of minimal FES (i.e., producing external work) is not well understood or even known and needs further explanation to professionals (e.g., medical practitioners, family doctors, physiatrists, and physiotherapists) who have contact with persons in need. We believe that this is particularly true in the USA because we receive many inquiries from citizens of this country about the application of h-bFES. We have done our best to attract attention to valuable results by publishing in high impact journals. The continued dissemination of our results is now in the good hands of the editors of top medical journals and of the advisors of Granting Agencies. We are confident that they will share our desire to offer to people in need the chance to live a better life, as they deserve.

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Chapter 28
Preventing Muscle Atrophy Following Strokes: A Reappraisal

Sunil Munakomi

Abstract Muscle atrophy leading to muscle weakness accounts for major cause of disabilities among stroke survivors. It amounts to compromised gait and prevails to viscous cycle of diminished physical capacities and compromised participation in rehabilitative tasks. There is predisposition to recurrent strokes due to added risk of developing metabolic syndrome. Therefore, beyond the shadow of doubt, there is ripple effect of rehabilitation and thereby muscle protection in these subsets of patients. Herein, we highlight upon the newer insights with regard to preventing muscle atrophy following strokes.

Keywords Stroke · Muscle atrophy · Rehabilitation

28.1 Introduction

Stroke accounts to major proportion for embarking disabilities in the global front [1]. Its long-term consequences are lauded on the facts that more than 30 % of survivors from strokes ultimately require some assistance during walking [2]. Paradoxically a survey carried out in 2005 in the United States revealed that only 31% of such patients opted for any rehabilitative facilities [3]. Moreover, rehabilitation strategies seldom extend beyond one year of initiation among these groups [4]. This is alarming because there is uprise in the number of stroke survivors owing to the advancement in clinical medicine [5]. In the context of low-income nations, it can have ripple effects hampering the patients in multispectral fashion as well as jeopardizing the proper allocation of available limited resources allocated in the health sectors [6].

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28.2 Pathophysiology

There have been various explanations for the genesis of the muscle weakness following strokes such as de-innervation, fiber-type shifts, disuse atrophy, as well as associated activation of inflammatory cascade [7, 8]. Findings have demonstrated increased tumor necrosis factor (TNF-alpha) expression in paretic leg muscle, thereby governing upon the fact that inflammatory pathways are accelerated in stroke muscle [9]. There is also tropism toward anaerobic metabolism in such affected muscles [7, 10]. Stroke patients invariably have low endurance to exercise due to various factors. Foremost being the added burden of energy lost for counteracting the reduced efficiency of motion as well as associated spasticity. A study by Landin and colleagues found evidence of reduced blood flow, excessive lactic acid production, and a diminished fatty acids oxidizing capacity in these paretic muscles [11, 12].

From the physiological point of view, there is super-excitability of stretch reflex, under firing of the motor units in the agonist group whereas simultaneous co-activation of the motor units in the antagonist group [13]. There is sarcopenia along with deposition of noncontractile tissues like fat rendering them comparatively weaker than their healthy counterparts [7]. This has also been implicated for the coexistence of insulin resistance which increases the odds of metabolic syndromes and thereby risk of recurrent strokes in these patients [14, 15]. This can exacerbate the vicious cycle of reduced tolerance to physical activity with further deterioration in their independent functionality [16]. The synergistic ill effects of increased energy needs and low aerobic capacity compel these patients to execute even basic activities of their daily livings at the peak of their physiological limits. This prevails to viscous cycle of diminished physical capacities and thereby compromised participation [17].

Laboratory models of cerebral ischemia have implicated for the role of ubiquitin-proteasome pathway in muscle atrophy [18]. Myostatin, also known as growth differentiation factor 8 (GDF8), activates ubiquitin-proteasome system, thereby accelerating proteolysis, and inhibits the activity of myogenic factors like MyoD [19]. The surge in TNF-α reduces the expression of MyoD, thereby downregulating slow-twitch protein synthesis in the hemiparetic leg. It also accelerates oxidative stress via activating nuclear factor (NF-κB) transcriptional factor, thereby promoting formation of reactive oxygen species [20]. This is further aggravated with prevailing confounders like impaired feeding, sympathetic surge, and the prolonged immobility among such patients. Bulks of the affected muscle group were shown to be reduced by 6% and their strength by 16% in just 10 days of immobilization [20]. Difficulty in walking on a weakened limb amounts to increased oxygen consumption for any activities. There is also diminished physiological fitness reserve in these subgroups [15]. This leads to early fatigability and thereby prevailed tendency among such patients in avoiding performing any tasks [21]. This also encroaches upon their perceived involvement in rehabilitative processes [22].

28.3 Early Diagnosis

There have been major advances in this regard. Quantitative muscle ultrasound (QMUS) has been shown to be reliable in early detection of the architectural changes in the hemiparetic muscles as evident by the presence of fatty infiltration and muscle atrophy [23]. Recent studies have shown positive correlation between low serum *insulin-like growth factor* and its binding protein (IGF-1 and IGFBP-3) co-relating to ensuing muscle atrophy and diminished work performance in the hemiparetic side [24]. Likewise, C-terminal agrin fragment (CAF 22) has also been proven as a noble marker in predicting sarcopenia and the subsequent weakened muscular performance [25].

28.4 Management

First and the foremost, it is prudent to minimize neuronal damage following strokes. In cases of ischemic strokes, endovascular thrombectomy has shown potential to rejuvenate the penumbra zone [26]. Similarly, surgical removal of hematomas in hemorrhagic subtypes compressing upon the internal capsule has shown to accelerate motor improvement [27]. This can be facilitated by the application of diffusion tensor imagings like magnetic resonance (MR) tractography [28].

Exercise should be supervised with regard to frequency, intensity, duration, as well as proper transition for achieving its desired goal. Resistance training enhances neuronal cross talk and improves muscle bulk. Exercise also reduces TNF-α and thereby minimizes prevailing insulin resistance [29]. It also promotes aerobic capacity of the muscles, thereby facilitating endurance, balance, and mobility [30]. There is paradigm shift in attention paid to myo-protective therapy as it is more efficacious than the neuroprotective approaches in terms of functional outcome [31]. Targeted physiotherapy has been the workhorse in achieving this goal. The main determinants that govern the optimal functional recovery include motor relearning, repetition, positive feedback, and motivation. All current strategies on physical rehabilitation prevail on basically two theories [32]. Bobath theory focuses on concurrent facilitation as well as inhibition among the agonist and the antagonist groups of muscles, respectively. Brunnstrom theory, on the other hand, opts for encouragement for upright stance and maximizing mobilization.

Rehabilitation process should also focus on various aspects of muscle architecture such as its fiber length, pennation angle, tendon compliance, etc. so as to amplify recovery process [33].

Endurance exercise training has now become an integral component in rehabilitation process. This halts the relentless cycle of prevailing physical deconditioning and worsening disability as well as motivates participants in their task participation [34]. Exercise capacity is mostly limited owing to the generalized fatigue, thereby supporting the rationale for endurance training in this population [35, 36]. The

cornerstone in both these approaches is muscle reeducation wherein we are empowering the weakened agonist groups of muscles and minimizing the increased tone in the corresponding antagonist groups. Ideally this should be carried out in the position of slight muscle stretch and in a graded fashion depending on the stages of muscle recovery. In the acute phase, most rehabilitation strategies are individually tailored. In the later stages, focus is shifted in promoting group participation in supervised sessions among similar cohorts of participants.

During the acute phase, neurodevelopmental reflexes such as tonic neck and withdrawal reflexes can be utilized for facilitating muscle movements. Tactile stimulation, tapping, and stretch methods can be used as adjuncts for the same. Associated reactions can also be utilized and slowly tapered as the patients relearn and regain normal adaptive mechanisms. Gravity-eliminated positioning can further boost on the recovery. Gradual shift toward gravity-dependent positioning to facilitate weight bearing can then be undertaken followed by the implementation of normal resistance training. Sit to stand, bicycling, and forward stepping are few options available for promoting weight transfer and balancing gait. Studies have documented improvements in knee muscles with positive effects on gait performance and perceived participation. The sit-to-stand (STS) movement has significant impact on joint torque and range of movement, thereby promoting upright mobility and facilitating independent living [37]. Maneuvers to enhance loading on paretic limb also augment the same. It has been proven that single-leg stance in the affected limb augments gait function via improvement in weight bearing during the stance phase [38]. Weight-bearing exercise for better balance (WEBB), overground walking with balance training, and body weight-supported treadmill training are also being utilized for gait control and bodily balance [39]. Bridging exercises (BE) are therapeutically used for lumbo-pelvic stabilization [40]. Treadmill training improves exercise capacity by maximizing oxygen uptake (VO_{2max}), lowers the energy cost, and increases peak ambulatory workload capacity [41]. Task-oriented aerobic exercise improves cardiovascular performance profile [42].

Motor rehabilitation focuses in facilitating motor learning by virtue of change in behavior through continuous practice. This adaptive process of relearning is facilitated by neuroplasticity. This involves myriad of processes such as sprouting of dendritic collaterals to pruning of neural circuits. It has been shown that aerobic exercises promote activity-dependent secretion of brain-derived neurotrophic factor (BDNF) [43]. This helps in facilitating long-term potentiation by promoting interneural cross talk. It is also postulated that aerobic exercises in close temporal proximity to behavioral training prime the central nervous system (CNS) adaptive learning. As per current consensus, 30 min of aerobic and resistive exercises with targeted intensities of approximately 70% heart rate for 4 days per week is recommended. Furthermore, to harness the benefits of BDNF in facilitating motor relearning, bouts of aerobic exercises segregated within 1-h time frame in between the resistive exercises are justified [44].

There are various armamentariums to facilitate above processes. Electrical stimulation in conjunction with biofeedback has shown to increase the strength and range of motion, thereby minimizing disability. It also improves posture well as

weight bearing abilities. It helps gaining voluntary movement provided patient attempts for the same at the surge of electrical input. It also improves sensory feedback [45]. Similarly, intrathecal baclofen (ITB) therapy can augment walking speed [46]. Pharmacotherapy can also aid in motor rehabilitation [47]. Mirror therapy has shown to improve motor function as well as promote activities of daily living [48]. It is a way of puzzling the brain circuit in perceiving the movement of normal arm as that of the paretic arm [49]. Likewise, virtual reality intervention and interactive video gaming focusing on movement visualization via immersion in an artificial man-machine interface have added new paradigm in rehabilitative strategies. It is also capable of rewarding the performer as well as capable of analyzing their performance [50]. Similarly, robots can be mobilized in the labor-intensive phases of physical rehabilitation, thereby allowing ample time for the physiotherapist to focus and supervise on the functional aspects of the sessions. Such guided-force training increases the efficacy as well as efficiency of such programs [51]. Branched-chain amino acids have been used in conjunction to resistance aerobic exercise to improve the muscle capabilities [52]. Long-term use of edaravone, a free radical scavenger, has shown to minimize atrophy in the femoral muscle atrophy, thereby ameliorating movement. From functional outlook, myo-protective therapy seems to have the upper hand compared to neuroprotective approaches [31]. Recently SB623, a mesenchymal stem cell, restored motor function in selected patients providing newer therapeutic avenues in managing strokes. It seems to rejuvenate the damaged brain circuits. Furthermore, there is no need for immunosuppressive therapy [53]. An anti-myostatin approach has also emerged as a novel approach in combating skeletal muscle loss and weakness in stroke patients [54]. Repetitive transcranial magnetic or direct current stimulation can also modify cortical excitability [55]. Similarly, repetitive transcranial magnetic inhibition can minimize spasticity [56].

28.5 Monitoring and Novel Future Perspectives

Isokinetic knee muscle strength and gait performance tests are reliable and sensitive methods in detecting clinical improvements in stroke survivors. Simple bathroom scales can be a simple feedback tool in determining objective progress. Reduced compound motor action potentials (CMAPs) in the acute phase normalize in the chronic phase following collateral sprouting from the neighboring normal motor axons. Muscle ultrasound is a simple and noninvasive method to assess muscular integrity and thereby monitor recovery and effects of therapy. A key-form recovery map can also be a helpful tool for monitoring the rehabilitative process [57].

In the context of low-income nations, people with disabilities are socially discriminated. They are invariably marginalized and therefore bound to become socially aloof. So considerations need to be made upon the prevalent cultural competence with reinforcement on capacity building through cost-effective, appropriate, and sustainable approaches [58]. The geographical barriers can be minimized by teletherapy so that there is maximum inclusion of such affected cohorts [59].

There has been major success seen through the application of compact robot gym system [60]. The key highlights were its transportability, cost-effectiveness, and sustainability as well as its high safety profile. Moreover, the haptic and regular feedback ensures adaptive control as well as constant monitoring of the progress. The application of gaming motivates participation, whereas facility of multi-stations ensures therapist to monitor many patients simultaneously. Therefore, focus needs to be on promoting community-based fitness programs [61, 62]. The risks of long-term consequences of cardiovascular comorbidities and the increased odds of recurrent strokes in stroke survivors can be minimized by their indulgence in active physical activities [63]. A water-based exercise program can be a cheaper alternative as it has shown to improve VO2max by 22% in chronic stroke survivors [64]. Group participation promotes self-respect [65]. It may be the missing link in the puzzle amidst care and resurrection of stroke survivors and thereby provide newer insights to strategies aimed for the same [30].

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Part VII
Future Prospects

Chapter 29
Muscle Atrophy: Present and Future

Richard Y. Cao, Jin Li, Qiying Dai, Qing Li, and Jian Yang

Abstract Muscle atrophy is the loss of muscle mass and strength, and it occurs in many diseases, such as cancer, AIDS (acquired immunodeficiency syndrome), congestive heart failure, COPD (chronic obstructive pulmonary disease), renal failure, and severe burns. Muscle atrophy accompanied by cachexia worsens patient's life quality and increases morbidity and mortality. To date there is no effective treatment on that. Here we summarize the diagnosis methods and cellular mechanisms of muscle atrophy. We also discuss the current strategies in muscle atrophy treatment and highlight the potential treatment strategies to resist muscle atrophy.

Keywords Muscle atrophy · Present · Future

29.1 Introduction

Muscle atrophy results from a variety of common diseases, including cancer, AIDS (acquired immunodeficiency syndrome), congestive heart failure, COPD (chronic obstructive pulmonary disease), renal failure, and severe burns [1, 2]. Muscle atrophy is a complex and highly regulated phenomenon. It is characterized by a decrease in muscle fiber cross-sectional area, myonuclear number, protein content, muscle strength, an increase in fatigability, and resistance to insulin [3, 4]. It is also associated with an increased risk of morbidity and mortality.

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Despite decades of research, no effective treatments have been proven to prevent muscle mass loss. Here we will provide a brief overview of researches in the field of muscle atrophy. We will discuss about the new progress in the field as well as its limitations and highlight the future direction of muscle atrophy therapy.

29.2 Diagnosis Methods

Diagnosis is important for clinical management of muscle atrophy. Skeletal muscle mass index (SMI) is the most common indicator to diagnose muscle atrophy. It can be measured by image or laboratory functional test. Dual-energy X-ray absorptiometry (DXA), magnetic resonance imaging (MRI), and computerized tomography (CT) are used in SMI detection. Also, anthropometry (which means by directly measuring the muscle mass) and bioelectrical impedance analysis (BIA) are useful tools in muscle atrophy diagnosis [5, 6]. Lab tests mainly focus on detecting creatinine and urea. Levels of these two chemicals correlate with muscle injury and muscle loss [7, 8]. Strength of handgrip and exercise capacity reveal muscular function. Finally, muscle biopsies could directly show evidence of muscle atrophy but are seldom used due to its invasiveness.

Several technical improvements have been made in lab testing for muscle atrophy. Transcript profiling showed a subset of universal upregulated genes in rat muscle atrophy model, such as muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFbx). Especially the latter one could be potential therapeutic target for muscle atrophy [2].

Current tests to evaluate muscle atrophy are time-consuming, invasive (as biopsy is the only confirmatory test), and complicated. However, the biggest disadvantage is that no tests could detect atrophy at the early stage.

Noncoding RNAs (ncRNAs) are a group of RNAs that is not translated into proteins. They function as gene regulators and are widely detected in tissue or in peripheral blood. Noncoding RNAs include microRNAs (miRNAs), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), etc. Previous studies have found several miRNAs could be candidate serum markers for muscle atrophy. Muscle-specific miRNAs have been proven to regulate muscle metabolism under different conditions [9]. In aging-related muscle atrophy, Let-7 family members including Let-7b and Let-7e were found to be increased compared to young individuals. Meanwhile the expression of cell cycle regulators was significantly downregulated [10]. A study discovered that miR-431 influenced muscle mass through promoting myoblast differentiation and modulating TGF-β downstream effectors [11]. miRNAs are also reported to involve in other muscle wasting conditions, such as regular catabolism, dexamethasone-induced atrophy, denervation injury, and even cancer [12]. Functional miRNAs in muscle atrophy mainly include miR-23a/206/499, miR-1, miR-133, miR-23a, miR-206, miR-27, miR-628, and miR-21 [13–15]. Among them, miR-29b was found to be commonly upregulated in different muscle wasting conditions, including denervation-induced, dexamethasone-induced,

fasting-induced, cancer cachexia-induced, aging-induced, and immobilization-induced muscle atrophy. Moreover, the expression of miR-29b is positively correlated with the degree of denervation-muscle atrophy [16]. Thus, ncRNAs might also be used to diagnose muscle atrophy.

Exosome was also shown to play important roles in muscle atrophy. Exosomes are vesicles measuring from 30 to 100 μm and able to carry many factors (RNA and protein) in the blood. They mediate cell–cell and tissue–tissue communication in an autocrine, paracrine, or endocrine manner [17]. Exosomes are nature reservoirs for signal factors, and they are detectable in the peripheral blood, which makes them ideal disease markers. In dexamethasone-induced muscle atrophy model, miR-23a is reported to participate in muscle atrophy through calcineurin/NFAT pathway. Dexamethasone increases concentration of miR-23a in the exosomes while it does not affect the number of exosomes [18]. Other studies showed a connection between exosomes secretion and malignancy-related muscle loss. Exosomes secreted by cancer cells carried miRNAs that function as apoptosis factors. miRNAs like miR-21, miR-182, and some other miRNAs from heart shock family were found to induce apoptosis in myocytes [19, 20]. Other noncoding RNAs, such as lncRNAs and circRNAs, were also reported to be contained in exosomes and contribute to various processes [21].

29.3 Pathways Regulating Muscle Atrophy

The major process during muscle atrophy is myofiber reduction, which is the result of excessive protein degradation. Current theory for these degradation pathways was the ubiquitin–proteasome system and the autophagy–lysosome pathway. Studies have been carried out to explore the regulating factors of these two pathways. Both of them could be triggered by stimulation like chronic inflammation and acute metabolic changes.

Ubiquitin–proteasome system (UPS) could degrade sarcomeric proteins in response to catabolic stimulate. UPS works through a series of enzymatic reactions involving activating (E1), conjugating (E2), and ligating (E3) enzymes [22]. Among them, atrogin-1/MAFbx (muscle atrophy F-box) and muscle RING finger 1 (MuRF1) are the main E3 ubiquitin ligases that play important roles in muscle atrophy. Genetic deficiency of either of these two genes showed a significant resistance to atrophy [2]. Likewise, their expressions were elevated in almost all types of muscle atrophy [23]. Other E3 ligating enzymes, such as Trim32 [24], TRAF6, ZNF216, USP14, and USP19 [25], were identified to function in muscle atrophy.

IGF1-PI3K-AKT pathway is the dominant pathway that mediates protein degradation. Catabolic signals inhibit this pathway by reducing the protein phosphorylation levels and then promote the proteolysis and depress protein synthesis. In addition, IGF1–PI3K–AKT–mTOR pathway and IGF1–PI3K–AKT–FoxO pathway also regulate the autophagy–lysosome systems [26–29].

Chronic inflammation influences myocyte metabolism through the interactions between different cytokines. Studies have found that interleukin 6 (IL-6) deficiency is associated with muscle atrophy [30, 31]. On the other hand, IL-6 induces myocyte proliferation through STAT3 signaling pathway, which occurs exclusively in the nuclei of satellite cells [32]. Other inflammatory pathway like IKKbeta/NF-kappaB/MuRF1 pathway was also found to regulate muscle atrophy [33].

Another way to disturb muscle volume is to inhibit muscle growth. Myostatin is the major autocrine inhibitor of muscle growth. It binds to the activin A receptor type IIB (ActRIIB) in skeletal muscle cells and activates transcription factors SMAD2 and SMAD3, thus suppressing muscle growth [34–37].

Catecholamine axis also contributes to the balance of muscle atrophy and growth. Deficiency of β2-adrenoceptors worsens skeletal muscle atrophy in patients with heart failure [38]. In cardiac muscle, sympathetic neurons control cardiomyocyte size by a β2-AR-dependent mechanism [39]. Further study showed this could be a result of its suppression effects on atrogin-1/MAFbx, which has been known as a muscle-specific ubiquitin ligase [40, 41].

Noncoding RNAs like miR-1, miR-1331a/b, miR-206, miR-146a, miR-221, miR-499, miR-208b, miR-486, and miR-29b, several long noncoding RNAs, and circRNAs are reported to contribute to muscle atrophy as well [42–44]. The fruitful achievements in the nucleic acid studies have led us to understand disease in a new way.

Even with these accomplishments, challenges still exist in the muscle atrophy field. First, functional noncoding RNAs are still to be studied. Second, epigenetic genes involving a serious of histone and DNA modifying enzymes have emerged as novel targets for the therapeutics purpose. They are widely studied in various fields, but little is known in muscle atrophy [45]. Third, current studies are mainly focused on the muscle cell itself, neglecting the cross talk between muscle cells and other factors, such as extracellular matrix, stem cells, and immune cells. Muscle atrophy always represents as a complication, which means it happens along with other diseases. For example, in cancer-induced muscle atrophy, cancer cells release exosomes which specifically interfere muscle cell growth. While under the condition of inflammation, muscle cells are influenced by inflammatory factors. Also, the biological process of muscle atrophy varies in different external conditions. For example, autophagy was considered as defense mechanism in fasting-induced muscle atrophy, but it causes damages in other scenarios [25, 46, 47]. Understanding this difference may be important for treatment of muscle atrophy. Finally, almost all previous study has stayed at the animal level. Translational research and clinical research need to be carried out in the future.

29.4 Therapeutic Approaches and Limits

Although a lot of basic research has been invested to treat muscle atrophy, there are no efficient drugs for neither prevention nor treatment of muscle atrophy [5]. Current standard treatments for muscle atrophy are nutritional supplement, physiologic therapy, and drug treatment.

29.4.1 Nutrition Treatment

Nutrition supplement provides energy for muscle activity directly and helps to maintain muscle mass. Increased consumption of calorie and protein could bring beneficial effects. In severely ill patients or those who suffer from muscle atrophy, some trials have shown that nutrition treatment improved life quality and long-term survival [48, 49]. In fact, many nutritional components were found to be beneficial to muscle atrophy (Table 29.1). But the effects might be only limited within patients who have primary muscle wasting [50].

29.4.2 Exercise Training

Physical therapy has been well studied to be effective in maintaining muscle strength [64, 65]. Exercise has also been considered as an effective way to promote muscle hypertrophy and muscle regeneration [66, 67]. In heart failure-induced muscle atrophy, aerobic exercise alleviates the process by reducing inflammatory reactions and decreasing ubiquitin-proteasome activities [68, 69]. Malignancy-related muscle

Table 29.1 Nutrition treatment used in muscle atrophy

Component	Muscle atrophy type	References
Protein	Sarcopenia	[51]
	Heart failure	[52]
Essential amino acid	Sarcopenia	[53]
	Heart failure	[52]
β-Hydroxy β-methylbutyrate (HMB)	Cancer	[54]
	AIDS	[55]
	Chronic obstructive pulmonary disease	[56, 57]
	Sarcopenia	[58]
	Immobilization	[59]
Vitamin D	Sarcopenia	[60]
	Cancer cachexia	[61]
Allopurinol	Sarcopenia	[62]
	Unloading	[63]

atrophy could also benefit from exercise therapy. Apart from suppressing inflammation, exercise promotes the mitochondrial biogenesis via peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) pathway [70–74]. In addition, exercise training inhibits myocyte autophagy [73]. Unfortunately, exercise therapy cannot be applied to everybody. It has limited effects on patients who are immobilized on the bed or patients who have nerve injury. Moreover, certain patients with severe muscle atrophy cannot tolerate exercise therapy.

29.4.3 Drug Treatment

Based on the prior studies, current drug treatment for muscle wasting mainly focused on improving appetite, modulating inflammation, and interfering with anabolic and catabolic reactions. Table 29.2 summarized the candidate medications and its therapeutic targets. However, no medications have been approved to be effective in clinical trials so far.

29.5 New Therapeutic Strategy

Due to the advance of new technologies and theories, novel treatment strategies have sprung up.

29.5.1 Noncoding RNAs

With the development of next-generation deep sequencing, the research on gene regulation transfers from genome to transcriptome. Researches on RNA field have been developed unprecedentedly. Unlike protein-coding genes, noncoding RNAs are the ones which lack the ability to code protein. They were once considered as "evolutionary junk," until later on it was discovered that these group of RNAs had tremendous effects on regulating gene expression. Current well-defined noncoding RNAs include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), long noncoding RNAs (lncRNAs), microRNAs (miRNAs), circular RNAs (circRNAs), and other small RNA-related molecules. Great achievements have been made in exploring the functions of these RNAs. Some of the noncoding RNAs have already been studied in clinical trials. For instance, liposomal miR-34 mimic was used to repress oncogene expression, and its ability to shrink tumor size has been proved [102]. On the other hand, miRNA antagomirs, such as anti-microRNA oligonucleotides (AMOs) and N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine ("ZEN"), are used to downregulate certain miRNAs [103, 104]. The use of antisense RNA in long noncoding RNA interference has showed a significant value in

Table 29.2 Studies of agents with potential efficacy in muscle atrophy

Disease process	Drug/compound	Target	References
Cancer cachexia	Thalidomide	TNF-α	[75]
	ALD-518	IL-6	[76]
	RC-1291	Ghrelin mimetic	[77]
	RC-1291	Ghrelin receptor agonist	[78, 77]
	Celecoxib	COX-2	[79]
	BYM338	Myostatin and the activin type II B receptor (ActRIIB)	[26]
	MG132	Ubiquitin–proteasome system	[80]
	Myostatin-specific antibody	Myostatin	[81, 82]
Heart failure	JA-16	Myostatin	[83]
	Salbutamol	β2-Agonists	[84]
	Clenbuterol	β2-Agonists	[85]
	Testosterone	Testosterone	[86, 87]
	Selective androgen receptor modulators (SARMs)	Hormonal	[88]
	Ghrelin agonist	Ghrelin	[89] [90]
Sarcopenia	Metformin	/	Clinical Trials NCT01804049
	Incretins	Enzyme dipeptidyl peptidase IV	[91]
	Statins	Glucose oxidation	[92]
	Allopurinol	Xanthine oxidase (XO)	[62]
	Formoterol	β2-adrenoceptor	[93]
	Myostatin-specific antibody	Myostatin	[94]
Chronic obstructive pulmonary disease (COPD)	Ghrelin/GH/IGF-axis Ghrelin	Stimulates GH secretion	[95]
	SUN11031	Synthetic ghrelin	[96, 97]
	NAC	ROS scavenger	[98]
	α-lipoic acid	ROS scavenger	[99]
Renal failure	Myostatin-specific peptibody	Myostatin	[100]
	C188-9	STAT3	[101]

treating myocardial hypertrophy and fibrosis [105–107]. Besides, miR-1, miR-133, miR-23a, miR-206, miR-27, miR-628, miR-431, miR-21, and miR-29b are considered to be the therapeutic target for muscle atrophy. miR-29b was an increased miRNA in multiple types of muscle atrophy, and miR-29b inhibition could relieve muscle atrophy [11, 108–113].

29.5.2 Gene Therapy

In the last few years, targeted genome-editing technology has developed. Among them, clustered regularly interspaced short palindromic repeats (CRISPR) are well studied and applied in clinical trials. This is a highly versatile system, which is derived from a prokaryotic adaptive immune system. In bacteria, CRISPR/Cas system captures and avoids the invasion of foreign DNA via RNA-guided DNA cleavage [114]. The recently developed CRISPR–Cas9 system has two biological components: the RNA-guided DNA endonuclease Cas9 and a chimeric single guide RNA (sgRNA) [115–119]. The guide RNA binds Cas9 with one end, and the other end recognizes the target DNA sequence by base pairing. This system has been applied to modify endogenous genes in a wide range of organisms, including bacteria, yeast, plants, fruit flies, zebrafish, frogs, rabbits, mice, rats, pigs, dog, sheep, goat, monkeys, and human cells [120].

This technique can be applied to various research fields. In cancer, CRISPR/cas9 was used to produce the next-generation chimeric antigen receptor T cells (CAR-Ts), which have potential effects in cancer treatment [121, 122]. CRISPR/Cas9 was also used to disturb HIV duplication by targeting LTR sequence [123]. Additionally, CRISPR/Cas9 disrupts rs1421085 of FTO region and thus restores thermogenesis and opposes obesity [124].

CRISPR is widely used in muscle atrophy studies as well. CRISPR was used to knock out myostatin in dog, goat, pig, sheet, and rabbit and thus induce typical muscle hyperplasia or hypertrophy in vivo [125–132]. This highlights the hope in muscle atrophy treatment. Interestingly, CRISPR/Cas9 was used to target myostatin in cancer-related cachexia [133]. Insulin-like growth factor-1 (IGF1) and FGF5 are also potential targets for muscle atrophy treatment [134, 135].

Another strategy used in gene therapy is gene transfer vectors. Vectors transport genes to target cells. They are usually adeno-associated virus (AAV) – a group of viruses that cause low risk of genotoxicity [136]. Plus, they have long-term stable transgene expression [137]. Preclinical and clinical studies have been carried out using AAV as tools to deliver therapeutic genes [138–140]. In muscle atrophy, AAVs like rAAV6 and AAV2/9 have been used to deliver microutrophin to improve muscle function [141, 142]. In neurogenic muscle atrophy, AAVs containing neurotrophin3 were injected in the mouse model. Reevaluation showed an increased muscle fiber size as well as a change in oxidative state [143]. In malignancy-related striated muscle wasting, Smad7 gene delivery by rAAV6 was able to inhibit the expression of atrophy-related ubiquitin ligase MuRF1 and MAFbx through ActR2b pathway [144, 145]. Similarly, other studies with therapeutic genetic molecules carried by AAVs validated their efficacy by checking downstream factors like vascular endothelial growth factor (VEGF), sarcoplasmic reticulum Ca^{2+} ATPase 1 (SERCA), and β2-adrenoceptor or associated Gα proteins [146–148].

Lack of clinical trials is the main disadvantage of gene therapy. Safety issues with these therapies remain unknown since current studies mainly focus on the positive effects on muscle atrophy. More studies need to be carried out for safety and capability.

29.5.3 Stem Cell Therapy

Stem cell therapy (also called cellular therapy or cytotherapy) refers to a process during which cellular material is injected to treat disease. The effectiveness of stem cell therapy has been studied in a variety of diseases [149–155].

Satellite cell is the original stem cell in muscle tissue. These cells are usually located between muscle fiber or in basal lamina. Under normal conditions, they are naturally quiescent. They start to actively proliferate and differentiate to compensate muscle fibers loss in response to stimuli. In a healthy individual, the compensation is usually adequate. However, in patients with muscle atrophy, the self-renewal capacity of satellite cell was significantly decreased [156, 157]. Hence, increasing satellite cells or enhancing the functions of them could potentially solve the problem of atrophy. Studies have been conducted to transplant myogenic stem cells into atrophied muscle. Promising results have been observed in some studies, showing the tremendous capacity of regenerating new muscle fibers and fusion with the host myofibers after transplantation [158–161]. Unlike skin or adipose tissue transplantation, technical difficulty complicates muscle fiber grafting and makes it difficult to apply in clinical practice. Other stem cells, such as mesenchymal stem cells [162, 163], iPSCs [164], pericytes [165], and endothelial cells [166], could also be used as stem cell therapy.

29.6 Conclusions and Remarks

Muscle atrophy is one of the most common and devastating events in chronic diseases. Unlike the diseases that cause muscle atrophy, muscle atrophy itself is not life-threatening. But it can lead to devastating consequences including but not limited to osteoporosis, blood clot, pressure ulcer, and, more importantly, psychological effects. Preventing muscle atrophy can prolong the patient's life span and improve life quality. However, studies exploring the biology nature and molecular mechanisms of muscle atrophy only started in the recent two decades. Our knowledge in this field is way lag behind compared to other diseases.

We have made a great number of achievements in learning this disease in the recent years. Challenges still exist. Lacking appropriate markers make it hard to monitor muscle atrophy. As we have discussed in this chapter, either proteins or noncoding RNAs could be a candidate to indicate muscle atrophy, but more clinical trials need to be conducted. The causes of muscle atrophy are multifactorial which makes the treatment more complex. In the future, gene therapy and stem cell therapy will be applied in muscle atrophy treatment.

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