

Why Age Matters: Inflammation, Cancer and Hormones in the Development of Sarcopenia

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Abstract

In an aging population, the decline in muscle mass and strength in combination with a high prevalence of osteoporosis and cancer leads to a multitude of clinical manifestations. In the recent years, mouse models of wasting in cancer and inflammation, including xenograft, genetic and chemically induced models, allowed to uncover several key mechanisms underlying muscle loss. These include inflammation, hormone alterations and deregulated protein degradation. Inflammation is associated with increased expression of tumor necrosis factor α (TNF- α), nuclear factor κ B (NF- κ B), and interleukin (IL)-6 and is therefore linked to inflammatory bowel diseases or chronic obstructive pulmonary disease (COPD). Moreover, active NF- κ B signaling and IL-6 secretion commonly occurs in malignancies and cancer-induced cachexia. The ubiquitin proteasome-mediated degradation of proteins represents a second pathway underlying sarcopenia and is partially initiated by inflammatory signaling. Consequently, increased levels of the E3 ligases Muscle RING-Finger Protein-1 (MuRF1), Atrogin-1/Muscle Atrophy F-box (MAFbx), and tumor necrosis factor α receptor adaptor protein 6 (TRAF6) are associated with high rates of protein degradation. Furthermore, hormonal alterations, such as the aging-related decline of growth hormone (GH) and insulin-like growth factor 1 (IGF-1), lead to a reduction of muscle mass.

Interestingly, experimental targeting of several of those sarcopenia-associated factors *in vivo* resulted in a rescue of muscle mass and function. While therapeutic options nowadays still need to be evaluated regarding their clinical practicability, IL-6 antibodies, inhibition of cyclooxygenases and inhibitors of myostatin appear promising.

Keywords: Osteoporosis; Sarcopenia; Cachexia; Myostatin; Muscle wasting; Inflammation; Baicalein

Introduction

Definitions, epidemiology and costs

Loss of neuro-muscular function [1,2] leads to falls and fall-related injuries that protract the encumbrance for the elderly [3]. This is especially important in a population with a high prevalence of osteoporosis, an age-related loss of bone density and strength [4,5]. On the neural side, the basic functional unit of the neuromuscular system, the motor unit, and its neural inputs profoundly change with age (for review see [6]). On the muscular side, there are three major types of loss in muscle mass described, i.e., atrophy, and cachexia and sarcopenia. In atrophy, muscle fiber size decreases while the total number remains unchanged. In cachexia both adipose and muscle tissue are decreased, leading to diverse consequences dependent on the concomitant medical condition. In contrast, sarcopenia leads to a decrease in fiber size and number. Interestingly, the sarcopenia-related decline in muscle mass during aging is frequently accompanied by an increase in adipose tissue [7-10]. This correlation between obesity and sarcopenia, referred to as "sarcopenic obesity" (SO), was investigated in detail and confirmed in the InCHIANTI study in 1998 [11]. In addition, muscle wasting is often concomitant with miscellaneous severe diseases such as cancer, sepsis, liver cirrhosis, chronic

obstructive pulmonary disease (COPD [12]) or chronic heart failure [13-17].

The forfeiture of muscles due to cancer cachexia has been estimated to affect >1.3 million [18,19] in the US population alone. On a worldwide prospect, more than 7.4 million deaths can be attributed to muscle wasting annually [20] with currently about 1% overall prevalence of cachexia in industrialized countries [21]. Intriguingly, 30% of cancer patients die due to cachexia, while more than 50% of patients die with cachexia being present [22]. The healthcare costs were reported around 18.5 billion dollar in the US in 2000 [23] and it was estimated for Europe that sarcopenia leads to increases in hospitalization costs between 34% and 58% [24].

These estimations are complicated by the fact that there is still no consensus on how to diagnose sarcopenia.

Diagnosis

In contrast to osteoporosis, which can routinely be defined by declining bone mineral density since 1994 [25], diagnosis of sarcopenia and muscle wasting remains challenging. Generally, muscle mass, strength and physical performance are evaluated to detect sarcopenia. Methods include computed tomography, magnetic resonance imaging, the assessment of handgrip strength and gait speed as recently reviewed by Choi [26]. According to the International Working Group on Sarcopenia (IWGS), the loss of muscle protein mass and function [27] can be further defined as a low relative

appendicular skeletal muscle mass (RASM, lean mass divided by height squared [kg/m^2]) [28-30]. In contrast, the criteria defined by the European Working Group on Sarcopenia in Older People (EWGSOP) include a decline in muscle mass plus either low muscle strength or low physical performance [31]. The latter takes handgrip strength or grip strength as parameters of muscle strength, which are interestingly long-term predictors of mortality [6,32].

Models of muscle wasting

In aging, cancer, inflammation and hormone alterations are major contributing factors of muscle wasting. Accordingly, several strategies were described to investigate the mechanisms of muscle wasting in mice based on these three aspects. Generally, the loss of muscle mass was observed in xenograft and genetic mouse models as well as after chemical treatments (Figure 1).

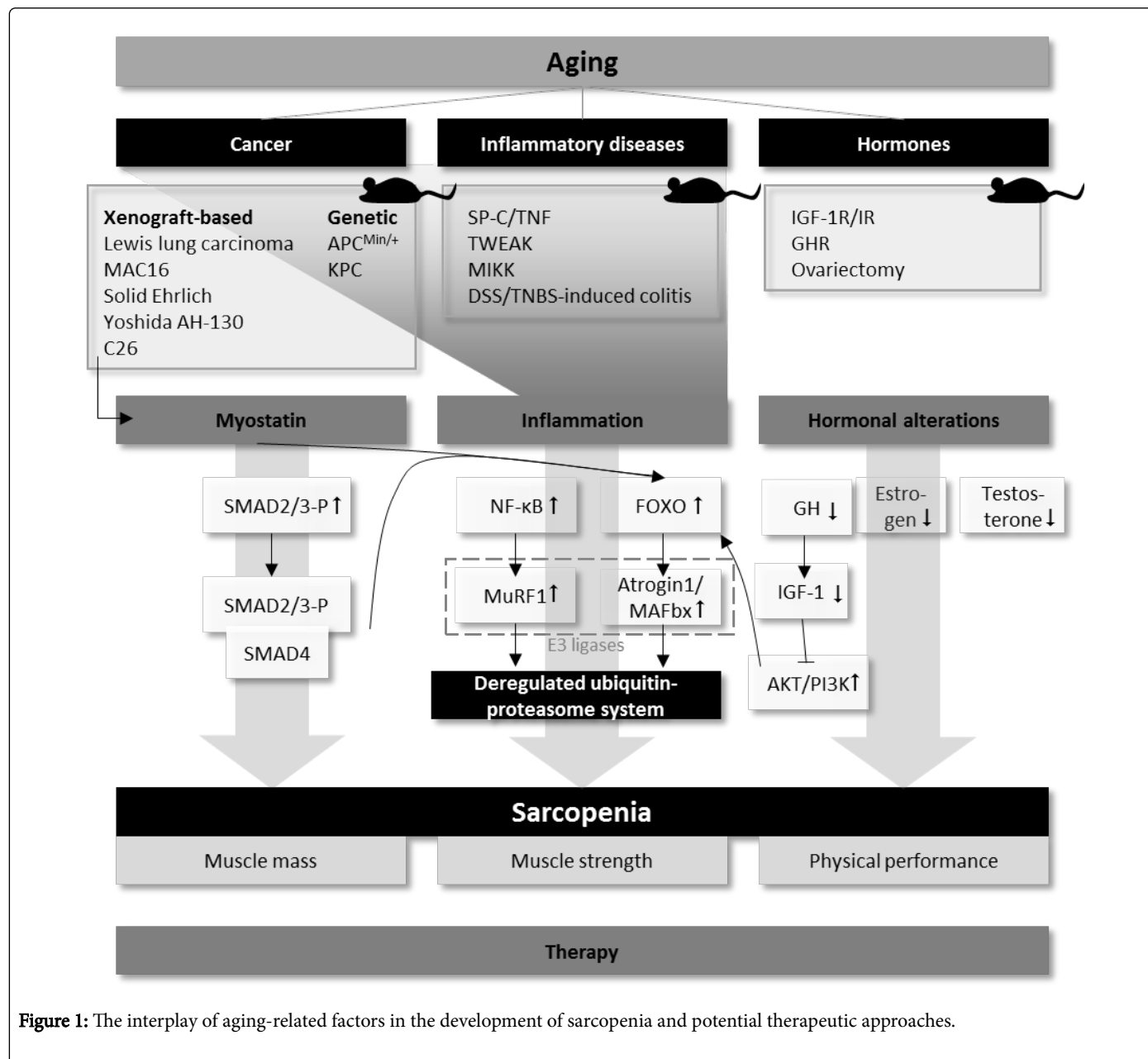


Figure 1: The interplay of aging-related factors in the development of sarcopenia and potential therapeutic approaches.

The progress of aging is frequently accompanied by the development of cancer, inflammatory diseases, and hormonal alterations. These aspects contribute to the increased inflammatory signaling, however, also changes in myostatin levels can be observed during aging. In inflammatory processes, nuclear factor kappa B (NF-κB) is commonly activated which leads to elevated synthesis of the E3 ligase Muscle RING-finger protein-1 (MuRF1). In addition, Forkhead-Box-Protein O (FOXO) levels are elevated which induce the E3 ligase

Atrogin1/Muscle Atrophy F-box (Atrogin1/MAFbx). Consequently, the ubiquitin-proteasome system is deregulated resulting in high levels of protein degradation. Moreover, aging is frequently accompanied by low levels of testosterone, estrogen, Growth hormone (GH), and Insulin-like growth factor 1 (IGF-1), which normally inhibits Phosphatidylinositol-3-Kinase (AKT/PI3K) signaling. Accordingly, the decrement of GH and IGF-1 leads to potent AKT/PI3K activity inducing FOXO. Similarly, myostatin was described as a further factor

inducing FOXO, for instance by being involved into the phosphorylation of SMAD2/3 and thereby facilitating their binding to SMAD4. Therefore, cancer, inflammatory signaling, hormonal changes, myostatin, and deregulations in the ubiquitin-proteasome system can contribute to the development of sarcopenia which is characterized by a decrease in muscle mass, strength and physical performance. Several mouse models reflect symptoms of this disease and various potential therapeutic measures were successfully tested in these experimental animals. So far, no potent drug to reduce the burden of sarcopenia was generated for clinical practice.

Cancer

Xenograft models describe the transplantation of cells, such as cancer cell lines, subcutaneously into the flanks of immunodeficient mice, thereby frequently developing large tumors until muscle wasting becomes apparent. For instance, in Lewis lung carcinoma (LLC) mice, LLC cells are transplanted subcutaneously [33]. Studies showed that on day 21 and 25 after inoculation, fat mass and lean mass were significantly reduced [34]. In this model, the skeletal muscle-isoform of gp130 (skm-gp130) seems to regulate muscle mass signaling crucially through STAT3 and p38 [35]. In another xenograft model, the Murine adenocarcinoma 16 (MAC16) mouse, UCP2 and -3-expression both seem to protract muscle wasting, whether inflammatory cytokines seem not to interfere [36]. Twenty days after injecting MAC16 colon adenocarcinoma cells, lean body mass was significantly reduced, independent from the cumulative food intake [37]. Another well-established model is the C26 colorectal carcinoma model [38], in which a C26-tumor fragment is subcutaneously transplanted. After fifteen days, mice lost approximately 3g; after another one week, the animals lost 12g in comparison to control groups, especially by wasting of adipose and muscle tissue [39,40]. Another xenograft model for muscle wasting is the Solid Ehrlich carcinoma in which Ehrlich-Lettre ascites (EAC) carcinoma tumor cells are injected subcutaneously. 28 days after injection, handgrip strength and body weight were significantly reduced, whereas food intake was not altered [41,42]. Finally, the Yoshida AH-130-model is generated by transplantation of AH-130 ascites hepatoma cells [43]. Through activation of the myostatin-system, the ubiquitin proteolytic system is activated via Atrogin-1, MuRF-1 and E214k. Muscle loss is mainly detected in *M. gastrocnemius* after seven days. While xenograft transplantations reflect a relatively simple approach which can lead to the desired outcome of muscle loss, they do not reflect the human situation properly, for instance, due to the immunodeficiency [13].

This problem can be overcome by using genetic mouse models in which frequently certain mutations or knockouts are induced to study the role of a specific gene *in vivo*. The Adenomatous Polyposis Coli (APC) multiple intestinal neoplasia (*Apc*^{Min/+}) model, for example, is characterized by a mutation in the APC tumor suppressor. The heterozygous *Min* mutation leads to the formation of numerous adenomas, forming throughout the intestine and consequently causing muscle wasting and fatigue. Regularly, the animals die at an age of four to six months. In these mice, lean muscle mass and single muscle mass decreases significantly between week 13 and 22 [44,45]. Intriguingly, in this model weight loss seems to be accompanied by an increase in IL-6 levels, thereby especially affecting *M. gastrocnemius* [46-48].

Furthermore, pancreatic adenocarcinomas often correlate with a cachectic phenotype [32,33] which indicates the potential of the common LSL-KRAS^{G12D/+}; LSL-TRP53^{R172H/+}; Pdx-1-Cre (KPC) mouse model for studying muscle wasting, especially in *M. quadriceps*

femoris. In these KPC-mice, pancreatic adenocarcinoma develops with median survival of five months [13]. Underlying molecular mechanisms involve increased levels of Atrogin-1 and MuRF1 [49-52].

Inflammation

As previously mentioned, inflammatory signaling is highly associated with sarcopenia. Mechanistically, increased tumor necrosis factor (TNF)- α levels lead to inflammation via NF- κ B activation and finally result in muscle wasting. For example, the SP-C/TNF model is characterized by increased TNF- α level in lung and serum due to the lung-specific surfactant protein c-promoter (SFTPC). Consequently, pulmonary inflammation and thus wasting and impaired muscle regeneration were observed in these animals [33,34], preferably in male mice. Similarly, the increase of the TNF-like weak inducer of apoptosis (TWEAK) protein exacerbates overall muscle atrophy, whereas transgenic ablation decreases muscle loss. This effect was primarily observed in *M. soleus* which showed reduced fiber size after 6 months and mostly increased fast-type fibers. As expected, these mice were characterized by an increased abundance of the E3 ligase MuRF1, however, MAFbx levels remained unchanged [35,36]. Moreover, the muscle-specific expression of I κ B kinase (IKK) in MIKK mice is required for ubiquitination and degradation of I κ B α [37], which activates NF- κ B. Under the control of the muscle creatine kinase promoter, constitutively active IKK β via its phosphorylation of S177 and S181 leads to muscle wasting by impaired skeletal muscle mass. Mostly, number of fibers is normal, but fiber diameter, area and function are significantly reduced [38,39]. Other genetic models such as muscle creatine kinase-Cre mice on a Cre-LoxP system have been described, that make it possible to knockout specific proteins like TRAF6 [40,41] or TGF β R-II [42] in the muscle, leading to prevented cancer cachexia in an experimental model [41]. Besides these genetic models for inflammation, there are also chemical models to induce inflammatory signaling. Colitis induced by chemical treatments reflects the human situation in inflammatory bowel diseases (IBDs) and can serve as model for wasting [49]. For instance, it was demonstrated that dextran sodium sulfate (DSS)- and trinitrobenzene sulphonic acid (TNBS)-mediated colitis deregulated the expression of smooth muscle contractile proteins *in vivo*, thereby impairing muscle function [43]. In addition, muscles of TNBS-treated mice were characterized by increased Atrogin-1 and MuRF1 mRNA levels and elevated protein degradation rates [44].

Hormones

In contrast to cancer- and inflammation-related experimental animals, mouse models for hormonal deficiency and resulting effects on muscle mass are rare. For instance, due to its major function in muscle development and function, mice with a global reduction of IGF-1 were generated. Unfortunately, these mice were characterized by impaired tissue development and subsequent postnatal lethality [45]. It was possible to overcome this problem by the generation of mice with a muscle-specific IGF-1R/IR (insulin receptor) knockout. These animals showed a severe decrease in muscle mass [46]. In addition, one of the main contributing component of sarcopenia is the growth hormone receptor (GHR). It could be demonstrated that GHR loss in mice resulted in a lower muscle mass in *M. soleus* and *M. tibialis anterior* [47] and, therefore, GHR knockout can serve as a model of age-related sarcopenia. Finally, the reduction of estrogen levels via ovariectomy was shown to be a valuable model for sarcopenia [48], especially when housing animals in an enriched environment [49].

Finally, the administration of Angiotensin II via osmotic minipumps over two weeks leads to a weight-reduction of 56% after one week and 41% after two weeks. Mechanistically, reactive oxygen species and IGF-1 lead to apoptosis and protein degradation and therefore muscle wasting [50,51], which reveals the Angiotensin II-Infusion as an uncommon model for muscle wasting.

While mouse models do have several limitations, such as the high effort to generate them, heterogeneous knockout efficiencies, and a more rapid muscle loss compared to humans, they give us the opportunity to dissect molecular pathways.

Pathogenesis

Based on these models, four main pathways involved in the development of muscle wasting have been described: Inflammation, ubiquitin proteasome-mediated degradation, hormonal alterations and malignancies. Molecular key players in these pathways were defined and reflect potential therapeutic targets.

Inflammation

Inflammatory signaling, one of the key mechanisms underlying muscle loss, frequently involves the activation of the NF- κ B via TNF, finally resulting in the production of pro-inflammatory cytokines including interleukins [52]. For instance, when investigating sarcopenic obesity, it was demonstrated that inflammatory signaling is frequently induced in adipose tissue in which adipocytes and infiltrating macrophages secrete pro-inflammatory cytokines. In fact, high levels of C-reactive protein (CRP), IL-6 and TNF- α [53] were associated with decreased muscle mass and strength in patients. Interestingly, TNF- α secretion was shown to promote apoptotic signaling with age. Since muscle fibers are multinucleated, apoptosis does not necessarily interfere with fiber integrity, however, it was found to contribute to sarcopenia [54,55]. Moreover, inflammatory bowel diseases were associated with sarcopenia. In fact, besides IBD-related malnutrition which is associated with weight loss, inflammatory signaling was shown to significantly contribute to sarcopenia. Generally, IBDs such as CD and ulcerative colitis (UC) are characterized by activated NF- κ B signaling [56] and up to 60% of Crohn's disease (CD) patients were affected by severe muscle loss [57]. Accordingly, treating CD patients with the TNF- α inhibitor Infliximab resulted in an increase in muscle volume and strength, as well as in a decrement in IL-6 levels [58]. Similarly, chronic obstructive pulmonary disease (COPD) is associated with increased NF- κ B activation [59] and 15% of affected individuals develop sarcopenia [12]. Furthermore, COPD patients were more likely to present SO resulting in a severe systemic inflammation and reduced exercise capacity compared to individuals without COPD [60].

Ubiquitin proteasome-mediated degradation

Besides inflammatory signaling, the homeostasis between protein synthesis and degradation is not maintained in sarcopenia. In addition to its function in cytokine production, activated NF- κ B can translocate into the nucleus to mediate the induction of MuRF1. MuRF1 is an E3 ligase which conjugates protein substrates with an ubiquitin molecule, thereby potentially targeting its substrates for proteasomal degradation. Interestingly, MuRF1 activation interferes with the sarcomere integrity by degrading the myosin heavy chain and further filament components [61,62]. Similarly, the E3 ligase Atrogin-1/MAFbx is induced upon constitutive FOXO 3 activation [63]. In

addition, myostatin, a protein exclusively expressed in smooth muscle cells, was shown to increase FOXO levels by assisting during the phosphorylation of the transcription factor SMAD2/3 and thereby facilitating its binding to SMAD4 as reviewed in detail elsewhere [64]. Consistently, MuRF1 mRNA levels were increased in limb muscles of cachectic COPD patients [65] and MAFbx in smokers [66]. Interestingly, the deletion of MuRF1 and MAFbx *in vivo* could prevent muscle loss [67] and treatment of myoblasts with IGF-1 *in vitro* was able to inhibit the expression of MuRF1 and Atrogin-1 [68]. Moreover, the E3 ligase tumor necrosis factor α receptor adaptor protein 6 (TRAF6) is upregulated in skeletal muscle wasting through the activation of JNK1/2 [41]. Consequently, the downstream signal molecules MAFbx and MuRF1 were upregulated [69]. Interestingly, upon cytokine stimulation TRAF6 was shown to be involved in the activation of NF- κ B and Protein Kinase B(PKB/AKT)/PI3K signaling [70,71]. In addition, it was described to be critical for homeostasis of satellite stem cell function and therefore myofiber regeneration [72]. Accordingly, inhibition of TRAF6 resulted in the preservation of cancer cachexia [41]. Interestingly, TRAF6 loss exacerbates DSS-induced colitis in mice [73].

Hormonal alterations

Aging is accompanied by changed levels of hormone production. For instance, secretion of growth hormone (GH) and IGF-1 decline with age and, interestingly, this phenomenon is associated with sarcopenia [74]. Generally, binding of IGF-1 to its receptor leads to the activation of the AKT/PI3K pathway. Decreased AKT/PI3K signaling results in impaired phosphorylation of FOXO members resulting in the nuclear translocation of this transcription factor family. Nuclear localization and constitutive activation of FOXO3 induced transcription of Atrogin/MAFbx and thereby severe muscle atrophy [63].

Besides the effect on GH and IGF-1, the decline in testosterone and estrogen levels were correlated with aging and sarcopenia [75]. Interestingly, testosterone supplementation increases IGF-1 expression *in vitro* [76] and exerts a positive effect on muscle mass and strength in older men [77]. In contrast, the effect of estrogen was highly dependent on the route of administration since IGF-1 levels increased after transdermal and decreased after oral treatment [78].

Malignancies

Muscle wasting was described as comorbidity in several human malignancies, including colorectal, pancreatic, and lung cancer [79]. Similar to sarcopenic obesity and hormonal alterations, malignancies frequently develop in elderly individuals. In fact, 50% of all cancer diagnoses and 70% of cancer-related deaths are attributed to an age \geq 65 years [80]. As recently reviewed by Gordon and colleagues, inflammatory responses, including active NF- κ B signaling, significantly contributes to cancer-related cachexia. For instance, lung cancer patients are characterized by highly elevated NF- κ B activity in limb muscles [81] and in a mouse model for lung cancer, IL-6 secretion modulated muscle mass via inducing FOXO3, Signal transducer and activator of transcription 3 (STAT3), and the kinase p38 [82]. Moreover, myostatin was shown to be secreted by C26 colon cancer cells *in vitro* and to upregulate MuRF1 and Atrogin-1 levels [83]. The increased abundance of MuRF1 and Atrogin-1, thus, deregulated ubiquitin proteasome-mediated degradation could reflect another mechanism associated with cancer cachexia. Accordingly, myostatin inhibition increased skeletal muscle mass and strength *in vivo* [84].

Therapy

Therapeutic options in sarcopenia consist of physical training [27], sufficient nutrition and pharmacotherapy [85-87]. Pharmacotherapy is based on the highly heterogeneous underlying molecular pathways associated with muscle wasting. Thus, possible therapeutic options aim at different targets.

Since inflammation is one of the key players mediating the development of sarcopenia, several studies sought to inhibit underlying pathways in order to prevent muscle loss. For instance, the inhibition of TNF- α , an activator of NF- κ B signaling, using Thalidomide was able to reduce the severity of muscle loss in rats with biliary cirrhosis [88]. However, as described in a Cochrane review, the effects of Thalidomide in the treatment of cancer-associated cachexia are not completely clear. Therefore, its suitability for clinical practice still needs to be elucidated [89]. As mentioned earlier, treating IBD patients with Infliximab, a potent anti-TNF- α agent, resulted in an increase in muscle mass and strength [58]. Similarly, administration of IL-6-neutralizing antibodies could restore tumor-induced cachexia in mice [90]. Moreover, inhibition of cyclooxygenases (COX), mediators of inflammatory responses, represents a potential therapeutic strategy [91]. Interestingly, we recently demonstrated the beneficial effect of the lipoxygenase inhibitor baicalein on muscles [48]. Generally, this agent is a potent inhibitor of inflammation by interfering with *COX-2* gene expression [92]. During our study we discovered that baicalein treatment increased muscle fiber area and diameter and led to elevated number of capillaries per fiber in non-ovariectomized as well as ovariectomized rats. Together, a protective effect of baicalein on muscle cells was suggested due to the stimulation of angiogenesis in skeletal muscle and by reducing the muscle loss mediated by decreased estrogen levels [48]. Studies like this indicate the potential of anti-inflammatory agents in the treatment of sarcopenia.

Furthermore, the success of drugs interfering with hormone deregulation was reported. Hormone supplementation is FDA-approved for several syndromes associated with muscle wasting. However, while testosterone therapy was able to increase muscle strength in several studies, GH supplementation failed to induce changes in muscle function [93] but could increase muscle mass [94,95]. Likewise on the hormone level, encouraging effects of vitamin D on muscle strength [96] in vitamin D-deficient individuals [97,98] have been discovered.

Another apparent target is myostatin, which inhibits protein synthesis and, accordingly, systemical administration of myostatin *in vivo* resulted in severe muscle loss. Antibodies targeting myostatin were generated and *in vivo* data show increased muscle mass and grip strength in mice [84]. Furthermore, myostatin-inhibitors such as propeptide-Fc (GDF8 propeptide-Fc) increased muscle mass *in vivo* [99]. The glycoprotein Follistatin inhibits myostatin and therefore leads to muscle hypertrophy *in vivo* [100,101]. Similarly, soluble myostatin receptors reduced muscle atrophy [102] and siRNA-mediated myostatin silencing showed promising results in mouse models [103]. Similarly, adeno-associated virus serotype 8 (AAV8) vectors can be used to transfect myostatin propeptides and subsequently raise muscle growth [69].

Another potential target is the E3 ligase TRAF6 which is involved in protein degradation via the ubiquitin-proteasome system. Interestingly, anti-TRAF6 siRNA administered in a mouse model rescued muscle atrophy *in vivo* [69]. Taken together, several molecular players involved in the development of sarcopenia were defined and inhibition

of these factors was shown to have a beneficial effect on muscle mass and function in several *in vivo* studies. However, while several promising drugs were generated, the ultimate objective to use a therapeutic agent in clinical practice to lower sarcopenia burden in patients still requires further investigations.

Conclusion

Sarcopenia reflects a major health problem by affecting the individuals' quality of life as well as by causing a significant economic burden for society. Over the last decade, several molecular players involved in the development of this disease were defined in mouse models as well as in patient samples. A number of *in vivo* studies indicated promising therapeutic strategies, however, no suitable drug was generated for clinical practice so far. Thus, further extensive research is required in this field.

Genetic mouse models offer attractive experimental setups since they reflect the human situation more closely than xenograft models. In future projects, molecular players of muscle loss could be identified by next generation sequencing using sarcopenia patient-derived muscle samples. Subsequently, the function of potential mediators of this medical condition could be defined by performing muscle-specific knockouts in mice. Subsequently, methods to rescue these phenotypes could include treatment with anti-inflammatory drugs, specific small-molecule inhibitors or physical training.

Finally, inflammation seems to be one of the major mechanisms involved in muscle loss and, importantly, inflammatory signaling accompanies a variety of further pathological conditions including cancer, IBDs and COPD. Therefore, muscular targeting of anti-inflammatory agents would be highly promising for future studies.

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