# Role of Apoptosis in Myopathies

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**Abstract**
Muscle fiber atrophy and loss is a characteristic of a wide range of neuromuscular affections. Over the past years, there has been increasing evidence that apoptotic cell death mechanisms contribute to this loss. While the initiating stimuli of apoptotic muscle fiber death such as homeostatic dysregulations and oxidative stress seems to be manifold and assumedly disease-specific the up-regulation of the mitochondria-associated factors bax and bcl-2 as well as caspases such as caspase-9 and -3 indicate that predominant effectors involve permeability transition pores in the mitochondrial membrane and subsequent caspase activations which confer the typical morphological and biochemical features of apoptosis such as TUNEL-positive DNA-fragmentation and formation of apoptotic bodies. Deeper insights in muscle fiber apoptosis revealed that apoptosis of multinucleated muscle fibers with individual nuclei controlling successive muscle fiber segments is different from that seen in mononucleated cells. It is likely that apoptotic degradation of nuclei and contractile elements is a localized event in muscle fiber segments leading to muscle fiber atrophy and finally loss in various diseases. In the absence of effective primary treatments, there is hope that interventions in muscle fiber apoptosis will bear promising therapeutic strategies, especially in slowly progressive diseases such as muscular dystrophies and denervating disorders.
**Key words**: apoptosis, caspases, mitochondria, muscle fibers, myopathies.

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A characteristic of many progressive neuromuscular disorders is muscle fiber loss with muscle wasting as the leading clinical presentation. Identification of a growing number of players and mechanisms in muscle fiber apoptosis during the past years provided increasing evidence that apoptotic cell death mechanisms contribute significantly to muscle fiber atrophy and loss in a wide spectrum of neuromuscular affections [79, 97].

Generally speaking, apoptotic cell death is considered to be a triphasic process [42]. In a pre-mitochondrial initiation phase, extra- or intracellular damaging apoptosis-initiating stimuli (e.g. oxyradical production or cytosolic calcium influx) activate very different pathways towards cell death [12]. These initiating events, the mechanisms of which are poorly understood, converge on the mitochondrion where the switch to die is set by a dysbalance between pro- and anti-apoptotic factors, notably members of the bcl-2 family such as apoptosis-promoting bax and anti-apoptotic bcl-2 [109]. In this decision phase, increased permeability of the mitochondrial membrane through permeability transition pores causes a release of a number of pro-apoptotic factors such as cytochrome c [53] which mediate activation of a proteolytic caspase cascade in the effector phase [31, 46]. Caspase-9 seems to be the most upstream member of this apoptotic protease cascade. Its activation has been shown to be triggered by cytochrome c and APAF-1 which binds caspase-9 to form the 'so-called' apoptosome [2]. Once activated, caspase-9 induces cleavage and activation of downstream effector caspases such as caspase-2, -3, -6, -7, and -8 which are responsible for the morphological and biochemical changes in the post-mitochondrial degradation phase of apoptosis such as re-organization of the cytoskeleton, DNA-fragmentation, membrane blebbing, and formation of apoptotic bodies [69, 91]. They act by targeting and destructing a select set of proteins with cleavage occuring at specific protein sites which is genetically programmed and systematically carried out to ensure a proper demise of the cell and disposal of cell debris. An important feature of apoptosis is the 'silent' and rapid phagocytosis of these apoptotic bodies by resident cells preventing an inflammatory reaction which could be harmful to neighbouring cells.

## Specific Aspects of Apoptosis in Muscle Fibers

There is evidence that apoptosis encompasses distinct isotypes in different cell lineages of the same organism. A special feature of muscle fibers compared to other cells is that they are multinucleated and successive muscle fiber segments are controlled by individual nuclei. Hence, apoptosis of multinucleated muscle fibers may be different from that seen in mononucleated cells [36, 57].

In normal postnatal and adult skeletal muscle fibers, TUNEL-positive apoptosis-associated DNA-fragmentation of myonuclei is rare (and may be due to methodical reasons) and there is no evidence of any expression of apoptosis-associated proteins such as members of the bcl-2 family or caspases [81, 82, 98, 99, 103]. This observation corroborates the finding that sarcoplasm of normal human skeletal muscle fibers is refractory to mitochondrial cytochrome c-dependent activation of type-II caspases and lacks the apoptosis protease activator protein-1 (APAF-1) [10].

However, in various neuromuscular disorders such as muscular dystrophies, mitochondrial myopathies or cases of muscle denervation, the strong expression of mitochondria-associated factors such as bax and bcl-2 as well as caspases indicates that under pathological conditions muscle fibers are capable of mitochondria-mediated events involving permeability transition pores in the mitochondrial membrane which can lead to apoptosis (Fig. 1A-F) [38, 81, 82, 99, 100, 103]. It is likely that an imbalance of pro-apoptotic bax and anti-apoptotic bcl-2 is one of the determining factors. However, the role of APAF-1, which mediates activation of the proteolytic caspase cascade, seems to be in a way disease-specific. While skeletal muscle of patients with mitochondrial encephalomyopathies display distinct expression of APAF-1 [38] dystrophin-deficient skeletal muscle lack any upregulation of APAF-1 which indicates that different and other pathways of caspase-9 activation than through the 'apoptosome' may exist in skeletal muscle in specific diseases [95, 96].

As mentioned previously, a particular attribute of skeletal muscle fibers is being multinucleated. In this regard it has been shown that not all nuclei of a muscle fiber display apoptotic DNA-fragmention at the same time. TUNEL-labeled nuclei exist alongside more non-labeled intact nuclei which maintain cellular integrity and function [98, 104]. These first hints of partial and segmental muscle fiber degradation were further substantiated by a segmental expression of cell-degrading caspases in dystrophinopathies (Fig. 1E). It may be speculated that analogical to segmental necrosis also apoptotic events are restricted to single segments of large multinucleated muscle fibers. It is likely that apoptosis of individual nuclei and degradation of their associated sarcoplasmic segments leads not to immediate loss of the whole muscle fiber but contribute to the long-time process of muscle fiber atrophy which occurs in primary myopathies, after denervation which removes the myotrophic influence of the nerve [8, 104], and in inactivation atrophy that is associated with spinal cord lesions [20]. The segmental expression of degrading caspases may, therefore, not only explain loss but also muscle fiber atrophy. Furthermore, dystrophin-deficient muscle fibers with segmental necrosis display consistently an up-regulation of caspases in adjacent non-necrotic muscle fiber segments. It is not conclusively known if apoptosis and necrosis in muscular dystrophies represent two independently initiated events or whether necrosis occurs as a result of apoptosis, however it seems likely that apoptotic events may be followed by necrotic processes [80, 106]. It could be envisaged that minor damage may trigger activation of the intrinsic apoptotic cell death programme but segmental energy depletion during early phases of apoptosis can preclude caspase activation, and consequently switch execution of cell death toward necrosis [64, 73].

An up-regulation of caspases has been noted in several neuromuscular conditions (Fig. 1E-G) such as denervating disorders [103], muscular dystrophies [10, 62, 81, 95, 96], a congenital myopathy [39], mitochondrial myopathies [38], acute quadriplegic myopathy [16] as well as in skeletal muscle associated with chronic heart failure [51, 113] and burn injury [120]. Caspase-3 especially seems to be a major contributor to apoptotic muscle fiber degradation. The apoptosis-specific protein (ASP), a marker for late apoptotic stages and also known as c-Jun/AP-1, has also been shown to be up-regulated in apoptotic muscle fiber degeneration seen in distal myopathy with rimmed vacuoles [118]. However, it is unclear if expression of ASP is related to 'pure' apoptosis or rather to autophagy with the formation of rimmed vacuoles.

To date it is not clear by which procedure degraded nuclei and contractile elements are removed. It has been shown in infantile spinal muscular atrophy that neighbouring muscle fibers phagocytize apoptotic bodies consisting of degraded chromatin and contractile elements of apoptotic muscle fibers [26, 27]. It has also been suggested that apoptotic muscle fibers are removed by resident macrophages as well as by autophagocytosis which plays a role in the formation of rimmed vacuoles [118]. Furthermore, there is evidence that degradation of sarcoplasmic segments with their contractile elements may be a far earlier event than degradation of the nuclei and that this cytoplasmic degradation may involve mechanisms of proteolysis [52].

## Muscular Dystrophies

Various sarcolemmal proteins have been identified during the past two decades allowing a new classification of the muscular dystrophies based on their underlying protein defects [35, 55, 107]. The first evidence of apoptotic muscle fiber loss in dystrophin-deficient muscle was provided in mdx mice in which apoptotic events in muscle fibers were reported to precede muscle fiber necrosis [80,

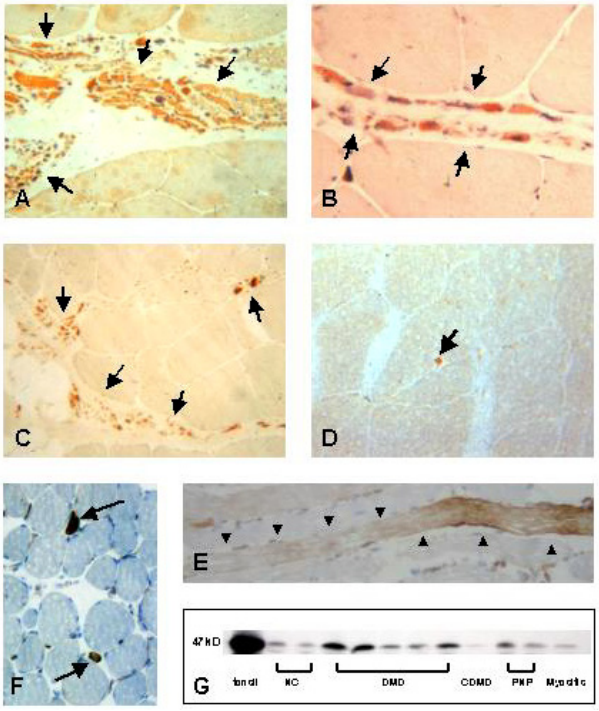


*Figure 1. Denervated atrophic muscle fibers (arrows) display strong up-regulation of the pro-apoptotic bax in spinal muscular atrophy (A, x 300) and of the anti-apoptotic bcl-2 in neurogenic muscular atrophy associated with a axonal neuropathy (B, x 420). TUNEL-positive DNA-fragmentation (arrows) in denervated muscle fibers in a patient with a peripheral neuropathy (C, x 200) and in dystrophin-deficient muscle fibers in a case of Duchenne muscular dystrophy (D, x 300). Distinct expression of caspase-9 in Duchenne muscular dystrophy segmental (E, x 200, muscle fiber marked with arrowheads) and in small atrophic muscle fibers (F, x 200, arrows). (G) Westernblot analysis confirmed up-regulation of caspase-9 in Duchenne muscular dystrophy (DMD) and neurogenic muscular atrophy due to peripheral neuropathy (PNP) while expression levels of a carrier of Duchenne muscular dystrophy (CDMD) and a patient with myositis resembled that of normal controls (NC) which reveal physiological expression of caspase-9 in vessels (as seen in figure 1 F).*

83, 106]. This observation corroborates findings in patients with Duchenne muscular dystrophy whose muscle fibers display apoptosis-associated features such as up-regulation of mitochondrial factors and caspases, TUNEL-positive DNA-fragmentation as well as ultrastructural features of apoptotic nuclear degradation [81, 82, 90, 95, 96, 100]. Muscle fiber apoptosis has also been reported in sarcoglycan-and merosin-deficient mice, both *in vitro* and *in vivo*, and in patients with such sarcolemmal protein defects [32, 34, 60, 62, 100, 111, 112].

The triggering events for muscle fiber apoptosis in the muscular dystrophies are ill-defined although it is generally accepted that exercise-induced damage plays a role. Muscle fibers are subjected to continual stress from contractile activity. The sarcolemmal dystrophin-glycoprotein-complex provides an anchorage-strengthening function and contributes to sarcolemmal intergrity and stability. Sarcolemmal protein defects cause membrane instability and disruption with changes in membrane permeability followed by homeostatic dysbalance and enhanced cytosolic Ca2+-influx, both known to be initiating events of apoptosis [41].

The up-regulation of the mitochondrial factors bax and bcl-2 in muscular dystrophies with sarcolemmal defects is confined to regenerating muscle fibers, contributing there to apoptotic events during new muscle fiber formation which is in accordance with numerous TUNEL-positive apoptotic myonuclei [13, 25, 100] and with findings in experimentally induced muscle fiber necrosis and regeneration [66]. It is striking that non-regenerating muscle fibers show neither expression of bax and bcl-2 nor of APAF-1 which indicates that other pathways of caspase-9 activation than through the 'apoptosome' may exist in dystrophinopathies and other sarcolemmopathies. These observations imply that mitochondria-mediated events exert no trigger effect on apoptotic muscle fiber degradation in dystrophinopathies compared to e.g. mitochondrial myopathies. Furthermore, as regenerating muscle fibers show co-expression of bax and bcl-2 it is likely that apoptotic events during muscle fiber regeneration which resembles embryonal muscle development [24] are different from muscle fiber apoptosis mediated directly through damage due to sarcolemmal protein defects.

Dystrophinopathies display clear up-regulation of the initiator caspase-9 and the effector caspases-3, -6, and –7 indicating that up-regulated expression of caspase-9 can initiate the proteolytic cascade involving the up-regulated downstream executioners caspase-3, -6, and -7 which mediate muscle fiber disassembly and loss [95, 96]. Especially caspase-3 seems to be a major effector of apoptotic muscle fiber degradation in Duchenne muscular dystrophy and facioscapulohumeral muscular dystrophy [81]. Murine merosin-deficient muscle fibers also show activation of caspase-3 apoptotic pathways [62]. This findings correlate with elevated mRNA levels for caspase-3 and -7 but disagree in terms of negative transcript levels of caspase-9 and -6, as previously reported [81]. An up-regulation of caspase-2 and -8 on the protein level could not be confirmed (own unpublished observation) although their transcripts have been likewise shown to be expressed in dystrophin-deficient skeletal muscle. However, such discordance between mRNA and protein levels have also been described for caspase-3 in rat and mouse adult skeletal muscle considering this as a fail-safe mechanism to avoid accidental cell death [78].

However, several studies using small numbers of cases have failed to demonstrate apoptotic events in the muscular dystrophies [6, 40, 58, 67]. These published discrepancies and differences in apoptotic features may

be attributable to the age of the patients and, therefore, stage of the disease as well as to the methodology to assess apoptosis which lacks a 'gold standard' assay.

It is not conclusively known if apoptosis and necrosis in muscular dystrophies represent two independently initiated events or whether necrosis occurs as a result of apoptosis, however it seems likely that in muscular dystrophies marked by sarcolemmal protein deficiencies apoptotic events may be followed by necrotic processes [106]. It could be envisaged that minor damage may trigger activation of the intrinsic apoptotic cell death programme in some muscle fiber segments and ATP depletion during such early phases of apoptosis preclude downstream caspase activation, consequently switching execution of cell death towards necrosis [48, 66, 73]. Interestingly, exercise (which promotes sarcolemmal damage and leakage with calcium influx) seems to be a prime contributor to both apoptosis and necrosis of muscle fibers in Duchenne muscular dystrophy [11, 56, 68, 80].

Also in muscular dystrophies due to other defects than sarcolemmal protein deficiencies, there are hints that apoptotic cell death is involved in muscle fiber loss. In muscular dystrophies with calpain defects, perturbations of the IκBα/NF-κB pathway with abnormal subsarcolemmal localization of NF-κB have been related to apoptotic muscle fiber loss [4, 5, 72]. Also a secondary calpain 3 deficiency in patients with 2q-linked muscular dystrophy due to primary titin mutations has been linked to apoptosis of myonuclei which seems to be likewise mediated through altered distributions of NF-κB and its inhibitor IκBα [33]. In oculopharygeal muscular dystrophies, mutated PABPN1 was associated with apoptotic cell death [23]. Apoptotic DNA-fragmentation has been also described in myonuclei in myotonic dystrophies with decreased levels of the myotonin protein kinase [7, 117].

## Metabolic Myopathies

Although reports of apoptotic cell death mechanisms in mitochondrial disorders disclose some controversy [86] there is increasing evidence that muscle fiber apoptosis contributes to the pathology of mitochondrial myopathies. Apoptotic features were found in cytochrome c oxidase-negative muscle fibers with single mtDNA deletions, and ragged-red-fibers revealed TUNEL-positive DNA-fragmentation as well as up-regulation of the apoptosis-associated proteins bax and bcl-2, APAF-1, and caspase-3 [17, 18, 38, 59, 61, 110, 122]. As ragged-red-fibers displayed also distinct expression of the apoptosis inhibiting protein XIAP it is suggested that this may contribute to a suspension of the apoptotic process in mitochondrial myopathies [38]. In contrast, muscle fiber degeneration in patients with progressive external ophthalmoplegia and mutated adenine nucleotide translocator-1 gene is nonapoptotic [21].

Nevertheless, acute phases of metabolic myopathies (often due to excessive exercise) are predominantly marked by rhabdomyolysis with single segmental muscle fiber necrosis. As previously mentioned the progression to either necrosis or apoptosis depends, at least in part, on the cellular ATP level. It is therefore likely that in acute metabolic insufficiency with ATP depletion (caused by metabolic glycolytic or respiratory defects) affected muscle fibers fail to manifest any sign of apoptosis, but, instead, manifest a necrotic phenotype [87]. As mitochondrial dysfunction is a common trigger for both apoptosis and necrosis common pathways of apoptosis and necrosis may be used in metabolic myopathies [14, 49].

## Inflammatory Myopathies

In polymyositis and inclusion body myositis, the appearance of cytotoxic T8-lymphocytes attacking intact muscle fibers were supposed to represent typical examples of apoptotic muscle fiber death. But the majority of studies did not find any evidence for muscle fiber apoptosis in inflammatory myopathies [6, 15, 37, 40, 58, 59, 63, 67, 87, 102] although contradictory findings of TUNEL-positive muscle fiber nuclei in poly- and dermatomyositis [93, 116] have not resolved this issue. In contrast, there is no doubt that apoptosis is often related to inflammatory cells and might play a role in the regulation of the inflammatory cellular response. There are also contradictory results on the expression of apoptosis-associated factors. As described in other necrotizing myopathies such as muscular dystrophies, regenerating muscle fibers displayed distinct up-regulation of the mitochondria-related factors bax and bcl-2 [65, 70]. Although, there were no features of apoptotic muscle fiber degradation, some studies demonstrated expression of the apoptosis-promoting factor Fas in muscle fibers. However, co-expression of bcl-2, FLICE-inhibitory protein (FLIP), and inhibitor of apoptosis proteins (IAP)-like protein suggested that up-regulation of these anti-apoptotic factors confers a protective effect on muscle fibers in inflammatory myopathies [1, 6, 22, 28, 29, 40, 50, 63, 93]. Moreover, Fas and Fas ligand were both linked to muscle fiber regeneration [15, 84]. In summary, the established cell death mechanism in inflammatory myopathies is likely to be necrosis and not apoptosis.

As it has been shown that apoptosis and necrosis share common cell death factors and pathways [49, 71] it is likely that the expression of these mentioned 'apoptosis-associated' factors is not related to apoptosis but to other signals, e.g. up-regulation of TNF in inflammatory myopathies [101]. Up-regulated cytokine expression may also be a key to cell death triggering events in inflammatory myopathies as it has been shown that up-regulation of cytokines such as TNF-α, IL-1α, and IFN-γ enhance the autocrine NO production and oxidative cell damage [115]. Nevertheless, up-regulation of NOS isoforms and the presence of nitrotyrosine [101, 119] can be also related to cytoprotective functions and a benefit from antioxidative thereutical strategies may be questionable [92].

## Denervating Disorders

It is well established that apoptotic events contribute to muscle fiber atrophy and loss in denervated skeletal muscle [8, 9, 58, 85, 104, 108, 121].

One of the most extensive studied denervating disorder in muscle fiber apoptosis is spinal muscular atrophy. The first evidence of apoptosis as the pathogenic mechanism of muscle fiber loss was provided at the ultrastructural level. In a child with acute infantile spinal muscular atrophy (Werdnig-Hoffmann disease), numerous muscle fibers revealed apoptotic degradation with formation of apoptotic bodies and phagocytosis by neighbouring muscle fibers [26]. Subsequent studies demonstrated apoptotic DNA-fragmentation as well as up-regulation of apoptosis-associated factors such as bcl-2, bax, and caspase-1 [58, 98, 99]. Genes regulating apoptosis have been shown to play a major role in the underlying cause of spinal muscular atrophies, the loss of motoneurons [47, 77], and it is likely that some of these genes such as the SMN gene are also involved in the loss of denervated muscle fibers indicated by a significant reduction of the telomeric SMN gene product expression in muscle fibers [30]. However, muscle fiber apoptosis is not specific to denervation in spinal muscular atrophies, but is also found in other denervating disorders such as peripheral neuropathies and amyotrophic lateral sclerosis indicating that a defect in or lack of innervation prompt muscle fibers to activate an 'intrinsic' suicide programme [98, 103, 105]. Muscle fibers may be programmed to die if they are not adequately stimulated. This is established by myonuclear TUNEL-positivity as well as up-regulation of bax and bcl-2 in denervated, atrophic muscle fibers [9, 104]. Interestingly, denervated apoptotic muscle fibers display not only apoptosis-promoting factors but also apoptosis-inhibiting proteins such as bcl-2 indicating that during the long process of muscle fiber atrophy and degradation denervated muscle fibers activate anti-apoptotic strategies. Nevertheless, denervated muscle fibers seems over a longer period not able to escape apoptotic muscle fiber atrophy and loss because strong up-regulation of anti-apoptotic factors, such as bcl-2, seems finally not to be able to neutralize high bax levels and prevent apoptotic cell degradation. Concerning the expression of Fas in denervated muscle fibers contrary results have been announced. While a survey of spinal muscular atrophies and peripheral neuropathies revealed no significant expression of Fas [99] others reported up-regulation of Fas in skeletal muscle of patients with amyotrophic lateral sclerosis and peripheral neuropathies which, however, was not associated with muscle fiber apoptosis [89].

That DNA-fragmentation and expression of apoptosis-associated factors are related to denervation was also confirmed in experimentally denervated and reinnervated rat facial muscle [8, 104]. Denervated rat muscle fibers displayed strong up-regulation of bax and bcl-2 in the decision phase and apoptotic DNA-fragmentation and expression of caspase-1 in the degradation phase. All these features of apoptosis recede and turn towards physiological levels after successful reinnervation. However, little is known about degradation and resorption of the contractile sarcoplasmic elements in denervated muscle fibers. Matrix metalloproteinases may be putative candidates of muscle fiber proteolysis althought there are conflictive findings [44, 88].

In chronical long-standing skeletal muscle denervation, apoptosis seems to contribute also to the depletion of satellite cells which display increased susceptibility to apoptosis [43]. However, others favour rather a exhaustion of the satellite cell pool by increased proliferation than loss by apoptosis in long-term denervated muscle fibers [76].

## Miscellaneous Neuromuscular Disorders

Features of muscle fiber apoptosis have been reported in a broad range of primary and secondary neuromuscular diseases. Apoptosis-typical DNA-fragmentation has been reported in a distal myopathy [118], congenital myopathies [39] as well as myopathies with rimmed vacuoles [94]. Nevertheless, other neuromuscular diseases such as myofibrillar myopathy have so far failed to provide evidence of apoptotic processes [3].

Apoptotic events take also place in a number of secondary neuromuscular conditions such as thyroid-associated ophthalmopathy [45, 116], chronic heart failure-associated myopathy [113] as well as critical ill myopathy [16]. Apoptotic mechanisms and up-regulation of apoptosis-associated factors have also been linked to aging processes [19, 54].

*Future perspectives*

It is likely that apoptotic events are a common and wide spread mechanism for muscle fiber loss and there is increasing knowledge on the players and effectors of this apoptotic process which may offer the prospect of therapeutic interventions, especially in slowly progressive muscle diseases. Such therapeutic interventions could comprise blocking pro-apoptotic mediators and effectors as well as enhancement of anti-apoptotic mechanisms. For instance, higher levels of bcl-2 may sustain cell survival by neutralizing high apoptosis-promoting bax levels until reinnervation. There have been some efforts in utilizing the therapeutic potential of caspase inhibitors in other than muscle diseases [74, 75, 114]. However, it is essential, that cytoprotective drugs must act at the pre-mitochondrial or mitochondrial stages of apoptosis. Compounds that only interfere with post-mitochondrial effects such as inhibition of caspases may turn out to be ineffective. Nevertheless, therapeutic blocking or inhibition of apoptosis may have profound implications, as this effect may not remain restricted to muscle fibers and as persistence of damaged, but 'non-dead' and unremoved cells may cause unknown side effects. Therefore, further research is necessary to gain deeper insight into mechanisms leading to muscle fiber loss and to give information for therapeutic interventions to modify the rate of muscle

fiber death and loss in view of other lacking primary treatments.

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