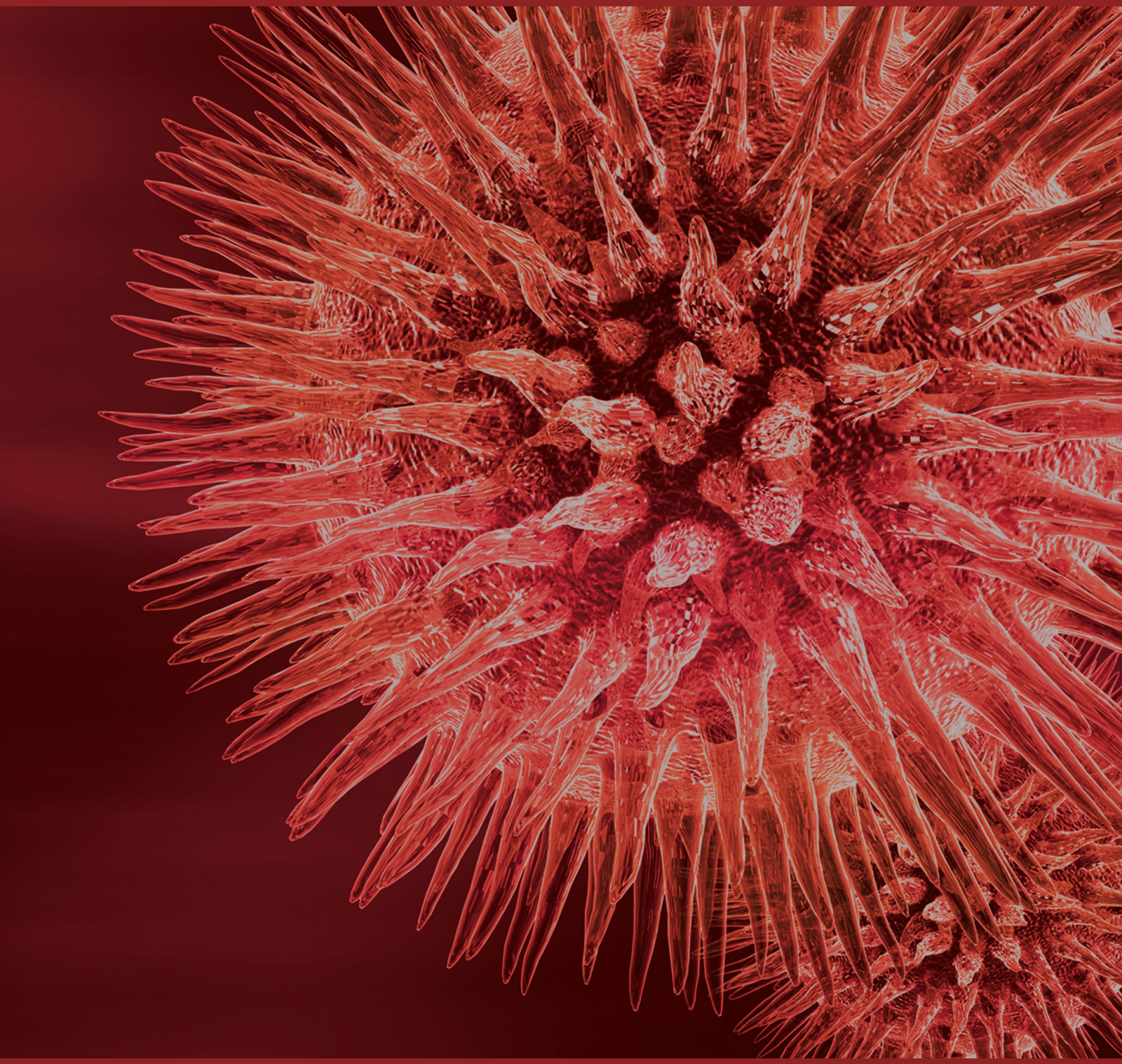
BioMed Research International

Nutrients and Muscle Disease

Guest Editors: Enzo Nisoli, Robert W. Grange, and Giuseppe D'Antona



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Editorial
Nutrients and Muscle Disease

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Myopathies, classified as either hereditary or acquired, lead to common, clinically relevant complaints including fatigue, progressive strength loss, myalgias, and cramps. Important progress has been made in the comprehension of the molecular mechanisms underlying muscle diseases but, in the majority of cases, their clinical management is symptom-oriented and includes physical therapy, physical exercise, orthopaedic corrections, pharmacologic interventions, and artificial ventilation.

Previously, there has been a paucity of efficacious available treatments for myopathies, particularly related to the notion that nutrients may exert a variety of positive effects on skeletal muscles. However, more recently there has been an increase in studies that have explored the effects of nutritional interventions. These include changes in macronutrient composition and nutritional supplements to ameliorate the decrements in skeletal muscle structure and function. Indeed, the emerging consensus is that multiple strategies including nutrition and physical exercise may be successful in certain cases. This approach has been mainly supported by the acquired and consolidated knowledge on the ergogenic, trophic, anti-inflammatory, and antioxidant effects of selected nutrients (i.e., amino acids, creatine, carnitine, ω3-polyunsaturated fatty acids, vitamin D, and polyphenols) on skeletal muscles that, in part, explains their widespread use within the general population and, more so, by recreational and professional athletes.

Notwithstanding this potential, a number of variables can significantly impact the muscular outcome of nutrients including type, combination, timing of administration, duration of treatment, as well as sex, age of intervention, and genotype of the subjects to be supplemented. Thus, confident conclusions about their use cannot yet be drawn under either normal physiological or pathophysiological conditions. Furthermore, experimental evidence to support both short- and long-term effects and the safety of supplements in the different myopathies are still not well-defined. These limitations have encouraged researchers to investigate the regenerative and anti-inflammatory properties of selected nutrients under simplified experimental settings, such as exercise-induced adaptation to muscle injury due to cycles of repetitive eccentric and concentric contractions. Overall these efforts led to the idea that nutrients may positively impact muscle regeneration by acting at different levels and partly through common mechanisms (e.g., the mTOR pathway) which could be related to degenerative/regenerative responses to damage, including early inflammation, satellite cell activation, proliferation and fusion, neuromuscular junction formation and, finally, maturation of newly formed myofibers.

The special issue includes two original research articles and five comprehensive reviews. What emerges from this special issue is that selected compounds or nutritional approaches could be useful in certain types of muscle pathologies, but, considering the multifaceted etiology of myopathies, there is also the likelihood that each compound or nutritional intervention could be ineffective or even potentially harmful under certain conditions. We hope that this special issue may represent a useful reference and encourage additional studies by investigators to expand knowledge of

nutrition as an alternative/supplementary approach to treat muscle diseases.

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Research Article

Improvement of Endurance of DMD Animal Model Using Natural Polyphenols

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Duchenne muscular dystrophy (DMD), the most common form of muscular dystrophy, is characterized by muscular wasting caused by dystrophin deficiency that ultimately ends in force reduction and premature death. In addition to primary genetic defect, several mechanisms contribute to DMD pathogenesis. Recently, antioxidant supplementation was shown to be effective in the treatment of multiple diseases including muscular dystrophy. Different mechanisms were hypothesized such as reduced hydroxyl radicals, nuclear factor-κB deactivation, and NO protection from inactivation. Following these promising evidences, we investigated the effect of the administration of a mix of dietary natural polyphenols (ProAbe) on dystrophic mdx mice in terms of muscular architecture and functionality. We observed a reduction of muscle fibrosis deposition and myofiber necrosis together with an amelioration of vascularization. More importantly, the recovery of the morphological features of dystrophic muscle leads to an improvement of the endurance of treated dystrophic mice. Our data confirmed that ProAbe-based diet may represent a strategy to coadjuvate the treatment of DMD.

1. Introduction

Muscular dystrophies (MDs) are a heterogeneous group of disorders characterized by muscular wasting and inflammation that ultimately cause reduction of force and premature death [1]. The role of inflammatory cells is not completely understood but it is known that they could exacerbate muscular wasting, either directly or by secreting mediators, like cytokines and complement's component [2], and by reactive oxygen species (ROS) production [3–5]. ROS were shown to increase membrane permeability in muscle fibres, most likely through lipid peroxidation [6]. Different groups demonstrated that increased ROS production was a primary feature of dystrophic muscle damage and not simply a consequence of muscular degeneration [7, 8]. Others showed that ROS-mediated effect on dystrophic muscles increased protein oxidation, which could cause a wide range of deleterious effects on muscle contractile function [9], both in mdx mice [10] and in DMD patients [11]. It is now known that the intracellular increase in ROS formation typical of the pathological conditions of the MDs is mainly due to mitochondria [12]. Even if the precise mechanism by which oxidative stress causes mitochondria dysfunction is not clear, numerous studies reported that ROS could negatively modify both expression and structural conformation of the proteins that are involved in normal mitochondria functions [13, 14].

In latest years, antioxidant supplementation was shown to be useful in the treatment of different diseases like atherosclerosis, autoimmunitary diseases, diabetes, and chronic diseases including muscular dystrophies [15–18]. The beneficial effects of antioxidants were correlated with their ability to reduce oxidative stress, deactivate nuclear factor-κB (NF-κB) pathway, and vascular effects like vasodilatation and antihypertension through NOS pathway [19]. Many natural antioxidants were tested in animal model of muscular dystrophies and these studies reported amelioration in muscle morphology and function [20].

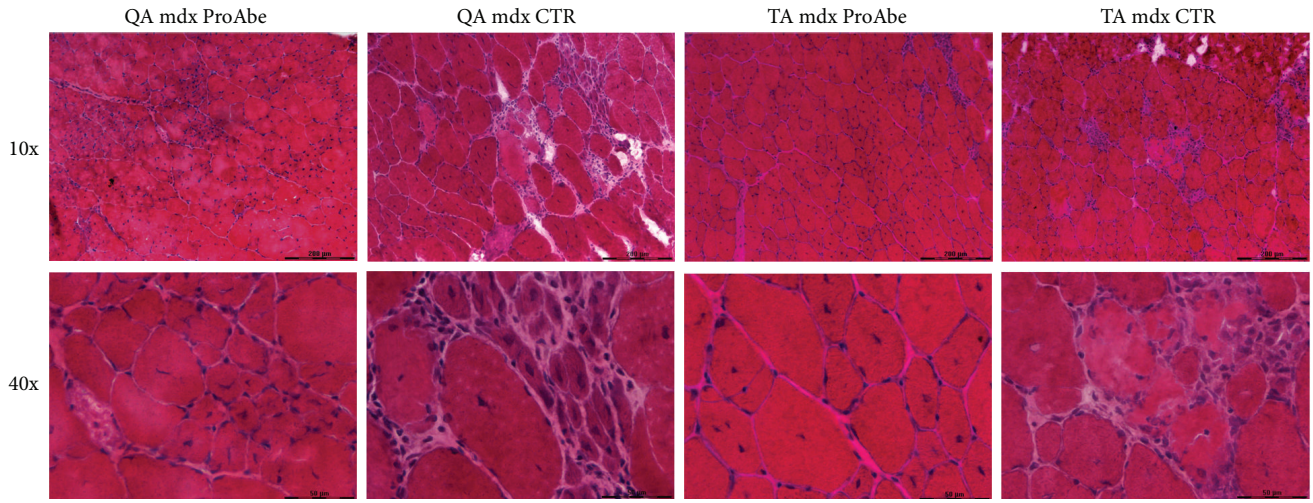
As an example, green tea is rich in antioxidants such as catechins and also in minerals and vitamins so that it was demonstrated to be useful in the reduction of the risk of cardiovascular diseases and of fibrosis development [21]. Interestingly, *in vivo* studies on mdx mice fed with antioxidants derived from green tea showed reduced signs of muscular damage and ameliorated hydroxyl radicals content, oxidative stress, and fibrosis in treated muscles [22]. According to the emerging role of vitamins in the prevention of chronic diseases [23], Murphy and Kehrer observed similarities between the development of pathological signs in muscular dystrophies and the pathology of muscles exposed to oxidative stress in vitamin E deficiency [24]. Messina and coworkers demonstrated that a synthetic vitamin E analogue, IRFI-042, possessing strong antioxidant properties, improved mdx muscle function and reduced the activation of NF-κB [25]. NF-κB is a key regulator of several genes such as the proinflammatory cytokine TNF-α [26] and matrix metalloproteinases [27]. In this sense, Kumar and Boriek showed that passive stretch of mdx diaphragm increased activation of NF-κB, which was attenuated by the antioxidant N-acetylcysteine [28]. At the same time, it was demonstrated that systemic administration of the NF-κB inhibitor curcumin stimulated muscle regeneration after traumatic injury, suggesting a beneficial effect of curcumin supplementation in alleviating dystrophic signs [29]. A possible role for coenzyme Q10 (CoQ10) in the development of different diseases was investigated: especially in cardiovascular pathologies, relatively low levels of CoQ10 were assessed in myocardial tissue [30]. More recently, CoQ10 was added to prednisone therapy in DMD patients, increasing muscle strength [31]. Similarly, carnitine held much promise in neural disorders, allowing osmoprotection and modulating immune and inflammatory responses [32]. Several works showed that this compound regulated lipid metabolism in DMD patients, restoring muscle membrane fluidity [33], decreasing glucose oxidation, and reducing fatty acid [34]. Following previously published data [35], Fogagnolo and colleagues demonstrated that mdx mice fed with docosahexaenoic acid (DHA) decreased plasma creatine kinase levels and myonecrosis, reducing inflammatory area and the levels of TNF-α [36]. In the end, another natural flavonoid, the baicalein, was used as a potent anti-inflammatory agent to diminish the concentration of free radicals [37, 38].

Palomero et al. showed that muscular fibres during exercise produce ROS [39]. Interestingly, Reid et al. proposed a correlation between ROS levels and force production. They showed that the maximum force was achieved by unfatigued skeletal muscle when exposed to low levels of oxidants. As either an increase or a reduction in ROS levels determined a reduction in muscle force, they suggested that there was an optimal redox state for force production [40]. Reid proposed that ROS could affect muscle force production by oxidation of contractile and excitation-contraction (E-C) coupling proteins [41] and the role of ROS in mediating muscle fatigue was demonstrated by treatment with antioxidants [42, 43]. Recently Renjini et al. showed that oxidative damage in muscular dystrophy correlates with the severity of the pathology [44] while Selsby and collaborators proved that the overexpression of the antioxidant enzyme catalase improved muscle function in the mdx mouse, especially the resistance to fatigue [45]. Following these promising evidences, several clinical trials started using antioxidants in DMD patients. However, the results were disappointing due to a number of factors, which could account for the negative outcome [7]. First of all, DMD patients were chosen at an advanced stage of the disease, when significant muscle fibre loss had already occurred. Unfortunately, antioxidants would be expected to either reduce or prevent muscle damage and degeneration but not to replace lost fibres. Moreover, the antioxidants used in these trials—such as superoxide dismutase (SOD), vitamin E, and selenium—were not membrane-permeant and were ineffective in scavenging intracellular ROS [20]. Furthermore, several works demonstrated that the combination of different polyphenols might enhance their therapeutic effects, due to a synergic effect of different antioxidants or the contemporary targeting of multiple pathologic pathways [17, 46–48].

According to these evidences, we fed mdx mice with a mix of natural polyphenols (ProAbe), constituted by a liquid phase and a solid phase and we evaluated the amelioration of muscle histology, the oxidation damage, and the possible increase of muscle mass and endurance in dystrophic background. Our data confirmed that the treatment with antioxidants could open a new era in treating muscular diseases.

2. Results

2.1. Muscular Features of mdx Mice. Fibrosis is considered the most devastating consequence of the progression of disease in DMD patients: due to the lack of dystrophin, satellite cell proliferation cannot compensate constant myofiber breakdown so that inflammatory processes that follow muscular necrosis lead to fibrotic remodelling and finally fatty cell replacement. As in DMD children, the muscle pathology progressed in mdx mice as a function of age. This way, we fed 3-month-old mdx mice ($n = 5$) with ProAbe and we performed H&E analysis of muscle sections to verify whether this diet could delay the onset of the pathology. In tibialis anterior (TA) and quadriceps (QA) of treated mice, we observed the presence of degenerating and small centrally nucleated regenerating muscle fibers, such as in untreated mice; however reduced signs of degeneration (consisting in hypertrophic fibers, fiber splitting, and fat replacement) were seen in treated mice versus untreated ones ($n = 5$) (Figure 1(a)). To verify whether antioxidants supplementation could bear an effect on muscle mass we measured cross-section fiber area (CSA)



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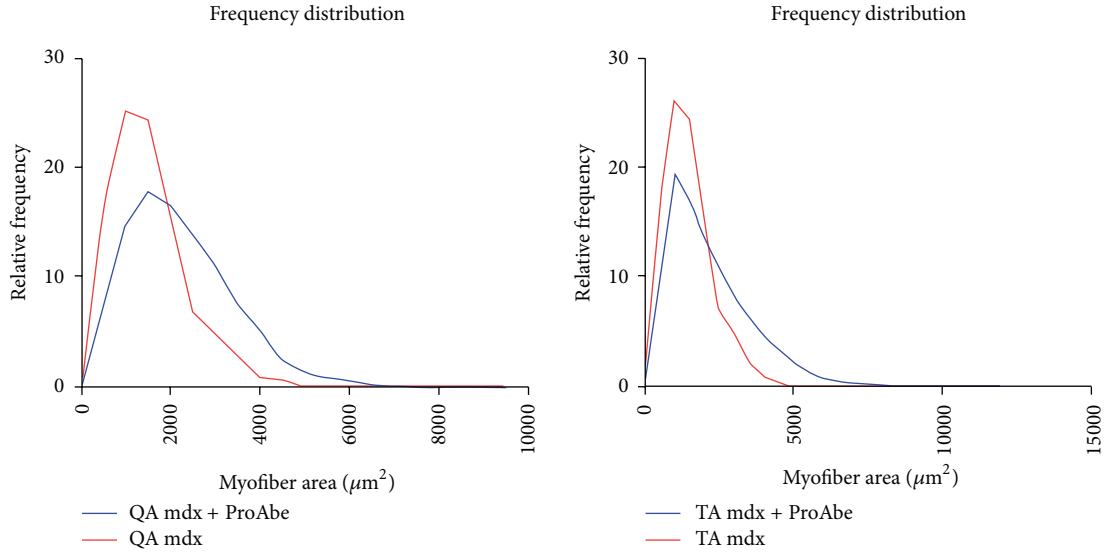

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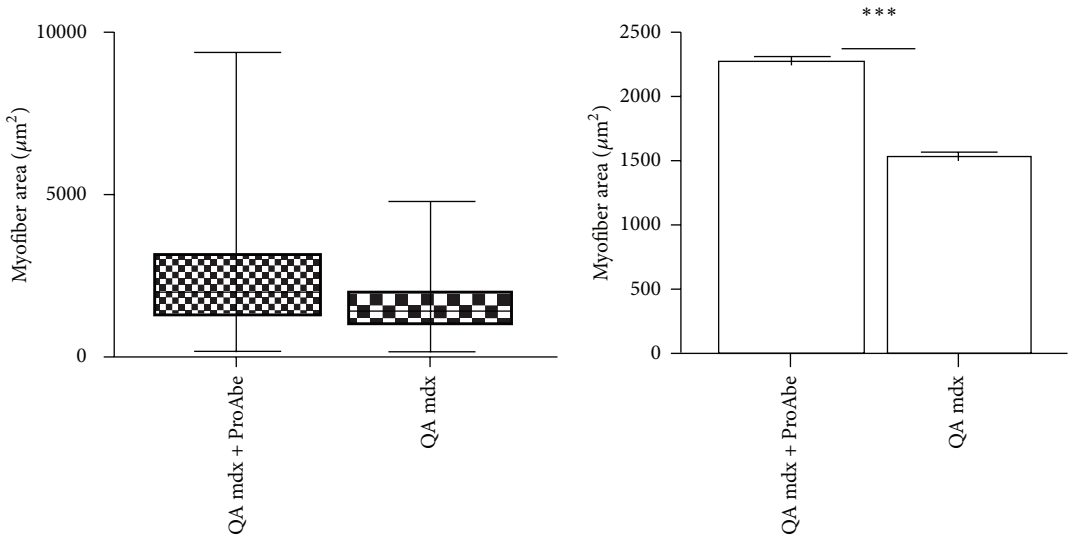

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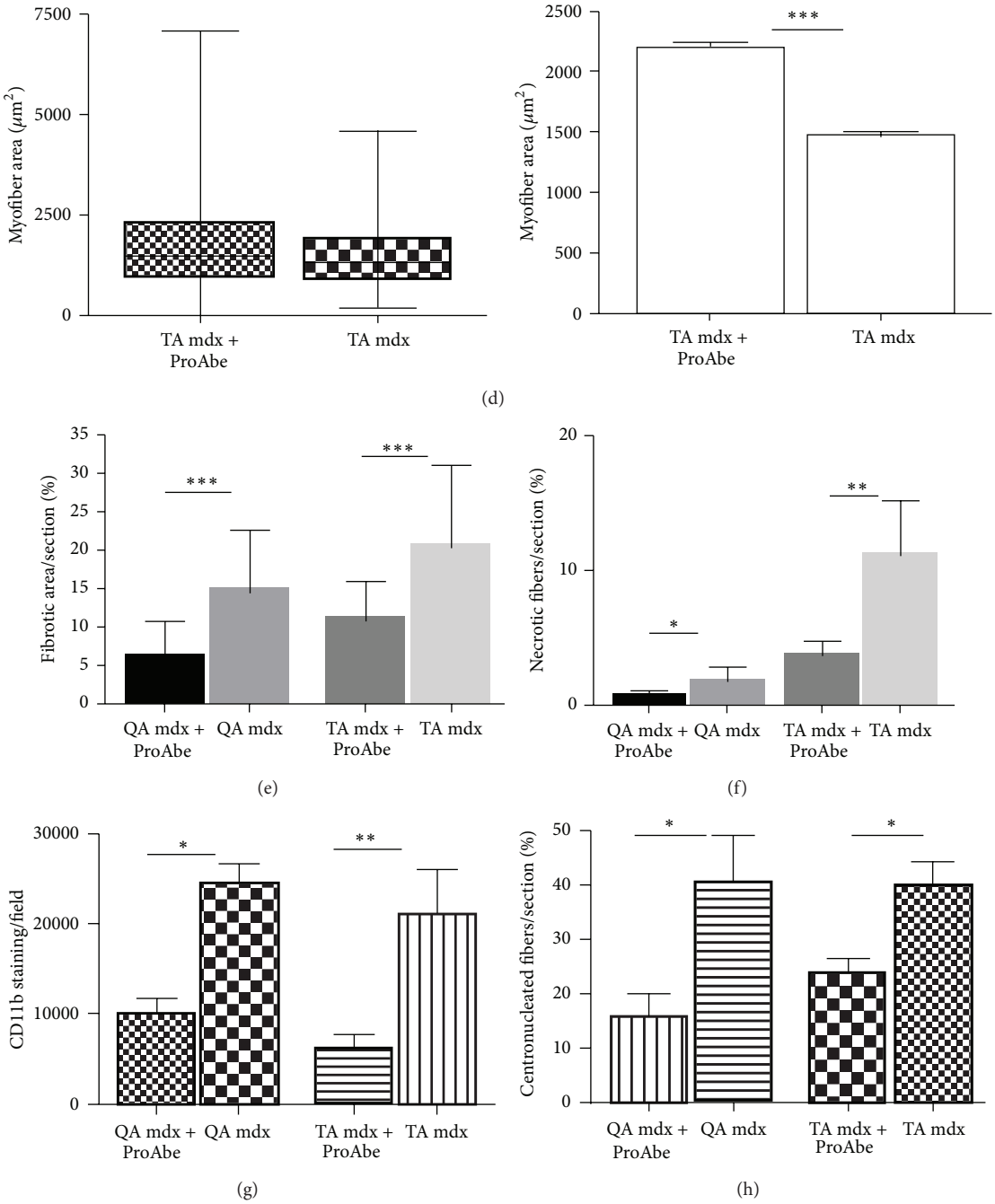


Figure 1: H&E analysis of treated and untreated mice. H&E staining was performed on 10 μm-thick frozen sections from TA and QA muscles (a). In treated mice reduced signs of muscular wasting were observed in comparison to untreated mice. In particular reduced inflammatory infiltrates between myofibers, reduced fat deposition, and reduced necrotic fibers were assessed. In both treated and untreated mice small centronucleated regenerating fibers were observed. For each muscle analyzed with H&E staining we showed the myofiber area and their distribution frequency (b). The curve of TA/QA muscles of treated mice shifted to the right related to untreated mice, thus demonstrating an increase in myofiber area of treated mice (CSA QA: 25° percentile of treated mice 1221,26 and of untreated mice 903,152; CSA TA: 25° percentile of treated mice 1120,39 and of untreated mice 874,068; CSA QA: 75° percentile of treated mice 3107,04 and of untreated mice 1946,06; and CSA TA: 75° percentile of treated mice 2978,59 and of untreated mice 1883,39). Moreover, we indicated the coefficient of variance (graphs showing Min and Max values and mean + SEM) ((c) for QA and (d) for TA). Amelioration of dystrophic phenotype following ProAbe treatment was demonstrated by decrease of fibrosis by measuring area of connective tissue (AM) (e), of the percentage of necrotic fibers (f), of macrophage infiltration area (CD11b staining) (g), and of the number of centronucleated myofibers per section (h).

of both treated and untreated mdx mice. We found that the distribution curves of treated mice shifted to the right in comparison to that one related to mdx control group, thus proving that there was a significant increase in fiber CSA in both muscles examined (TA and QA muscles) (F test to compare variance was significant for $P < 0{,}0001$) (Figures 1(b)–1(d)). In particular there was a reduction in the percentage of smaller fibers (CSA of QA in treated 2274 ± 32,59 and untreated mice 1535 ± 20,08; CSA of TA in treated 1681 ± 23,76 and untreated mice 1486 ± 19,44; t-test to compare mean was significant for $P < 0{,}0001$) (Figures 1(c)-1(d)). To better elucidate that ProAbe-dependent increase of muscle size was not due to fibrotic deposition, other morphological features of the muscles of treated mice were measured. We demonstrated a diminished percentage of fibrosis in both muscles treated with ProAbe (treated QA 6,255 ± 0,632 and untreated QA 14,67 ± 0,66 $P < 0{,}0001$; treated TA 11,29 ± 0,736 and untreated TA 20,62 ± 1,521 $P < 0.0001$) (Figure 1(e)) and we observed that the percentage of necrotic fibers was significantly smaller in both treated muscles (treated QA 0,8944 ± 0,06 and untreated QA 2,015 ± 0,376, $P = 0{,}045$; treated TA 3,867 ± 0,4884 and untreated TA 11,35 ± 1,926, $P = 0{,}0093$) (Figure 1(f)). As clearly described by different works [49–51] inflammatory cells in DMD can interact with resident muscular stem cells and cause the fibroadipogenic degeneration of muscular fibers. According to these evidences, CD11b staining was performed to identify macrophage infiltrates as an indicator of muscle inflammation and we assessed a reduction in inflammatory infiltrates both in TA and in QA muscles of treated mice (untreated TA: 21130 ± 4909 and treated TA: 6187 ± 1460, $P = 0{,}0154$; untreated QA 24610 ± 2217 and treated QA 10080 ± 1559, $P = 0{,}0017$) (Figure 1(g)). Moreover, we counted the number of centronucleated myofibers—that are the fibers in regeneration—and we demonstrated that their number was smaller in both treated muscles (untreated TA: 39,79 ± 4,571 and treated TA: 23,64 ± 2,833; $P = 0{,}0133$; untreated QA 40,42 ± 8,645 and treated QA 18,05 ± 3,981, $P = 0{,}0369$) (Figure 1(h)).

2.2. Force Measurement. To verify whether a natural polyphenols diet could improve muscular functionality we first test the endurance of treated and untreated mdx mice ($n = 10$ per each group). In these experiments we found that ProAbe administration significantly increased the endurance ability of mdx mice after 4 weeks of treatment (total average hours run: mdx treated 3,656 ± 0,728 versus mdx untreated 1,844 ± 0,426; $P = 0{,}0474$) (Figure 2(a)). We measured the total motor capacity relative to baseline performance and we observed an increase of 30% at day 30 of treatment (Figure 2(b)). Dystrophic animals assessed at 30 days of treatment showed an increased tolerance to exercise as demonstrated by the time to exhaustion (mdx treated 14,33 ± 0,817 versus mdx untreated 11,02 ± 0,849; $P = 0{,}0117$) (Figure 2(c)). Furthermore, we calculated the tetanic force of TA and DIA of treated and untreated mdx ($n = 5$ per each group) and we found a minimum but not significant improvement of the values in treated mdx muscles (Figures 2(d) and 2(e)).

2.3. Oxidative State Evaluation. To evaluate whether natural polyphenols diet could influence ROS production we quantified dihydroethidium (DHE) staining in muscle sections from treated and untreated dystrophic mdx mice ($n = 5$ per each group). DHE oxidation by intracellular ROS causes the formation of ethidium bromide, which fluoresces red, once intercalated within DNA. Thus nuclear fluorescence intensity directly correlates with ROS production. We measured DHE fluorescence intensity on sections of TA and QA (Figure 3(a)) and we found that the ProAbe-enriched diet significantly diminished ROS production (TA of treated 68450 ± 7782 and untreated mdx mice 95470 ± 5185; $P = 0{,}0277$) (Figure 3(b)). In QA muscle of treated mice we observed a similar decrease in ROS production that did not reach statistical significance (QA of treated 82940 ± 3434 and untreated mdx mice 95330 ± 5219; $P = 0{,}1183$). C57Bl mice were used as control (42380 ± 1378). As shown in Figure 3(a), DHE stained both myofiber nuclei and cell infiltrates: after ProAbe treatment, DHE staining was reduced both in inflammatory infiltrates and in myofiber nuclei (Figure 3(a)). To determine whether the reduction in DHE intensity was related to reduced inflammatory infiltrates (as previously shown in Figure 1(g)), we correlated both data and we showed that the reduction of inflammatory infiltrates was major than the corresponding reduction of DHE intensity (Figure 3(c)). As the number of myofiber nuclei was equivalent, ProAbe treatment influenced more the ROS produced by myofibers than those produced by infiltrating cells in particular macrophages.

2.4. Mitochondria. As cited before, we determined whether the effects of ProAbe on ROS production were exerted on mitochondria and we evaluated the activity of mitochondrial enzymes in QA and diaphragm (DIA) of treated and untreated C57Bl ($n = 5$) and mdx mice ($n = 5$) (Figure 4). We demonstrated that there were no significant modifications within the activity of these enzymes in treated mice.

2.5. Vascular Features. To test the influence of antioxidants upon muscle vascular architecture, we performed CD31+, α-sma, and laminin immunofluorescence staining on muscle sections from TA and QA of treated and untreated mice ($n = 5$) (Figure 5(a)). We counted the number of CD31+ vessels per fiber (capillaries) and of α-sma+ vessels per fiber (arterioles). The increase of CD31+ vessels in treated muscles was statistically significant in dystrophic mice (TA mdx treated versus TA mdx untreated: 1,553 ± 0,059 versus 1,272 ± 0,045; $P < 0{,}001$), but it was not significant in normal mice (TA C57Bl treated versus TA C57Bl untreated: 3,146 ± 0,1076 versus 2,835 ± 0,1289; $P = 0{,}0624$) (Figure 5(b)). Therefore, we found that all treated mice had a significant increase in α-sma+ vessels per fiber (TA mdx treated versus TA mdx untreated: 7,85 ± 0,2975 versus 3,233 ± 0,2108; $P < 0{,}0001$; TA C57Bl treated versus TA C57Bl untreated: 6,04 ± 0,34 versus 3,54 ± 0,2; $P < 0{,}0001$), thus demonstrating an amelioration of muscle perfusion (Figure 5(c)). QA muscles had similar but less significant amelioration of vascular compartment (data not shown).

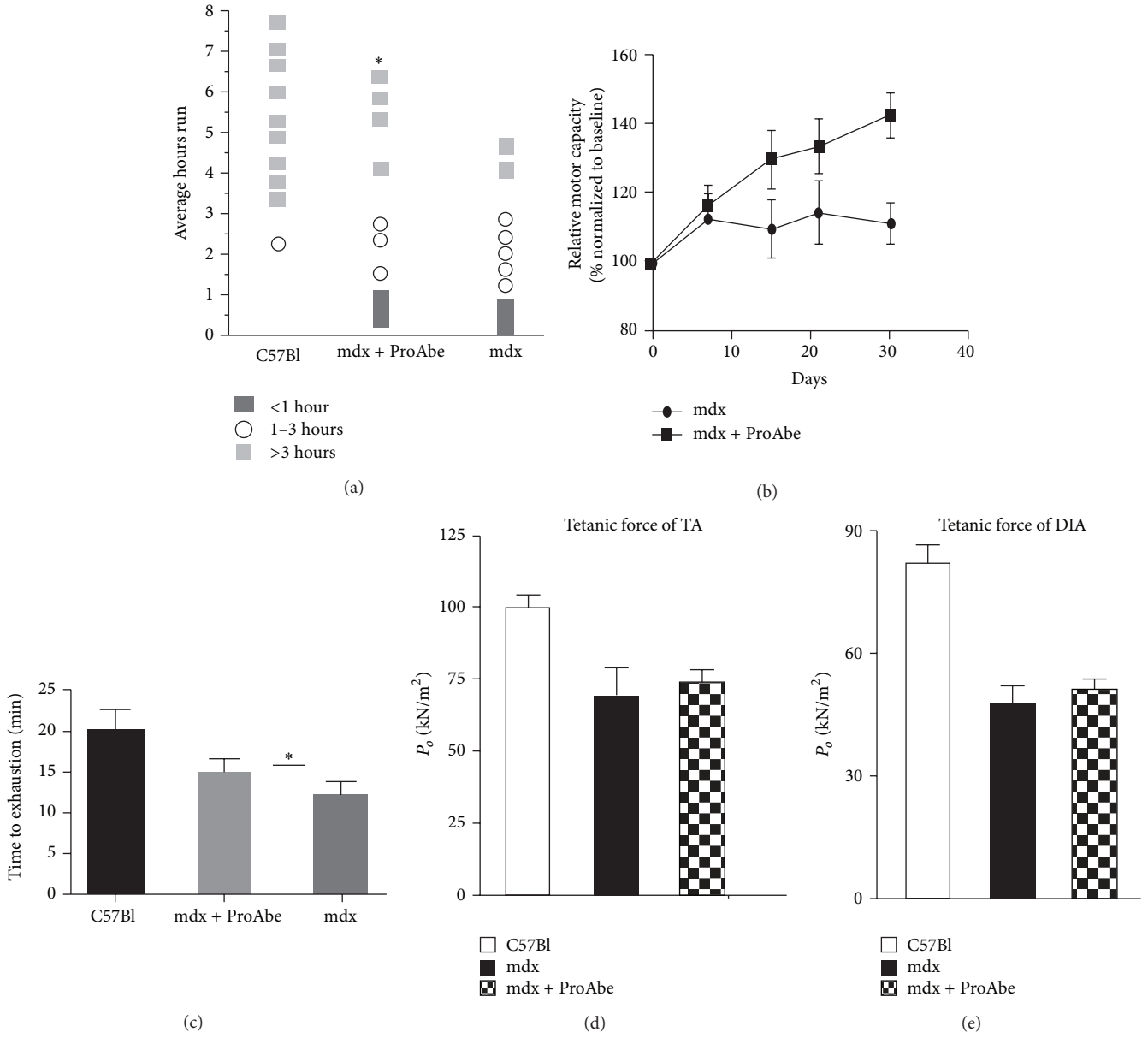


Figure 2: Endurance test. Histogram representing endurance test of treated mice. (a) Each symbol corresponds to a single animal performance; endurance test was repeated once per day for 30 days for each mouse. The average value corresponds to the total amount of time measured during the tests for 30 days. As demonstrated by the average number of hours run mdx-treated mice increased significantly their performance versus untreated mice, even if this value was far from that of C57Bl mice (a). (b) Graph showed relative motor capacity of mice (percentage normalized to baseline) at 4 different time points (each week for 30 days). (c) Graph showed the absolute value of T4 of graph in (b) (that is the average time to exhaustion at T4 (30 days)) that was higher in mdx treated mice related to untreated ones. Tetanic force of TA (d) and DIA (e) was performed in treated and untreated mdx mice.

2.6. Stem Cell Mobilization Mediated by ProAbe Treatment. To test the hypothesis that ProAbe could favour the mobilisation of bone marrow stem cells as demonstrated for other natural components [52–54], we collected the blood from C57Bl and mdx mice fed with ProAbe at different time points ($n = 5$ for each strain) and we investigated the expression of different cellular markers by FACS analysis. T0 (before treatment) was used as baseline. We chose CD34, a cell-cell adhesion factor, able to mediate the attachment of stem cells to bone marrow extracellular matrix; CXCR4 that regulates the mobilization of haematopoietic stem cells into the bloodstream as peripheral blood stem cells; and Sca-1 that is a classical stem cell antigen. Following the treatment with ProAbe, we observed a mobilization of stem cells from bone barrow to the peripheral blood (7-fold increase in peripheral CD34+ stem cells at 24 h of treatment related to baseline) and we assessed that the mobilization in the dystrophic mice was higher related to C57Bl mice. Values were normalized on

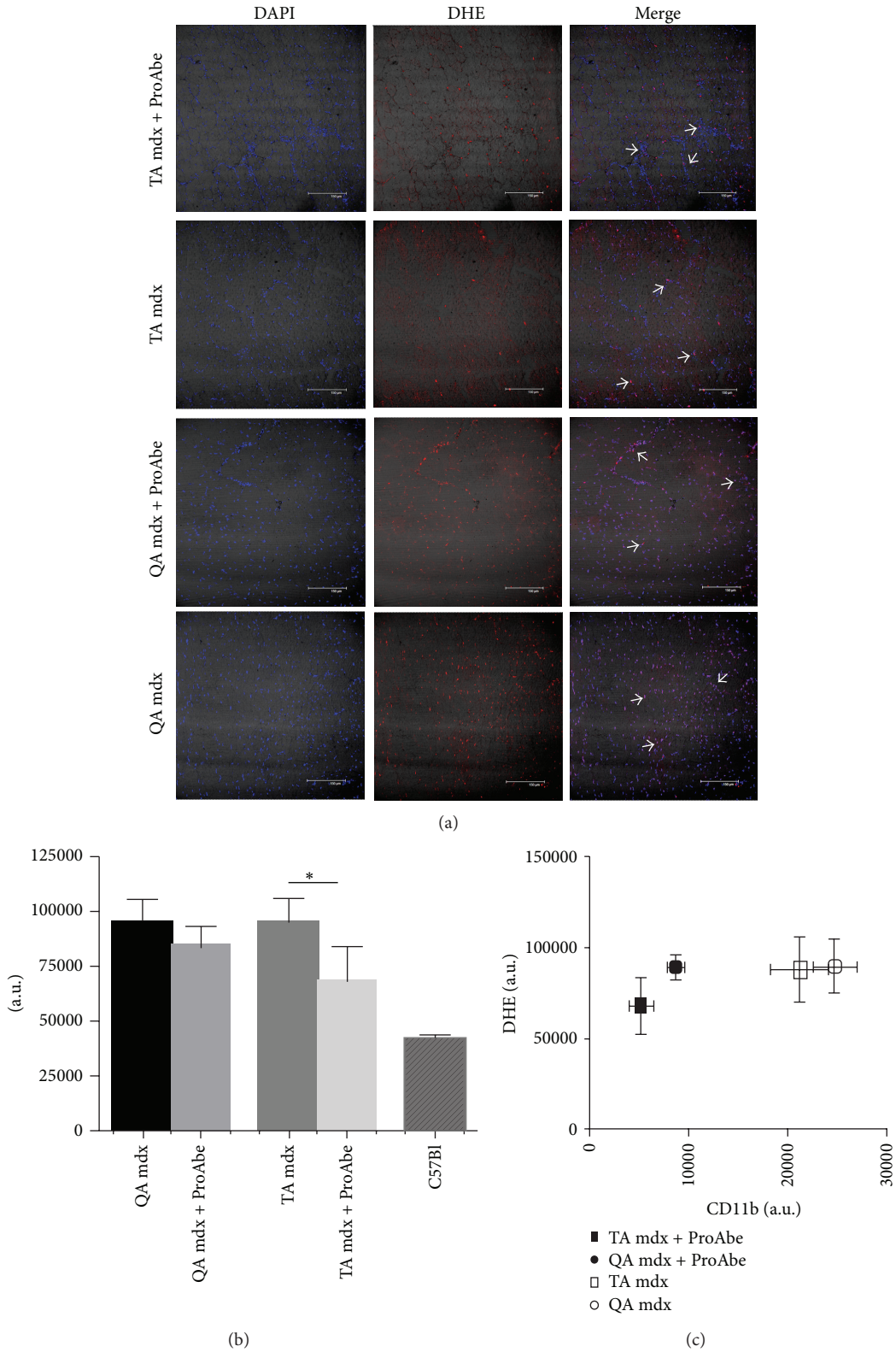
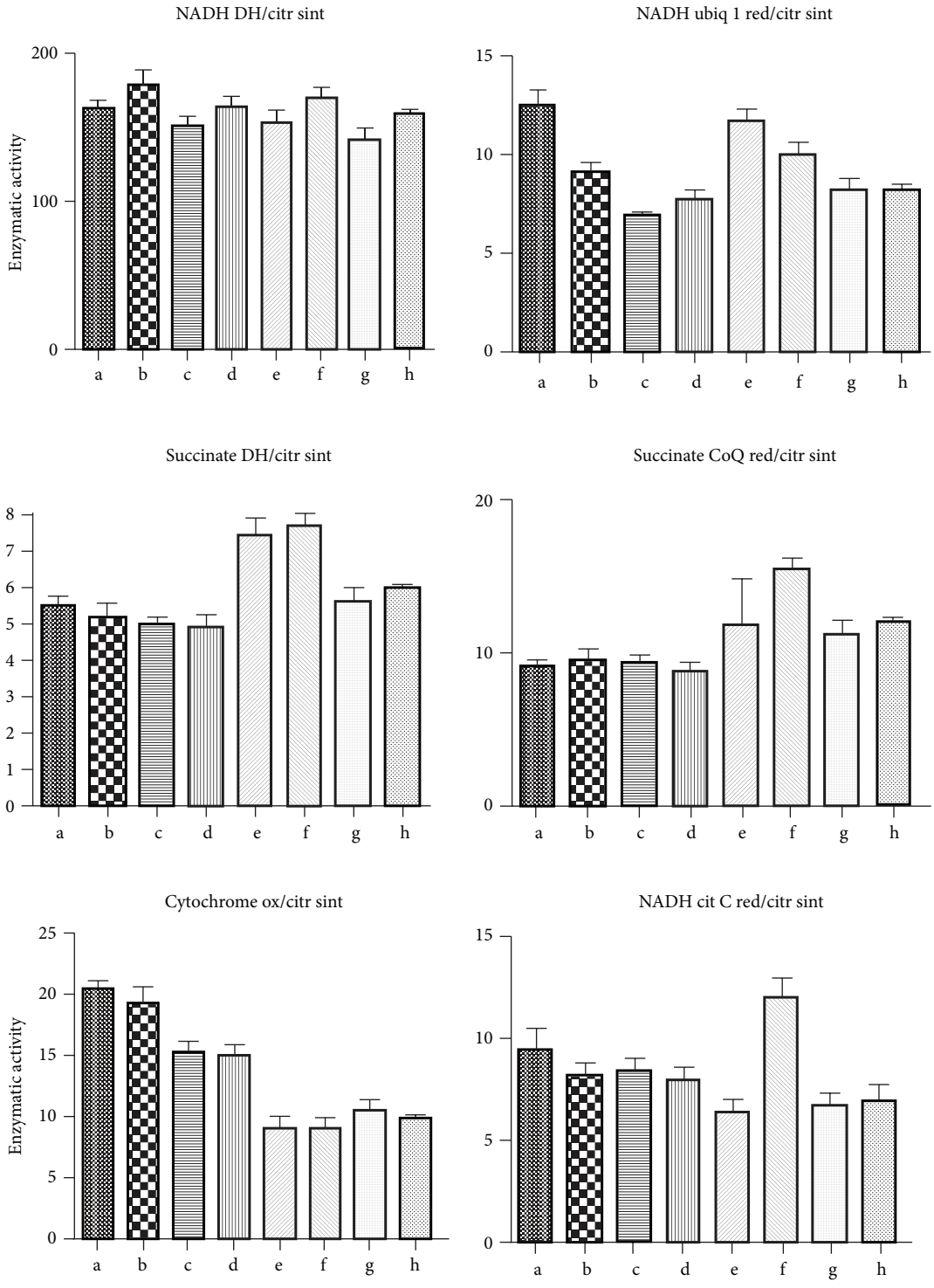


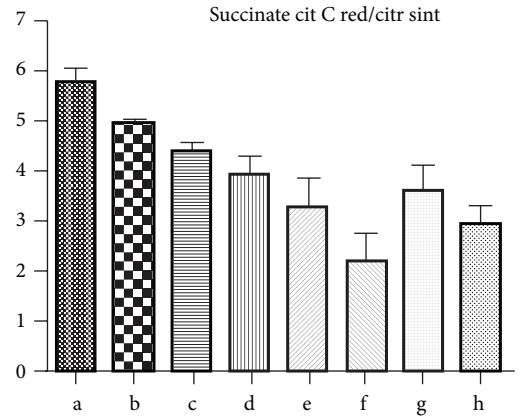
Figure 3: DHE staining and quantification. (a) DHE staining on muscle sections from TA and QA of mdx mice. DHE staining revealed anion superoxide production at nuclear level. Arrows indicated DHE staining on both cellular infiltrate and fibers myonuclei. (b) We quantified DHE staining intensity on muscle section. Histogram showed a reduced production of oxidative damage as measured by DHE staining in TA muscle of mdx-treated mice versus untreated ones ($P < 0,05$). (c) Graph showed the correlation between DHE staining intensity and CD11b+ infiltrate (per field). The mean and SD for each category are plotted. ProAbe treatment reduced CD11b+ infiltrates more than DHE intensity (as shown by right position of untreated mice versus left position of treated mice).



(a) QA C57Bl
(b) QA C57Bl + ProAbe
(c) QA mdx
(d) QA mdx + ProAbe
(e) DIA C57Bl
(f) DIA C57Bl + ProAbe
(g) DIA mdx
(h) DIA mdx + ProAbe

(a) QA C57Bl
(b) QA C57Bl + ProAbe
(c) QA mdx
(d) QA mdx + ProAbe
(e) DIA C57Bl
(f) DIA C57Bl + ProAbe
(g) DIA mdx
(h) DIA mdx + ProAbe

Figure 4: Continued.



(a) QA C57Bl
(b) QA C57Bl + ProAbe
(c) QA mdx
(d) QA mdx + ProAbe
(e) DIA C57Bl
(f) DIA C57Bl + ProAbe
(g) DIA mdx
(h) DIA mdx + ProAbe

Figure 4: Enzymatic activity of mitochondrial enzymes. Histogram representing enzymatic activity of a wide range of mitochondrial enzymes involved in respiratory chain complexes. No significant differences were evidenced between treated or untreated mdx mice in DIA and QA. The following abbreviations were used in the picture (NADH DH/citr sint: NADH dehydrogenase/citrate synthase; NADH ubiq 1 red/citr sint: NADH ubiquinone 1 reductase/citrate synthase; succinate DH/citr sint: succinate dehydrogenase/citrate synthase; succinate CoQ red/citr sint: succinate CoQ reductase/citrate synthase; Citrate ox/citr sint: citrate oxidase/citrate synthase; NADH cit C red/citr sint: NADH citrate C reductase/citrate synthase; and succinate cit C red/citr sint: succinate citrate C reductase/citrate synthase).

the total number of CD45+ stem cells and the significance of these values was related to the T0 (before treatment) value (% of CD34+ in mdx: T0: 0,8775 ± 0,2893; T24: 4,684 ± 0,6313 with $P = 0{,}028$; T72 h: 8,717 ± 2,309 with $P = 0{,}0104$. % of CD34+ in C57Bl: T0: 0,7462 ± 0,2366; T24: 3,554 ± 1,001 with $P = 0{,}0245$), (% of Sca-1+ in mdx: T0: 27,11 ± 2,053; T24: 64,8 ± 5,408 with $P < 0{,}0001$; T48 h: 64,95 ± 1,073 with $P < 0{,}0001$; T4 d: 65,96 ± 3,992 with $P < 0{,}0001$; T7 d: 64,16 ± 1,016 with $P < 0{,}0001$; T10 d: 62,82 ± 3,043 with $P < 0{,}0001$. % of Sca-1+ in C57Bl: T0: 36,95 ± 4,967; T24: 72,25 ± 2,785 with $P = 0{,}0003$; T48 h: 62,25 ± 0,8039 with $P = 0{,}001$; T4 d: 67,49 ± 3,772 with $P = 0{,}0012$; T7 d: 67,3 ± 1,214 with $P = 0{,}0003$; T10 d: 60,55 ± 1,746 with $P = 0{,}0021$), and (% of CXCR4+ in mdx: T0: 0,4009 ± 0,1396; T24: 2,805 ± 1,091 with $P = 0{,}0425$) (Figure 6(a)). Furthermore, we analysed the expression of stromal cell-derived factor 1 (SDF-1), the ligand of the CXCR4, by the cells isolated from QA of treated and untreated mice and we demonstrated that, after 7 days of ProAbe treatment, in mdx mice there was a significant mobilization of SDF-1+ stem cells from bone marrow (QA of treated mdx 9,65 ± 0,25 versus QA of untreated mdx 2,5 ± 0,1; $P = 0{,}0014$; QA of treated C57Bl 2,25 ± 0,25 versus QA of untreated C57Bl 1,5 ± 0,5) (Figure 6(b)). In addition skeletal muscle from mdx treated mice contained a higher fraction of a cell population CD34+Sca-1+CD45+ characterized by bone marrow origin and stemness (QA of treated mdx 0,026 ± 0,008645 versus QA of untreated mdx 0,00323 ± 0,001862; $P = 0{,}0419$) while in C57Bl the two fractions were similar (Figure 6(c)). In fact a subpopulation of cell isolated from skeletal muscle shared few peculiarities of bone-marrow-derived HSCs (expressing Sca-1 and c-kit); however muscle HSCs do not express CD45 while HSC derived from bone marrow express this antigen [55]. This way, the population CD34+Sca-1+CD45− representing resident stem cell population was analyzed in QA of treated and untreated mice and we found that it was unchanged (Figure 6(d)).

3. Discussion

Unfortunately extensive researches were not enough to build up efficacious strategies not only to correct the primary defect of DMD but also to alleviate the downstream pathologies. In the latest years, it was clear that membrane fragility caused by the absence of dystrophin led to inflammation and fibrosis of muscles so that several antioxidants and nutritional supplements were studied to inhibit the activity of certain molecules (NF-κB, TNF-α, and TGF-β) and, subsequently, to modulate these devastating phenomena. Several works demonstrated that, following supplementation in mdx mice, natural compounds were able to reduce fibrosis [22] and to ameliorate muscle strength [31]. Furthermore, they exerted antioxidant activity [25] and regulated the inflammatory cells [32] and lipid metabolism [33]. It is known that ROS could exacerbate muscular damage by oxidizing membrane phospholipids and proteins, thus increasing membrane permeability and

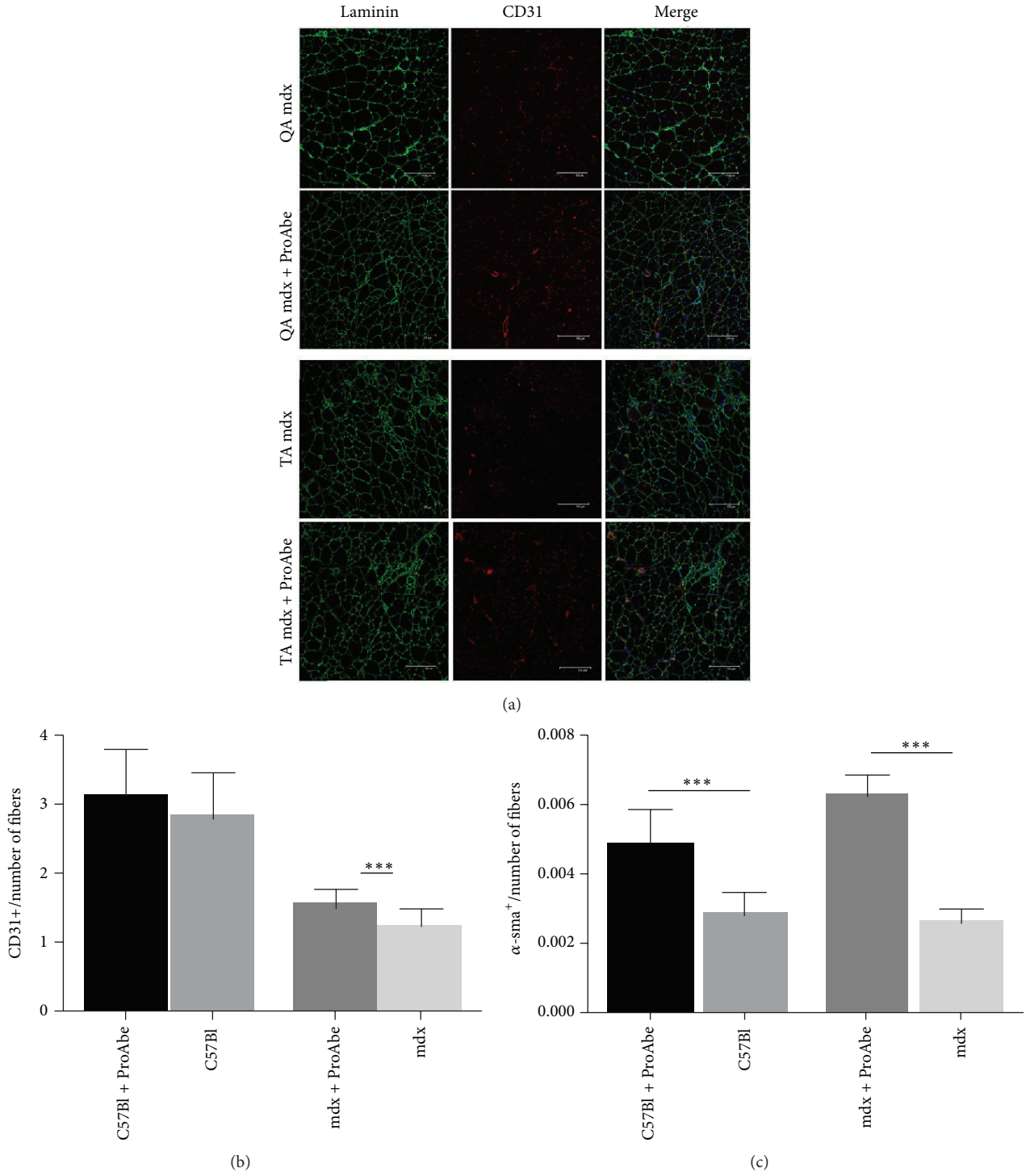


FIGURE 5: Vascular features of treated mice. (a) Panel representing staining for laminin and CD31 in QA and TA of mdx-treated and untreated mice. (b) The ratio CD31+ vessels/fiber was significant only for TA of mdx mice while (c) the number of a-SMA+ vessels/section was significant for TA of both mdx and C57Bl mice. Analysis of statistical significance was determined by unpaired *t*-test.

leakage [56, 57]. As expected, after ProAbe treatment, we observed a reduction in necrotic fiber and in mononuclear cells' infiltration among intact fibers, together with reduced fibrosis deposition. Furthermore small centronucleated fibers were diminished according to a reduction of percentage of smallest fibers and, conversely, a general increase in muscular mass (as shown by right-shift of frequency distribution curve) demonstrating that an antioxidant-based diet could

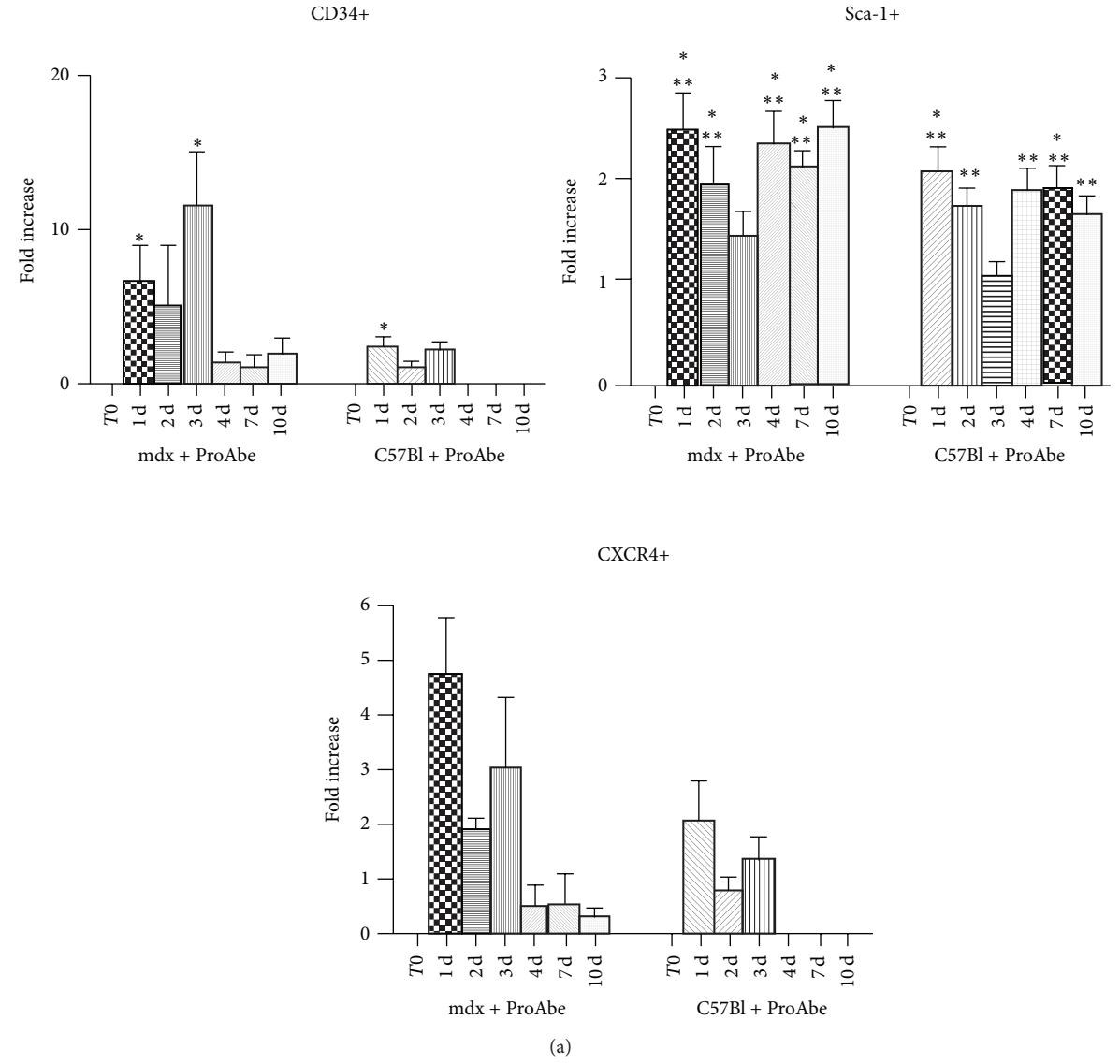
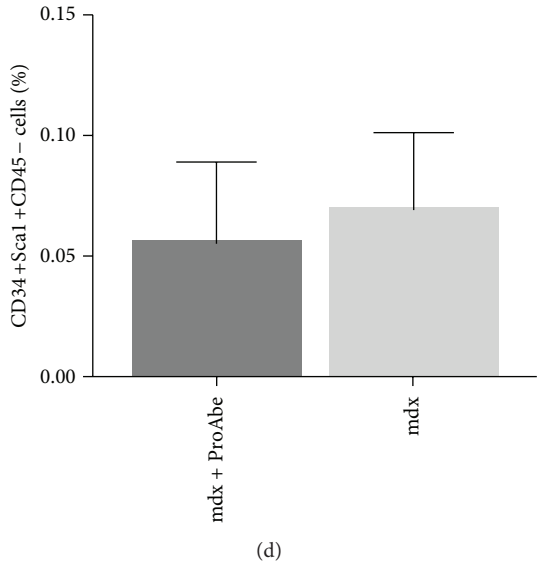


Figure 6: Continued.



(d)

Figure 6: Stem cell mobilization driven by ProAbe. (a) Histograms representing FACS analysis of percentage of stem cell and adhesion marker in PBMCs from treated C57Bl and mdx mice. T0 (before treatment) was used as baseline; bars represented the fold increase of cell's percentage relative to T0. At T24 h, both mdx and C57Bl mice showed an increase in the percentage of peripheral CD34+, Sca-1+, and CXCR4+ cells. After 72 h of treatment the percentage of peripheral CD34+ and CXCR4+ started to decline. After 10 days of treatment the percentage of markers analyzed was similar to T0 levels. (b) FACS analysis, showing the percentage of SDF-1+ cells in QA, was higher in both the mice fed with ProAbe; in particular, in dystrophic mice it was more than 3-fold higher. (c) FACS analysis showing that the percentage of CD34+Sca-1+CD45+ cells isolated from QA was significantly higher only in the mdx-treated mice. (d) FACS analysis showing the percentage of CD34+Sca-1+CD45− cells isolated from treated and untreated mdx mice.

modulate muscular damage. We also demonstrated that in treated mice there is a reduction of muscular anion superoxide production and consequently of DNA oxidation as observed by quantification of DHE staining, confirming that ProAbe supplementation could influence the oxidative state of the muscle. In addition, ProAbe could modulate ROS production both by reducing inflammatory infiltrate or by regulating inflammatory cells activity [58]. Skeletal muscle mass is regulated by a balance between protein synthesis and degradation, which is sensitive to many environmental triggers such as mechanical load, growth factors (myostatin), hormones (glucocorticoid), inflammatory cytokines (TNF-α and IL-6), oxidative stress (ROS and NO), metabolic stress (ATP levels), and nutrient availability. Even if we did not demonstrate an improvement of tetanic force in examined muscle, we found an amelioration in endurance test in mice fed with an antioxidant-rich diet. According to these data, it was previously demonstrated that resistance to fatigue was increased by a diet rich in antioxidants, probably because of the reduction of calcium overload [45, 59]. Successively, we tried to correlate the amelioration of endurance and the diminished production of ROS with possible role of mitochondrial enzymes, but we did not assess any significant difference in the activity of these enzymes between treated and untreated mice. On the other hand, as muscular performance could depend on vascular supply to each fibre, we demonstrated an increase in arteriolar supply by counting α-sma+ vessels and capillaries per fibers in all treated mice. The mechanism by which antioxidants are involved in vascular remodelling is not completely clarified, but it is known that polyphenols, like resveratrol, could protect NO from inactivation favouring its biologic activities [19].

Since the work of Gussoni that firstly described the presence of haematopoietic stem cells (HSCs) in adult skeletal muscle [60], it was demonstrated that these cells could be recruited according to specific molecular cues from bone marrow to muscle, where they actively participated in muscle regeneration [61]. Several papers showed that transplantation of bone-marrow-derived HSCs in animal models of DMD contributed not only to amelioration of skeletal muscle [62, 63] but also to endothelial cell formation [55]—probably due to their residing perivascular niche [64]. According to these evidences, transplantation of bone-marrow-derived HSCs obtained preliminary but promising results in DMD patients [65, 66]. Our data indicated an increase of the CD34+Sca-1+CD45+ stem cells whereas the resident population of CD34+Sca-1+CD45− remained unchanged suggesting a specific mobilization of bone-marrow-derived stem cells. These cells could be involved in the amelioration of the morphological features that we found in treated animals. Currently, mobilized cells are also the preferable and major source of stem and progenitor cells harvested for autologous and allogenic transplantations because of the higher yield of these cells, leading to faster engraftment and decreased procedural risks compared with harvested BM cells. Even if stem cell mobilization spontaneously takes place after tissue damage [67, 68], several enhancers were used for clinical purposes to obtain faster and significant results. It is known that granulocyte-colony stimulating factor (G-CSF) triggers stem cell mobilization [69] while SDF-1 attracts stem cells allowing

their extravasation [70]. The effects of these molecules were studied in DMD blood [71, 72], but, unfortunately, they were associated with severe adverse effects so that they were not feasible for human prolonged treatment [73]. However, more recently different works described the emerging role of natural compounds in mediating such kind of events [52–54]. Interestingly, as we observed a mobilisation of HSCs from bone marrow to peripheral blood in treated animals, we suggested that ProAbe could favour the migration of bone-marrow-derived progenitors and their participation in muscular regeneration and endothelial formation. Similar to the data reported by Brzoska and colleagues [74], we showed that the augmentation of SDF-1+ stem cells was associated with lower fibrosis rate and an improvement of muscle regeneration. According to our results and recently published data [75–77], we speculated that mobilized stem cells could participate in the formation of new myofibers while, alternatively, those cells could participate in angiogenesis in treated animals.

In conclusion an antioxidant-rich diet seems a promising approach to coadjuvate therapies concerning untreatable disease like muscular dystrophy, thanks to their ability to improve histological and functional features of muscles and to enhance the recruitment of stem cells. As it was shown that combination of different polyphenols/antioxidants could enhance their effects [17, 46–48], ProAbe could be useful for the administration of a lower dose of each antioxidant, thus limiting all the possible adverse events [78]. In addition, although the precise mechanisms of action of the majority of these compounds is not well understood, we suggested that ProAbe could be a feasible approach in addition to standard therapy as it can exert functional and morphological ameliorations in dystrophic muscles (increased CSA of myofibers, decreased amount of inflammatory cells and necrotic fibers, and restoration of muscle force) to counteract degeneration, thus allowing a reduction of necessary dose of chronic drugs and limiting their known side effects.

4. Material and Methods

4.1. Animal Ethics Statement. Procedures involving living animals were conformed to Italian Country law (D.L.vo 116/92 and subsequent additions) and approved by local ethics committees. This work was authorized by the National Institute of Health and Local Committee, Protocol number 6/13-2012/2013. Three-month-old normal (C57Bl) and dystrophic (mdx) mice were provided by Charles River (Calco, Lecco, Italy). The weight of C57Bl was approximately 19 g while the weight of mdx was 25 g. Animals were caged with comfort and safety, in controlled ambient (12-hour light, 12-hour dark) at a temperature between 21°C and 24°C: they were able to move freely within them and had access to clean water and food. After one month of treatment, mice were sacrificed by cervical dislocation according to Italian country law.

4.2. ProAbe Composition. The liquid formulation provides DHA plus derived from purified fish oil, deodorized and scented with lemon, and natural vitamin E. The powder formulation provides curcumin conveyed in phytosomes, coenzyme Q10, acetyl-L-carnitine, green tea extract, extract of *Scutellaria*, and vitamin C, whose functions in modulating dystrophic phenotype have been described above [21–23, 28, 31, 33, 34, 36, 38]. ProAbe was provided by Ystem (Milan, Italy) in collaboration with U.G.A. Nutraceuticals (Gubbio, Perugia, Italy).

4.3. Dosage. The recommended daily dosage for human is 1000 mg of curcumin; 750 mg of acetyl-L-carnitine; 200 mg of coenzyme Q10; 100 mg of green tea extract; 105,3 mg of extract of *Scutellaria*; 1250 mg of DHA; and 36 mg of vitamin E. Mice were proportionally fed (daily) with the appropriate quantity of ProAbe (human 70 kg; mouse 20 g) for one month for histological and endurance experiments while for mobilization experiments different time points were considered as described in detail in the next section.

4.4. FACS Analysis of Blood-Derived Murine Cells. Peripheral blood (100 μL) from the retroorbital sinus was taken from each mouse and the samples were lysed to allow cytofluorimetric studies. Blood samples were collected at different time points: T0 (before ProAbe administration) and T24 h, T48 h, T72 h, T4 days, T7 days, T10 days, and T14 days following ProAbe administration. For five-colour flow cytometry cells were incubated with 10 μL primary antibodies against CD34 FITC, SCA1 PE (BD Biosciences, San Diego, CA, USA), CD184 (CXCR4) APC (Miltenyi Biotech, Bologna, Italy), and CD45PE-Cy7 (eBioscience, San Diego, CA). QA were weighed and washed several times in PBS, finely minced with scissors, and incubated at 37°C for 45 minutes with 1 mg/mL collagenase type IA (Sigma-Aldrich), 80 μg/mL DNase I (Roche), and Trypsin 2,5% (1 : 3) (Gibco) in Dulbecco's modified Eagle's medium (Invitrogen). Most of the skeletal muscle stem cells were released from the tissue after this step. The cell extract was filtered with a 70 μm nylon mesh (BD Biosciences, Immunocytometry Systems, Mountain View, CA) and labelled for FACS analysis. After each incubation, performed at 4°C for 20 min, cells were washed in PBS 1% heat-inactivated FCS and 0.1% sodium azide. Isotype-matched immunoglobulins were added to each control sample. The cells were analysed using the Cytomics FC500 and CXP 2.1 software (BC, Beckman-Coulter). Each analysis included at least 50000–200000 events for each gate. A light-scatter gate was set up to eliminate cell debris from the analysis. The percentage of positive cells was assessed after correction for the percentage reactive to an isotype control conjugated to relative fluochromes. Bone marrow was collected from treated and untreated mdx and C57Bl mice by flushing femurs and tibias with saline solution and red blood cells were lysed with ammonium chloride. Nucleated cells were labelled in phosphate-buffered saline (PBS), 2% fetal calf serum (FCS) for 45 min at 4°C with CD184 (CXCR4) APC and SDF-1 antibody (R&D Systems). Goat anti-mouse 647 was used as secondary antibody.

4.5. Immunohistochemistry. From both treated and untreated mdx mice DIA, QA and TA muscles were removed, frozen in

liquid nitrogen-cooled isopentane, and sectioned on cryostat. We performed histological analysis on QA and TA as these muscles show similar level of dystrophic degeneration in mdx mice. Furthermore we investigated DIA muscle as it is the muscle that mostly recapitulates human pathology. Serial sections of 10 μm in thickness were stained with H&E and AM. Images were captured with LEICA AS LMD optical microscope. For immunofluorescence analysis, sections were incubated with antibodies: rabbit anti-laminin (1 : 50; Abcam), rat anti-CD31 (1 : 100; BD), anti-α smooth muscle actin (1 : 50 Sigma), and anti-CD11b (1 : 50, R&D). The slides were analysed using a fluorescent microscope (LEICA DMIRE2) and a confocal microscope (LEICA TCS-SP2). For quantitative analysis ImageJ Software (NIH) was used. For CD11b and DHE correlation study serial sections were utilised.

4.6. Endurance Test and Tetanic Force. Endurance performance test was conducted as previously described [79]. Briefly, 10 mice were placed on the belt of motorized treadmill (Columbus Instruments). The treadmill was run at an inclination of 0° at 5 m/min for 5 min, after which the speed was increased 1 m/min every minute. The test was then stopped when the mouse remained on the shocker plate for 20 s without any attempt to reengage the treadmill, and the time to exhaustion (expressed in minutes) was determined. This test was performed daily for 30 days [80, 81]. Regarding tetanic force measurement, the experiments were performed as previously described [79]. Relative motor capacity was analyzed at 4 different time points (each week for 30 days) while time to exhaustion at T4 (30 days).

4.7. Dihydroethidium Staining for ROS Detection. Dihydroethidium (DHE) is a commonly used indicator of ROS production, both *in vitro* [82] and *in vivo* [83]. DHE analysis was performed as previously described [84]. Briefly, ROS production was measured by incubating TA and QA cross-sections with 5 μm DHE in PBS at 37°C for 30 min. DHE intensity was analyzed by confocal microscope and quantified by counting the number of pixels exceeding a specified threshold, which was set in order to eliminate interference from any background fluorescence using ImageJ software.

4.8. Mitochondria. We collected DIA and QA of mdx and C57Bl mice fed with ProAbe and we prepared these samples for further analysis as earlier described [85]. Mitochondrial respiratory chain enzyme and citrate synthase activities were measured spectrophotometrically by described assays [85]. The specific activity of each complex was normalized to that of citrate synthase.

4.9. Statistical Analysis. Data were expressed as means ± SD. The fibres' counting in mdx mice was compared by Student's *t*-test. To compare multiple group means, one-way analysis of variance (ANOVA) was used. When only two groups were compared, the *t*-test was applied assuming equal variances. The difference among groups was considered significant at $P = 0{,}05$.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Novel Insights on Nutrient Management of Sarcopenia in Elderly

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Sarcopenia is defined as a syndrome characterized by progressive and generalized loss of muscle mass and strength. The more rationale approach to delay the progression of sarcopenia is based on the combination of proper nutrition, possibly associated with the use of dietary supplements and a regular exercise program. We performed a narrative literature review to evaluate the till-now evidence regarding (1) the metabolic and nutritional correlates of sarcopenia; (2) the optimum diet therapy for the treatment of these abnormalities. This review included 67 eligible studies. In addition to the well recognized link between adequate intake of proteins/amino acids and sarcopenia, the recent literature underlines that in sarcopenic elderly subjects there is an unbalance in vitamin D synthesis and in omega-6/omega-3 PUFA ratio. Given the detrimental effect of these metabolic abnormalities, a change in the lifestyle must be the cornerstone in the treatment of sarcopenia. The optimum diet therapy for the sarcopenia treatment must aim at achieving specific metabolic goals, which must be reached through accession of the elderly to specific personalized dietary program aimed at achieving and/or maintaining muscle mass; increasing their intake of fish (4 times/week) or taking omega-3 PUFA supplements; taking vitamin D supplementation, if there are low serum levels.

1. Introduction

Sarcopenia is defined by the European Working Group on Sarcopenia in Older People (EWGSOP) [1] as a syndrome characterized by progressive and generalized loss of muscle mass and strength. Sarcopenia is a physiological phenomenon that usually starts in the fifth decade. van Kan (2009) has investigated the prevalence of sarcopenia in the population aged 60–70 years: in this age group, the prevalence ranged from 5 to 13% but increased to 11–50% in subjects aged >80 years [2].

Sarcopenia becomes responsible not only for the reduction of mobility and the level of autonomy of the elderly, but also for their ability to maintain good health. The functional reduction of the quadriceps muscle predisposes to a limitation in walking, with risk of falls and fractures of the femoral neck. A survey conducted in the USA has estimated the cost-related health consequences of sarcopenia to be 20–30 billion dollars [3].

In most elderly patients, the onset of sarcopenia is multifactorial. Like in all body tissues, muscle proteins are subjected to a constant process of synthesis and degradation; in healthy adults (with an adequate protein intake) this turnover is in balance, allowing the maintenance of a positive nitrogen balance and a constant muscle mass [4, 5].

In elderly, one of the pathogenic mechanisms leading to sarcopenia is altered muscle protein metabolism: the proteolytic processes are not accompanied by an adequate protein synthesis within the physiological turnover, and muscle cells lose progressively the sensitivity to the anabolic stimulus induced from the essential leucine and IGF-1 (insulin-like growth factor), thus manifesting the so-called "anabolic resistance" [6].

This phenomenon may be associated with other hormonal, functional, and nutritional factors, each of which may contribute to a greater or lesser extent—depending on gender, age, and clinical condition of the patient—to the progression of disease, defined as secondary sarcopenia [7].

Table 1: Nutrients and drugs that have been shown to present an activity of stimulation in increasing the mass and/or muscle strength in humans or in the animal model.

Nutrients	Proteins and amino acids (BCAAs) and creatine.
	Antioxidants (vitamin E, vitamin C, carotenoids, and resveratrol)
	Vitamins: vitamin D
	Long-chain omega-3 fatty acids
Drugs	Antagonists of mineral corticoids (Spironolactone)
	ACE inhibitors
Hormone replacement therapy	Testosterone (T)
	Growth hormone (GH)
	Combination therapy: T and GH
	Estrogen
	DHEA-S

With respect to nutritional causes, about 40% of subjects >70 years do not assume the current RDA (Recommended Dietary Allowances) of proteins (0.8 g/kg/day) [8]. The phenomenon depends upon several factors, each with a variable contribution: these include odontostomatological problems, capable of altering the masticatory function (therefore influencing the choice of foods with reduced content of proteins); a reduced capacity of digestion and assimilation of proteins in enteric tube; delayed gastric emptying, associated with a reduced gallbladder contractility and higher serum levels of the hormone cholecystokinin (CCK) and neuropeptide Y (PYY) (facilitating a long-lasting satiety [9]); a higher blood concentration of leptin in the elderly (showing that the anorexigenic signal prevails over the orexigenic one [10]); a propensity, increasing with age, to take sweet foods, easily chewable and already ready to eat, but not always adequate in the amino acid content.

In addition, costs are an issue, since a greater adherence to the Mediterranean diet is inversely related to BMI but leads to higher cost, which in 2006 were estimated at 1.2 €/day [11], due to the higher cost of meat and fish, compared with carbohydrates. Lastly, it is necessary to add that, according to available evidence, current RDA that define the protein intake in the elderly population should be revised, because for a number of reasons—many of which have already been discussed—they are often inadequate in terms of quantity and quality [12–15].

The FAO and the WHO indicate that an intake of 0.75 grams of high quality protein per kilogram of body weight is safe and adequate; however, for elderly subjects, it has been proposed to increase this value to 1.25 g/kg/day in order to avoid sarcopenia [16]. It is also necessary to consider that elderly subjects frequently present subclinical nutritional deficits, in particular of vitamins and minerals useful for the muscular tropism, such as vitamin D [17].

The more rational approach to delay the progression of sarcopenia is based on the combination of proper nutrition, possibly associated with the use of supplements and/or foods for special medical purposes, and a regular exercise program. Alternative treatments which are based on administration of hormone preparations such as testosterone, GH, and estrogens are still not universally accepted and require further investigation [18, 19]. Table 1 lists the nutrients and drugs which have been shown to increase the mass and/or muscle strength in humans or animal models. Table 2 summarizes the studies (prospective cohort studies or randomized controlled trials) performed in elderly subjects to investigate the optimum dietary supplementation, other than proteins/aminoacids, for the treatment of sarcopenia.

Given this background, the aim of the present narrative review is to summarize the state of the art according to the extant literature about two topics: (1) the correct intake of protein and amino acids, in particular branched chain amino acids (BCAAs) need for prevention and treatment of sarcopenia; (2) the correct intake of other nutrients, such as antioxidant, vitamin D, and long-chain omega-3 polyunsaturated fatty acids, or dietary supplements, such as beta-hydroxy-methylbutyrate and creatine, in need for prevention and treatment of sarcopenia.

2. Methods

The present narrative review was performed following the steps by Egger et al. [20]. Table 3 showed the summary of methodology used. The step were (1) configuration of a working group: three operators skilled in endocrinology and clinical nutrition, of whom one acting as a methodological operator and two participating as clinical operators; (2) formulation of the revision question on the basis of considerations made in the abstract: "the state of the art on metabolic and nutritional correlates of sarcopenia and their nutritional treatment"; (3) identification of relevant studies: a research strategy was planned, on PUBMED (Public MedLine run by the National Center of Biotechnology Information (NCBI) of the National Library of Medicine of Bethesda (USA)), as shown in Table 3; (4) analysis and presentation of the outcomes: the data extrapolated from the revised studies were carried out in the form of a narrative review of the reports and were collocated in tables. The flow diagram of narrative review of the literature has been reported in Figure 1. At the beginning of each section, the keywords considered and the kind of studies chosen have been reported. Suitable for the narrative review were prospective cohort studies, randomized controlled trials (RCT), reviews, meta-analyses, cross sectional studies, and position paper which considered elderly with diagnosis of sarcopenia defined by the European Working Group on Sarcopenia in Older People (EWGSOP) [1].

3. Results

3.1. Aminoacids and Protein. This research has been carried out based on the keywords: "sarcopenia" AND "proteins" AND "aminoacids"; 113 articles were sourced. Among them, 1 observation study, 6 reviews, 2 cross sectional studies, 8 randomized controlled studies (RCT), and 1 position paper have been selected and discussed.

It is known that the amino acids, including branched chain amino acids (BCAAs), are necessary for the maintenance of muscle health in the elderly [21]. Approximately

Table 2: Studies (prospective cohort study or randomized controlled trial) performed in elderly subjects to investigate the optimum dietary supplementation, other than proteins, for the treatment of sarcopenia.

Nutrients	Author	Type of study	Results	Recommended treatment
Vitamin D	Snijder et al., 2006 [114]	Prospective cohort study	Poor vitamin D status is independently associated with an increased risk of falling in the elderly, particularly in those aged 65–75 yr.	
	Verhaar et al., 2000 [109]	Randomized controlled trial	Six months of alphacalcidol treatment led to a significant increase in the walking distance over 2 minutes.	Six months of vitamin D treatment (0.5 microg alphacalcidol)
	Gloth et al., 1995 [110]	Randomized controlled trial	In this cohort of homebound older people, improvement in vitamin D status was associated with functional improvement as measured by the Frail Elderly Functional Assessment questionnaire.	One month of therapy with either placebo or vitamin D (ergo-calciferol)
Beta-hydroxy-beta-methylbutyrate (HMB)	Flakoll et al., 2004 [50]	Randomized controlled trial	Daily supplementation of HMB, arginine, and lysine for 12 wk	Daily supplementation of HMB, arginine, and lysine for 12 wk positively altered measurements of functionality, strength, fat-free mass, and protein synthesis, suggesting that the strategy of targeted nutrition has the ability to affect muscle health in elderly women.
Long-chain omega-3 fatty acids	Smith et al., 2011 [131]	Randomized controlled trial	Omega-3 fatty acid supplementation had no effect on the basal rate of muscle protein synthesis but enhanced the hyperaminoacidemia-hyperinsulinemia-induced increase in the rate of muscle protein synthesis, which was accompanied by greater increases in muscle mTORSer2448 phosphorylation	1.86 g eicosapentaenoic acid (EPA, 20:5n23) and 1.50 g docosahexaenoic acid (DHA, 22:6n23), both as ethyl esters

Table 3: Summary of methodology.

Step	General activities	Specific activities
Step 1	Configuration of a working group	Three operators skilled in clinical nutrition: (i) one operator acting as a methodological operator (ii) two operators participating as clinical operators
Step 2	Formulation of the revision question	Evaluation of the state of the art on metabolic and nutritional correlates of sarcopenia and their nutritional treatment
Step 3	Identification of relevant studies on PUBMED	(a) Definition of the key words (sarcopenia, nutrients, and dietary supplement), allowing the definition of the interest field of the documents to be searched, grouped in inverted commas ("…"), and used separately or in combination; (b) use of the Boolean (a data type with only two possible values: true or false) AND operator that allows the establishments of logical relations among concepts; (c) research modalities: advanced search; (d) limits: time limits: papers published in the last 20 years; humans; languages: English; (e) manual search performed by the senior researchers experienced in clinical nutrition through the revision of reviews and individual articles on sarcopenia in elderly published in journals qualified in the Index Medicus
Step 4	Analysis and presentation of the outcomes	The data extrapolated from the revised studies were carried out in the form of a narrative review of the reports and were collocated in tables.

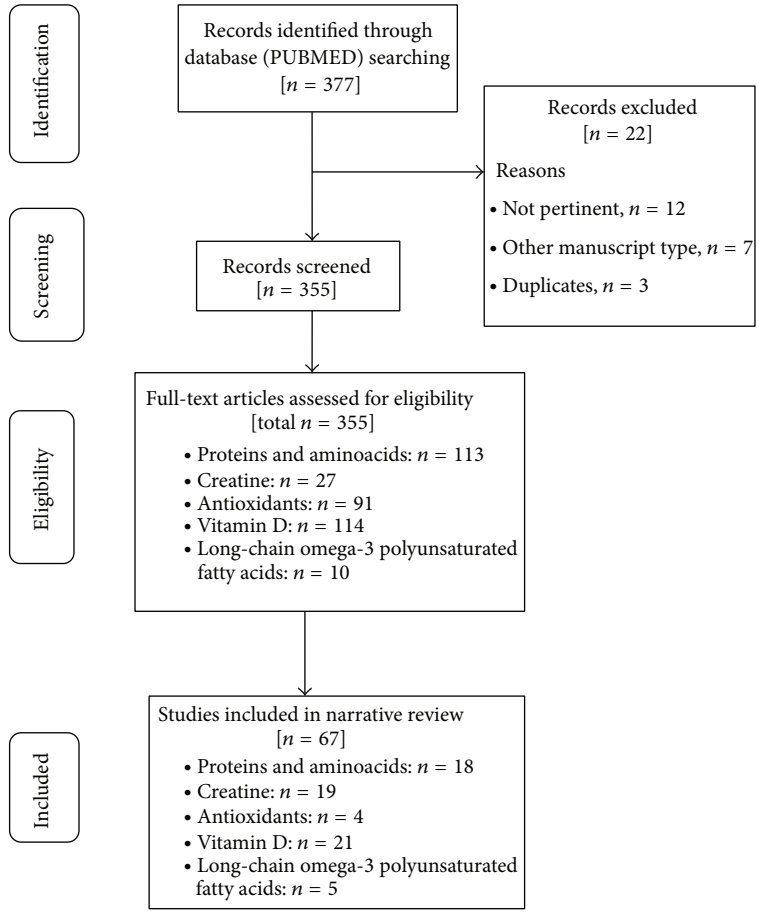

Figure 1: Flow diagram of narrative review of literature.

300–600 grams of muscle proteins is degraded and resynthesized daily, with complete renewal of the pool of muscle protein in the human body occurring every 3-4 months. Food intake stimulates muscle protein synthesis, resulting in a positive protein balance. After taking a protein-rich meal, the degree of protein synthesis remains elevated for more than 5 hours, with a peak of 2-3 hours after the intake [22]. It has been shown that in adult subjects a dose of approximately 15–20 grams of protein (or 7.5 grams of essential amino acids) is sufficient to maximize the synthesis of muscle proteins [23].

Probably the elderly, compared with younger subjects, would require a larger amount of protein to obtain the same maximization of protein synthesis, probably 30 grams as shown by Pennings et al. [24]. The bioavailability of amino acids plays a major role in the regulation of protein metabolism in elderly subjects and therefore a nutritional therapy must necessarily aim also at the recovery of muscle and sensitivity to the stimulus induced by the protein-synthetic amino acids, in order to contrast anabolic resistance. Over the past few years, the analysis of the different nutritional strategies has allowed the definition of some key concepts, recently discussed in a position paper by the PROT-AGE Study group [16], which include the recommended amount of protein intake for the healthy elderly; the recommended amount of protein intake for the elderly with acute or chronic conditions; the role of physical activity in association with dietary intake to maintain muscle strength and function in elderly; the practical administration of food proteins (source and quality of dietary protein, protein intake timing, and energy intake).

3.1.1. Recommended Amount of Protein Intake for Healthy Elderly. To maintain and recover the muscle, elderly subjects need to have a greater protein intake compared with younger subjects; older people should have an average intake of protein of 1.2/g/kg/day of body weight/day [16]. The threshold for anabolic meal intake of protein/amino acids must be greater in elderly subjects (i.e., 25 to 30 g of protein per meal, containing approximately 2.5–2.8 g of leucine), compared with young adults [24, 25]. The source of protein, timing of intake, and supplementation with amino acid supplements should be considered when making recommendations on the intake of dietary protein in the elderly.

3.1.2. Recommendations in Protein Intake in the Course of Acute and Chronic Pathology. In subjects with pathological conditions, the amount of additional proteins to be taken or the protein requirement depends on the specific disease, its severity, nutritional status of the subject before the onset of the disease, and the impact of the disease on the state of

nutrition. The majority of elderly patients who present an acute or chronic disease have an increased need for protein intake (1.2 to 1.5 g/kg body weight/day), while patients with critical illnesses or severe malnutrition have a need of protein equal to 2 g/kg body weight/day. Elderly subjects with severe renal impairment (estimated glomerular filtration rate < 30 mL/min/1.73 m^2) who are not on dialysis are an exception and, conversely, must limit their protein intake.

3.1.3. Quality of Protein and Specific Amino Acids. Not all dietary proteins have the same kinetic properties: the rate of absorption of dietary amino acids and their effect on the regulation of protein metabolism are dependent on the molecular characteristics of the protein. This characteristic gave rise to the distinction of dietary protein between fast and slow [26–28].

Previous work suggests that whey protein ingestion results in greater postprandial protein retention than does casein ingestion [29, 30].

The greater anabolic properties of whey than of casein are mainly attributed to the faster digestion and absorption kinetics of whey, which results in a greater increase in postprandial plasma amino acid availability and thereby further stimulates muscle protein synthesis [23, 26, 27, 31, 32].

Besides differences in protein digestion and absorption kinetics, whey and casein also markedly differ in their amino acid composition [26, 27, 32].

Whereas both proteins contain all the amino acids required to effectively stimulate muscle protein synthesis [33], whey has a considerably higher leucine content.

As regards differences between animal and vegetal sources, even if previous studies demonstrated that consumption of a meat-containing diet contributed to greater gains in fat-free mass and skeletal muscle mass with resistance training in older men than did a lactoovovegetarian diet [34, 35], more recent studies suggested that increases in muscle strength and size were not influenced by the predominant source of protein consumed by older men with adequate total protein intake [36].

3.1.4. Branched Chain Amino Acids and Leucine. It has been suggested that leucine, which is an essential amino acid belonging to the category of the branched chain amino acids (BCAAs; valine, and together with the isoleucine, whose average requirement is 40 mg/kg/day), is critical to maintaining a healthy muscle tissue and liver. The main sources of leucine are chicken and fish, cottage cheese, lentils, sesame, and peanuts. Unlike many other amino acids, BCAAs are metabolized only in skeletal muscle, since the BCAA aminotransferase enzyme is not present in the liver, the site in which the enzymes metabolizing all other amino acids are present in maximum concentrations, and up to 58% of all the amino acids ingested (except BCAA) can be oxidized in the liver on the first pass. Skeletal muscle is able to oxidize only 6 amino acids during exercise: in addition to BCAAs, asparagine, aspartate, and glutamate. When combined with exercise training, BCAA supplementation increases testosterone and decreases cortisol to create an anabolic environment [37]. BCAAs represent 14%–18% of the total amino acid content of skeletal muscles [38]. At rest, BCAAs, in particular leucine, have an anabolic effect by increasing protein synthesis and/or a reducing the rate of protein degradation, resulting in a positive net muscle protein balance [39]. The infusion of BCAAs in humans elevates the phosphorylation and the activation of p70S6 kinase and 4E-BP1 in skeletal muscle [40]. Both p70S6 kinase and 4E-BP1 are downstream components of the mTOR signaling pathway, which controls RNA translation and synthesis of proteins, and which is recognized as the central node to support muscle hypertrophy [41]. Leucine is involved in the direct phosphorylation and activation of mTOR in skeletal muscle, further enhancing the protein synthetic response [42]. According to the WHO, for healthy people the daily demand of BCAA to cope with the normal loss in protein metabolism and turnover are the following: valine: 10 mg/kg body weight; isoleucine: 10 mg/kg body weight; leucine: 10 mg/kg body weight [43].

It was recognized that the leucine content of the meal is an important regulator of the synthesis of muscle proteins and influences body composition in the long term [44]. Further research has compared the intake of 10 grams of protein with 18% of leucine with a similar beverage containing 35% of leucine, concluding that the beverage with the highest concentration of leucine determines a greater signaling of protein synthesis, resulting in an inferior muscle catabolism by cortisol [45]. Other studies on leucine show that once the minimum requirement of leucine for protein synthesis is satisfied, leucine can be used to activate various signaling pathways, including mTOR. mTOR is a major regulator of protein synthesis, energy sensors, and sensors of nutrients and the availability of amino acids, particularly leucine. The mTOR pathway is activated when ATP levels are high and is blocked when ATP levels are reduced. The activation of mTOR is vital for skeletal muscle hypertrophy. Leucine presents significant activity to stimulate insulin synthesis, which may increase the availability of amino acids for the synthesis of muscle proteins; in addition, leucine inhibits the destruction of muscle proteins with consequent increased balance over time [46]. Although leucine is described as the most important of the three BCAAs, isoleucine and valine also play a role, although they have not shown the same potential as leucine. In fact, the hypertrophy induced by leucine decreases to zero as soon as the presence of the other two BCAAs is poor; regardless of the amount of leucine available to the muscles, muscle growth does not occur if the concentration of the other two BCAAs decreases below a given level [47].

3.1.5. Beta-hydroxy-beta-methylbutyrate. Beta-hydroxy-beta-methylbutyrate (HMB) is a product of leucine metabolism that has been shown to slow protein breakdown in muscle tissue [48]. HMB may be effective at limiting the demands placed on the elderly subjects by acute stresses, such as sudden increases in physical activity, an immunologic challenge, or acute malnutrition [48, 49].

Daily supplementation of HMB (2 g/day), arginine, and lysine for 12 wk positively altered measurements of functionality, strength, fat-free mass, and protein synthesis, suggesting

that the strategy of targeted nutrition has the ability to affect muscle health in elderly women [50].

In conclusion, an adequate intake of proteins (1.2/g/kg/day) is essential to prevent sarcopenia and aminoacids supplementation; in particular branched chain amino acids (leucine 2.5 g/day) as well as the intake of beta-hydroxy butyrate (2 g/day) is a well documented intervention for treating sarcopenia.

3.2. Creatine. This research has been carried out based on the keywords: "sarcopenia" AND "creatine"; 27 articles were sourced. Among them, 3 reviews, 11 RCT, 1 single blind study, 1 control case study, 2 observational studies, and 1 cross over study have been selected and discussed.

Creatine is chemically known as a nonprotein nitrogenous compounds; it is a tripeptide composed of three amino acids (glycine, arginine, and methionine). In the human body, creatine is synthesized in the liver and pancreas from the amino acids arginine, glycine, and methionine. Moreover, creatine is present in foods (meat and fish) and is taken with the diet in the amount of 1-2 grams per day. Approximately 95% of the creatine in the body is stored in skeletal muscles, as phosphocreatine (PCr) for about two-thirds of the total content, while the remaining part is stored as free creatine. The energy provided for the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) during and after intense exercise largely depends on the amount of PCr stored in the muscle. With the depletion of PCr during intense exercise, the availability of energy decreases due to the inability to resynthesize ATP in the amount required to keep the high-intensity exercise [51]. Age-associated reductions of creatine/phosphocreatine in skeletal muscle have been reported in some studies [52, 53], although not all studies agree [54, 55]. The reduction of muscle creatine is biologically plausible, due to aging and, possibly, to certain comorbidities, such as sarcopenia, and/or changes with age in behavior (reduced physical activity and/or changes in dietary behaviors, such as decreased intake of meat for edentulous). The type II muscle fibers have a higher content of phosphocreatine compared with type I fibers (86 against and 74 mmol/kg dm) [56], and sarcopenia is characterized by a preferential atrophy of type II fibers [57]. The progressive atrophy of type II fibers may therefore partly explain the reduced muscle creatine in the elderly. Moreover, the reduction of creatine in the muscle of the elderly is in line with previous evidence that documents an increased oxidative process in aged skeletal muscles, for example, with decreased dependence on glycolysis [58] and a decrease of lactate dehydrogenase [59]. Smith et al. (1998) first reported an increase in muscle PCr (30%) in middle-aged adults (58 years) as a result of short-term intake of high doses of creatine (0.3 g/kg/day for 5 days) [52]. In a similar study, Rawson et al. (2002) reported a smaller increase in muscle phosphocreatine (7 versus 35%) in older (70 years) compared to younger subjects (24 years), in response to the ingestion of creatine (20 g/day for 5 days) [55]. However, the muscle PCr baseline was greater in the young compared with adult subjects described by Smith et al. (1998) [52], while the elderly subjects described by Rawson et al. (2002) had greater initial muscle PCr compared with younger subjects [55]. Brose et al. (2003) reported an increase in total muscle creatine (30% men, 17% women) in elderly subjects (70 years) who underwent 14 weeks of resistance training associated with intake of creatine in a dose of 5 g/day [60], a result that is similar to the increases reported in younger adults [61, 62]. Eijnde et al. (2003) reported an increase in total muscle creatine (5%) and free creatine (21%) following 6 months of exercise program for muscular endurance associated with the creatine supplementation (5 g/day) [63]. From these studies, although limited in number, it seems that the muscle creatine in the elderly can be increased with oral creatine supplementation in a dose of 5 g/day, but that the magnitude of the response can be significantly affected by the initial muscle creatine. Wyss et al. (1998) have suggested that the increase in extracellular creatine may decrease the absorption of creatine muscle by decreasing the activity of creatinine [64]. Although Rawson et al. (2002) have reported the presence of increased creatine in the blood of elderly subjects (elderly 68.5 mol/l, young 34.9 mol/l) [55], Tarnopolsky et al. (2003) showed no decrease in the activity of creatine after creatine ingestion in older men and women [65]. The most peculiar discovery was an improvement in fatigue resistance, which has been shown in many different studies using different exercise test [52, 66–70]. Some investigators have reported an increase in strength [68, 69], but this has not always been demonstrated [66, 67]. Importantly, in subsequent publications, researchers have begun to evaluate the performance of activities of daily living (activity daily living, ADL) and have shown that creatine supplementation may improve the performance of daily tasks identified in the ADL scale [69, 71, 72]. The improvement of the performance of activities of daily living is an important finding, because of the association between the performance of ADL, fall risk, and mortality. Among the studies that have evaluated the muscle mass, the majority showed a greater increase in lean mass accretion after ingesting creatine in combination with resistance training [60, 73, 74]. A further advantage given by combining creatine supplementation with resistance exercise is the increase in bone mineral content. Chilibeck et al. (2005) showed a greater increase (3.2 versus 1%) of bone mineral content in older men (71 years) after 12 weeks of creatine supplementation (0.3 g/kg for 5 days, 0.07 g/kg for 11 weeks), in combination with training against resistance compared to training alone [75]. Dalbo et al. (2009) have stated that creatine is an effective intervention to combat sarcopenia [76]. The timing of creatine ingestion (i.e., 0.03–0.5 g/kg before and after the sessions of resistance training) can be more relevant than the amount of creatine. These novel findings have immediate application for research and health professionals for the design of optimal creatine application strategies for older individuals [51].

In conclusion, an adequate creatine supplementation could represent an intriguing intervention to counteract sarcopenia, in particular fatigue related to sarcopenia, although double-blind, placebo-controlled studies have not been conducted.

3.3. Antioxidants. This research has been carried out based on the keywords: "sarcopenia" AND "antioxidant"; 91 articles

were sourced. Among them, 1 cross sectional study, 2 reviews, and 1 case control study have been selected and discussed.

Oxidative stress has been implicated as a central mechanism in the pathogenesis of sarcopenia [77].

Oxidative damage in skeletal muscle has been associated with the atrophy and loss of muscle function and fibers in sarcopenia [78].

Moreover, the accumulation of mitochondrial and nuclear DNA damage due to oxidative stress is thought to eventually compromise function, leading to the loss of myocytes [79].

Finally, reactive oxygen species can damage muscle tissue directly, but they also provide a trigger for the expression of inflammatory cytokines such as interleukin- (IL-) 1, tumor necrosis factor (TNF), and IL-6. In older age, a low-grade inflammatory state characterized by increased concentrations of inflammatory cytokines and acute phase proteins is common [80, 81].

Studies conducted among community-dwelling older adults suggest that the proinflammatory state does have a long-term consequence for sarcopenia. In the Longitudinal Aging Study Amsterdam, elevated IL-6 and CRP were associated with a loss of muscle strength over three years of follow-up [82].

Given this background, antioxidants (carotenoids, vitamin E, and vitamin C) should play an important role against sarcopenia.

Carotenoids inactivate free radicals and appear to modulate the transcription factors, such as the NFkB, which are involved in the regulation of IL-6 and other proinflammatory cytokines and have the ability, like alpha-tocopherol, to increase muscle strength [83, 84].

In the Women's Health and Aging Studies (WHAS) I and II, low serum carotenoid levels were associated with poor muscle strength [84]. Likewise in the InCHIANTI study, a low-carotene intake was associated with low physical performance [85]. These observations are consistent with a growing number of studies showing that a diet with high intake of fruits and vegetables is associated with a reduced risk of inflammation, hypertension, diabetes, cardiovascular disease, sarcopenia, and mortality [83].

Adherence to the Mediterranean diet, which is characterized by a high intake of fruits, vegetables, and whole grains, and lower consumption of red meat and saturated fats are associated with lower circulating IL-6 [86], and a recent trial showed the Mediterranean diet reduced IL-6 in adults [87].

In animal models, it was found that the ability of leucine to stimulate muscle protein synthesis is significantly decreased in aged rats compared to young adults. This defect was reversed when the animal was supplemented with antioxidants. The effects may be due to a reduction of the inflammatory state due to the antioxidants themselves [88]. In addition, the supplementation of vitamins E and C improves indices of oxidative stress associated with exercise in aged rats [89].

Concerning the specific vitamin E, in human studies, vitamin E has been shown to affect muscle strength of the elderly [90].

Several studies have shown the positive effects of vitamin E in reversing muscle damage during extensive muscle contraction (exercise) in healthy men. Vitamin E supplementation at a dose of 800 IU for 28 days resulted in lowering the expression of oxidative stress markers after a downhill run in both young and older men [91].

In another study, a longer supplementation period (12 weeks of vitamin E supplementation) lowered creatinine kinase level after exercise in young men, whereas older men showed decreased lipid peroxidation in both resting state and after exercise, indicating that vitamin E promotes adaptation against exercise induced-oxidative stress and reduced muscle damage [92].

In animal models, similar results were obtained [93, 94].

In conclusion, until today, despite the promising abovementioned animal studies and studies on subjects without sarcopenia, no RCT studies have evaluated the efficacy of an integration with antioxidants in the elderly patient suffering from sarcopenia. These studies are needed, given that recent epidemiological studies in community-dwelling older adults show that low serum/plasma carotenoids are independently associated with low skeletal muscle strength and the development of walking disability.

3.4. Vitamin D. This research has been carried out based on the keywords: "sarcopenia" AND "vitamin D"; 114 articles were sourced. Among them, 6 RCT, 5 reviews, 5 observational studies, 4 longitudinal studies, 2 population studies, 2 prospective studies, and 2 cohort analytic studies have been selected and discussed.

Vitamin D deficiency is common among geriatric patients (2–60%) [95, 96]. Vitamin D is hydroxylated in the liver to 25 (OH) D. This step is still well presented in the elderly, but it can be affected by liver disease [97, 98]. Further hydroxylation occurs in the kidney with the formation of 1,25-(OH2) D; however the activity of hydroxylation by the kidney may decrease with age, in parallel with the decline in renal function [99]. Consequences are the following: a low level of vitamin D, renal failure, and a low intake of calcium may result in mild secondary hyperparathyroidism. Increased levels of parathyroid hormone (PTH) cause an increase in bone turnover that is associated with bone loss, predominantly cortical; secondary hyperparathyroidism has been proposed as the main mechanism through which vitamin D deficiency contributes to the pathogenesis of hip fracture [95]. The presence of receptors for vitamin D was demonstrated in many organs [100], and the active metabolite, 1,25-2(OH)D, has been shown to be implicated in numerous systems that reduce the cell growth and inducing differentiation [101, 102]. Epidemiological studies have suggested that vitamin D deficiency is associated with cancer of the colon and breast [103, 104]. Furthermore, the status of vitamin D influences the immune system and insulin secretion [105].

Many studies have shown that low levels of 1,25-(OH) D and 25-(OH) D are associated with lower muscle strength, increased body instability, falls, and disability in older subjects [106, 107]. A significant association between the genotypes of the receptor for vitamin D with the strength of the quadriceps was also observed [108]. In addition, studies on

vitamin D supplementation in elderly subjects with vitamin D deficiency showed an improvement in physical function and isometric knee extension versus placebo [109, 110]. In parallel with the decline in muscle mass and function with aging, there is a reduction of the expression of the receptors VDR (vitamin D receptor) in skeletal muscle [111]. Previous research has linked some VDR polymorphisms with the reduction of muscle mass and function in the elderly [112], suggesting that vitamin D plays a role in the development and progression of sarcopenia [113]. Prospective analysis of LASA (the Longitudinal Aging Study Amsterdam) has shown that low levels of 25OHD are predictive of an increased risk of recurrent falls at 1 year [114], reduction of muscle mass and strength in 3 years [115], and admission to nursing homes in six years [116]. Limited data suggest that physical activity or hypertrophy of skeletal muscle may be an important source of vitamin D [113]. It has been shown that skeletal muscle is the main deposit of vitamin D in infant rats [117]. The outdoor exercise improves the levels of 25OHD in the elderly [118]. This suggests that physical activity may influence muscle hypertrophy independently of 25OHD. Pfeifer et al. (2002) suggested that the muscle building exercises can increase the levels of 25OHD [119], but further studies are needed [113]. Studies conducted to evaluate the effects of vitamin D supplements on functional abilities are partially contradictory. Some studies have shown that vitamin D intake did not improve physical performance [120, 121]. These studies were conducted in subjects with normal vitamin D. Conversely, studies conducted in 122 elderly subjects with low levels of vitamin D have been shown to benefit significantly from supplementation of vitamin D. In particular, Dhesi et al. [122] demonstrated in a group of subjects (mean age 77 years), history of falls and blood values of vitamin D less than or equal to 12 micrograms/liter of a daily supplementation with 600 UI ergo-calciferol induced a 3% improvement of physical performance, as assessed by the "Aggregated Functional Performance Time (AFPT)" [123], while in the control group a 9% deterioration was reported. With respect to the postural stability, which is related to the state of vitamin D [124, 125], the study showed 13% improvement, while the control group had a worsening equal to 3%. In terms of reaction time, the treated group had a 13% improvement compared with a deterioration of 3% of the placebo group. However, it was not demonstrated an improvement in muscle strength [126].

Recently, Muir and Montero-Odasso [127] performed a meta-analysis on a collection of studies that those aged over 60 years participate in randomized control trials of the effect of vitamin D supplementation without an exercise intervention on muscle strength, gait, and balance. The meta-analysis suggests that vitamin D supplementation (800–1000 IU) daily was associated with improvements of muscle strength and balance.

In conclusion, in vitamin D deficient sarcopenic subjects, dietary vitamin D supplementation (800–1000 IU daily) could be promising and interesting for treatment of sarcopenia.

3.5. Long-Chain Omega-3 Fatty Acids. This research has been carried out based on the keywords: "sarcopenia" AND "omega 3"; 10 articles were sourced. Among them, 2 RCT, 1 review, and 2 case control studies have been selected and discussed.

The ability of the skeletal musculature to use amino acids to build or renew constitutive proteins is gradually lost with age and this is partly due to a decline in skeletal muscle insulin sensitivity. Since long-chain omega-3 polyunsaturated fatty acids (LC*n*-3PUFA) from fish oil are known to improve insulin-mediated glucose metabolism in insulin-resistant states, some evidence in animal model suggests that polyunsaturated fatty acids might be a potentially useful therapeutic agent for the treatment and prevention of sarcopenia. It has been shown that providing feed enriched in fish oil to growing steers increases the activation (phosphorylation) of anabolic signaling proteins in muscle during administration of insulin and amino acids and increases the nonoxidative whole-body disposal of amino acids, an index of increased whole-body protein synthesis [128].

Furthermore, omega-3 fatty acid supplementation has been shown to prevent loss of muscle mass in burned guinea pigs [129].

In addition, omega-3 fatty acids present anti-inflammatory properties [130], which may also help alleviate the muscle anabolic resistance in older adults.

With respect to the effect of dietary omega-3 fatty acid supplementation on the rate of muscle protein synthesis and the anabolic signaling cascade in older adults, recently it has been demonstrated that omega-3 fatty acid supplementation had no effect on the basal rate of muscle protein synthesis but enhanced the hyperaminoacidemia-hyperinsulinemia-induced increase in the rate of muscle protein synthesis, which was accompanied by greater increases in muscle mTORSer2448 phosphorylation [131].

In conclusion, dietary LC*n*-3PUFA supplementation could potentially provide a safe, simple, and low-cost intervention to counteract anabolic resistance and sarcopenia.

4. Discussion

The more rational approach to delay the progression of sarcopenia is based on the combination of proper nutrition, possibly associated with the use of dietary supplements and a regular exercise program. An adequate intake of proteins (1.2/g/kg/day) is essential to prevent sarcopenia and amino-acids supplementation, in particular branched chain amino acids, is a well documented intervention for treating sarcopenia. Moreover, the current literature suggests that dietary LC*n*-3PUFA, vitamin D, and creatine supplementation could potentially provide a safe, simple, and low-cost intervention to counteract anabolic resistance and sarcopenia.

The author's nutritional recommendations have been showed in Table 4.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 4: Effect of nutrients or dietary supplementations on metabolic correlates of sarcopenia.

Nutrients or dietary supplementations	Recommendations	Specific effect
Proteins: average daily intake	It is recommended that the total protein intake should be 1–1.2 g/kg/day [16]	
Proteins: timing of intake	It is recommended to have 30 grams of protein of high biological value for each meal [25]	The elderly, compared with younger subjects, would require a larger amount of protein to obtain the same maximization of protein synthesis
Proteins: fast and slow	It is recommended to have whey protein ingestion because whey protein ingestion results in greater postprandial protein retention than does casein ingestion [31]	The greater anabolic properties of whey than of casein are mainly attributed to the faster digestion and absorption kinetics of whey, which results in a greater increase in postprandial plasma amino acid availability and thereby further stimulates muscle protein synthesis. Moreover, whey has a considerably higher leucine content
Proteins: animal and vegetal sources	When the total protein intake is adequate, the source of protein consumed (vegetal or animal) does not influence muscle strength and size [36]	Increases in muscle strength and size were not influenced by the predominant source of protein consumed by older men with adequate total protein intake
Branched chain amino acids (BCAAs),	It is recommended to have an adequate daily leucine supplementation (3 g/day)	A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly
Beta-hydroxy-methylbutyrate (HMB)	It is recommended to have a daily intake of beta-hydroxy butyrate (HMB-b, 2 g/day) because it can attenuate the loss of muscle mass and increase muscle mass and strength [50]	Beta-hydroxy-beta-methylbutyrate is a product of leucine metabolism that has been shown to slow protein breakdown in muscle tissue
Creatine	It is recommended to have an adequate creatine supplementation because it could represent an intriguing intervention to counteract sarcopenia and in particular fatigue associated with sarcopenia; the timing of creatine ingestion (i.e., 0.03–0.5 g/kg before and after the sessions of resistance training) can be more relevant than the amount of creatine [73, 76]	The ingestion of an adequate creatine supplementation determines the increase in muscle phosphocreatine (PCr) and the energy provided for the phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) during and after intense exercise largely depends on the amount of PCr stored in the muscle
Vitamin D	It is recommended to have a dietary vitamin D supplementation (800–1000 UI ergo-calciferol/day) in vitamin D deficient sarcopenic subjects [127]	Dietary vitamin D supplementation determines an increase of the expression of the receptors VDR (vitamin D receptor) in skeletal muscle
Antioxidants. vitamin E, vitamin C, carotenoids, and resveratrol	It is recommended to have a diet with high intake of fruits, vegetables whole grains, which is rich in antioxidant, and lower consumption of red meat and saturated fats, because it is associated with a reduced risk of inflammation correlated to oxidative damage [83]	Adherence to the diet rich in antioxidants is associated with lower circulating IL-6
Long-chain omega-3 polyunsaturated fatty acids (LC*n*-3PUFA)	It is recommended to have dietary long-chain omega-3 polyunsaturated fatty acids (1.86 g eicosapentaenoic acid and 1.50 g docosahexaenoic acid/day) supplementation [131]	Long-chain omega-3 polyunsaturated fatty acids (LC*n*-3PUFA) supplementation improves insulin-mediated glucose metabolism in insulin-resistant states and increases the activation (phosphorylation) of anabolic signaling proteins in muscle during administration of insulin and amino acids and increases the nonoxidative whole-body disposal of amino acids, an index of increased whole-body protein synthesis

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Review Article

Creatine, L-Carnitine, and ω3 Polyunsaturated Fatty Acid Supplementation from Healthy to Diseased Skeletal Muscle

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Myopathies are chronic degenerative pathologies that induce the deterioration of the structure and function of skeletal muscle. So far a definitive therapy has not yet been developed and the main aim of myopathy treatment is to slow the progression of the disease. Current nonpharmacological therapies include rehabilitation, ventilator assistance, and nutritional supplements, all of which aim to delay the onset of the disease and relieve its symptoms. Besides an adequate diet, nutritional supplements could play an important role in the treatment of myopathic patients. Here we review the most recent *in vitro* and *in vivo* studies investigating the role supplementation with creatine, L-carnitine, and ω3 PUFAs plays in myopathy treatment. Our results suggest that these dietary supplements could have beneficial effects; nevertheless continued studies are required before they could be recommended as a routine treatment in muscle diseases.

1. Introduction

The role of many foods/nutrients in maintaining good health and prolonging human lifespan has been clearly demonstrated over the past three decades. Particularly important are not only plant foodstuffs (i.e., fruits, vegetables, and legumes), but also animal foods (i.e., fish) and lipids (flaxseed and olive oils), that have been shown to have protective effects against several chronic pathologies such as age-related diseases, including cardiovascular [1], neurodegenerative [2], and inflammatory diseases [3], diabetes [4], and myopathies. Myopathies can be classified as either hereditary or acquired. Congenital myopathies are a group of inherited neuromuscular disorders whose main pathological features are distinctive and specific morphologic abnormalities in skeletal muscle. In contrast, differently acquired myopathies are caused by muscle fatigue, electrolyte imbalance, and dehydration or are induced by immune disorders that cause inflammation and pain [5]. In these pathologies the skeletal muscle is the fundamental target.

The deterioration of skeletal muscle structure and function leads to clinically relevant complaints, including progressive strength loss, fatigue, myalgias, and cramps. Important progress has been made in the comprehension of

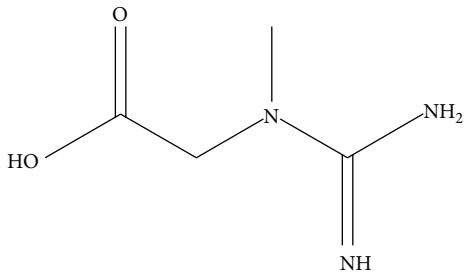

Figure 1: Chemical structure of creatine.

the molecular mechanisms underlying muscle myopathies. However, the treatment of muscle diseases is mainly symptom-oriented and includes physical therapy, physical exercise, orthopaedic corrections, artificial ventilation in cases of respiratory insufficiency, and pharmacologic interventions (i.e., corticosteroids). Considering the lack of therapies for myopathies, the idea that nutritional supplements might have beneficial effects in myopathy treatment is experiencing renewed interest. Conclusions about how beneficial nutritional supplements are for myopathy treatment are complicated by a lack of unequivocal results and flaws in the choice of supplements.

On the basis of their physiological roles in muscle biochemistry and bioenergetics, the nutrients creatine, L-carnitine, and ω3 polyunsaturated fatty acids (ω3 PUFAs) have been the focus of research aimed at verifying their safety and efficacy in treatment of a number of muscle diseases. Our aim here is to review the results of the most recent *in vitro* and *in vivo* research on the supplementation of creatine, L-carnitine, and ω3 PUFAs.

2. Creatine

2.1. The Nutritional Biochemistry of Creatine. Creatine (*N*-aminoiminomethyl-*N*-methylglycine, Figure 1) is an endogenous guanidine compound, which is synthesized by the kidneys, pancreas, and liver, starting from three amino acids: (1) methionine, which provides the methyl group through a transmethylation reaction, (2) glycine, which provides the acetic group and the nitrogen atom, and (3) arginine, which provides the amide group. Once produced, creatine is released into the bloodstream and then mainly captured by the cardiac and skeletal muscle and brain. To carry out its physiological role, creatine is converted to phosphocreatine by creatine kinase. The donor of the phosphate group is adenosine triphosphate (ATP), which is converted into adenosine diphosphate (ADP). Phosphocreatineis ahigh-energy reserve, availablefor theconversionof ADP to ATP, that is, essential during periods of high energy demand such as intense physical activity. Creatine kinase catalyzes the reversible transfer of the *N*-phosphoryl group from phosphorylcreatine to ADP to regenerate ATP. In this way creatine levels are restored [6]. In a 70 kg man, the total body creatine content is about 120 g, with a turnover of about 2 g/d, corresponding to 1.6% of total body creatine. On average, 50% of an individual's daily requirement of creatine is ingested from foods (approximately 1 g/d), while the remaining 50% is synthesized endogenously. Exogenous dietary sources of creatine include meat and fish (the concentration of creatine ranges between 4 and 5 g/kg of meat and 4 and 10 gr/kg of fish), and other animal products.

To date, the effects of creatine supplementation on muscle growth and muscle performance have been documented in more than 400 publications. Scientific evidence suggests that creatine supplementation (with a considered safe loading dose of 4 g of creatine monohydrate, 4 times per day), is an effective strategy to increase muscle creatine content by up to 10–40%, in less than a week [7]. This supplementation induces the increase of anaerobic performance, training volume, and capacity of human muscle to perform work during alternating intensity contraction [8].

Moreover, creatine also plays a pivotal role in brain energy homeostasis. It has been shown that in some psychiatric disorders, such as depression, the levels of creatine are low [9]. Therefore, recent studies have investigated the effect of creatine monohydrate supplementation in psychiatric patients suffering from posttraumatic stress disorders and depression. Creatine supplementation seems to also show neuroprotective effects in some neurodegenerative pathologies such as Alzheimer's and Parkinson's diseases. Recent evidence suggests that creatine could play important roles in the dysfunction of mitochondrial metabolism, which is recognized as a central causal factor in the pathogenesis of neurodegenerative disorders [10]. In a phase-II clinical trial, creatine monohydrate showed a delay in the progression of Parkinson's disease by 50%, compared to controls that received placebo. Then, in a subsequent followup study carried out 18 months later, creatine continued to show efficacy as a neuroprotective agent. The authors concluded that for its safety, tolerability, activity, and cost, creatine has many advantages in comparison with other drugs or food supplements potentially useful for Parkinson's disease [11, 12].

2.2. The Effects of Creatine Supplementation on Muscle Diseases. Creatine monohydrate supplementation, at dose of 0.3 g/kg/d for six days or 0.04 g/kg/d for 30 days [13], induces an increase in total creatine and phosphocreatine concentrations in skeletal muscle [13, 14], the magnitude of accumulation being inversely related to available endogenous stores [14]. It is accepted that the dosing regimen that significantly increase the intracellular phosphocreatine is a loading phase of approximately 20 g/d for 5–7 days followed by a maintenance phase of 5 g/d for several weeks [15, 16].

In healthy strength trained humans [17–19], creatine monohydrate supplementation has been shown to improve performance [14, 20], force output [20–22], and muscle free mass [13, 14, 23, 24]. In particular, creatine is an ergogenic aid (i.e., a temporal energy buffer) when supplementation is associated with high intensity exercise and the effect is more pronounced in untrained versus trained and in elderly versus young individuals [25], whereas similar changes in muscle performance have been found in males and females [14, 20]. The accepted mechanism explaining the positive

effect of creatine supplementation on performance consists of the temporal energy buffering due to the enhancement of the resting high energy phosphate levels (total creatine, phosphocreatine, creatine, and ATP), leading to a better match between ATP supply and the muscle fibers demands during physical exercise [26]. This change allows users to improve performance through increased total training volume. Creatine monohydrate supplementation is also well known for being responsible of a hypertrophic response determining an increased fat-free mass of about one kilogram [13, 14, 23, 24]. The hypertrophic potential following creatine administration has been mostly linked to fluid retention in myofibers due to swelling-induced osmotic potential of high intracellular creatine [24, 27]. An increased expression of myosin heavy chain isoforms [28] and myogenic regulatory factors [29, 30] and an improved mitotic activity of satellite cells have also been considered as key determinants of the net protein deposition following supplementation. The overall effects of creatine monohydrate supplementation on muscle structure have prompted researchers to investigate its efficacy in treating exercise-induced muscle injuries [26]. So far, contradictory results have been reported. While some studies on animal models and humans show that creatine supplementation does not decrease muscle damage or enhance recovery after high intensity eccentric contractions [31, 32], others have shown contradicting results as a greater isokinetic and isometric strength and a quicker amelioration of plasma creatine kinase (CK) levels during recovery were observed following creatine supplementation from an exercise-induced muscle damage [33]. Given the conflicting results from experiments investigating the efficacy of creatine treatment on skeletal muscle damage and recovery from eccentric-exercise damage we, and others [26], encourage further research to understand what, if any, role creatine treatment can play. Importantly, an anti-inflammatory effect of creatine has been observed when its supplementation was used in runners (with previous experience in running marathons) before a long distance race [34]. This protective effect has been confirmed in double blind trials when creatine supplementation (20 g d-1) was administered before high intensity endurance competitions [35, 36]. It is possible that the benefits of creatine supplementation in preventing muscle damage may relate to its antioxidant potential in endurance settings [26]. However, few studies have been published on the relationship between supplementation and oxidative stress, and controversial and inconclusive results have been obtained on indicators of oxidative damage [37–39]. Accordingly, creatine supplementation has been associated with either no change of lipid peroxidation, resistance of low density lipoprotein to oxidative stress or plasma concentrations of nonenzymatic antioxidants [39], increased free radical generation [37], and reduced oxidative stress [38]. In particular, Rahimi in [38] found a significant increase in athletic performance combined with attenuation of plasma malondialdehyde and urinary 8-hydroxy-2-deoxyguanosine levels in men who underwent 7 days creatine monohydrate supplementation (20 g/d) before a resistance exercise protocol. These results suggested a reduced training-induced oxidative stress and lipid peroxidation associated with supplementation [38]. However, no change [39] or an increase [37] in lipid peroxidation, resistance of low density lipoprotein to oxidative stress and plasma concentrations of nonenzymatic antioxidants has also been reported in adult males performing exhaustive incremental exercise trials combined with creatine supplementation. Taken together, these observations show that creatine supplementation may help in maintaining muscle integrity after intense and prolonged exercise, yet the mechanisms underlying the protective effect are only partially known.

The effects of creatine supplementation on muscle performance and protein metabolism and, possibly, muscle integrity may represent a rationale for its potential use to prevent or treat muscle disorders. Creatine monohydrate supplementation may benefit muscle disorders in a nonspecific fashion [40] by enhancing muscle strength and mass, reducing intracellular calcium accumulation and apoptosis [41], preventing oxidative stress, and attenuating cell death [42]. In fact, in myopathy, pathophysiological events lead to fiber necrosis, apoptosis, autophagy elevation in reactive oxygen species, mitochondrial dysfunction, increases in protein catabolism and degradation, and rise in intracellular calcium content [43], and all these elements may represent sites of attack for creatine. Furthermore a reduced creatine disposal may characterize neuromuscular disorders [44–49], due to the lower creatine transporter content or the impairment of energy charging capacity of the cells. This condition of relative creatine deficiency may boost the need for dietary supplementation. The efficacy of creatine supplementation in muscle diseases has been observed in animal models and humans. For instance, in mdx mice (a model of dystrophinopathy), an improved calcium handling resulting in lower intracellular calcium concentrations and enhanced cell survival have been shown in cultured cells [50]. In the same animal model an improvement of mitochondrial respiration and muscle function has been also observed [51]. In 1997, a prospective randomized study reporting the positive effects of creatine monohydrate supplementation in a neuromuscular disease (i.e. mitochondrial myopathy) was first published [52]. In this study creatine monohydrate was administered in 7 mitochondrial cytopathy patients using a randomized crossover design with the following scheme: 5 g for the first 14 days followed by 2 g of oral creatine monohydrate for the subsequent 7 days. Measurements included activities of daily living, isometric handgrip strength, basal and postexercise lactate, evoked and voluntary contraction strength of the dorsiflexors, and aerobic capacity at cycle ergometry. Creatine treatment resulted in significantly increased handgrip strength, with no changes in the other measured variables [52]. In 1999, Tarnopolsky and Martin [53] found an increase in high intensity power output following creatine monohydrate supplementation for 10 days (10 g daily for 5 days followed by 5 g daily for 5 days) in a heterogeneous group of people with neuromuscular disorders enrolled in two studies (Study 1, $n = 81$ open study; and Study 2, $n = 21$ single-blinded study). In the open trial, which aimed to test the clinical efficacy of creatine supplementation in a large cohort of patients, myopathies included muscle dystrophies ($n = 15$), mitochondrial cytopathies ($n = 17$), inflammatory myopathies ($n = 14$), and peripheral neuropathy disorders

($n = 18$), whereas the single-blinded study was conducted only on 21 heterogeneous patients ($n = 16$, miscellaneous myopathies, $n = 6$ muscle dystrophies, $n = 1$ peripheral neuropathy disorder, and $n = 1$ inflammatory myopathy [53].

Despite the results from [53] being based on a limited number of patients, the acquired knowledge has encouraged further research aimed at testing the effects of creatine supplementation in several conditions including sarcopenia of ageing [54, 55], dystrophies [56, 57], mitochondrial myopathies [58, 59], COPD [60–62], and chronic heart failure (where phosphocreatine/ATP ratio is a stronger prognostic factor than the degree of impairment [63]). To date, the results across all these studies remain inconclusive about a beneficial effect of creatine supplementation.

Meta-analyses of the growing literature base [40, 64, 65], highlighting the statistical under power of the majority of the studies, reveal that the efficacy for long- and short-term supplementation with creatine monohydrate is only seen in selected muscular dystrophies (i.e., dystrophinopathies and myotonic dystrophy type 2 and inflammatory myopathies, such as dermatomyositis and polymyositis) and in terms of increased muscle strength even under anti-inflammatory therapies (corticosteroids). Critical analysis of the literature shows we cannot draw safe conclusions in other myopathic conditions, partly due to methodological biases in setting up clinical trials and small sample sizes leading to lower power [43]. Importantly, in all analyzed studies across all tested myopathic conditions, creatine appeared well tolerated, apart from treatment of glycogenosis type V (McArdle disease). In this case treatment by creatine supplementation at high dose rates resulted in impaired activities of daily living and increased muscle pain (cramping) [43].

Our review of creatine supplementation has highlighted three important points. First, creatine monohydrate supplementation in healthy and diseased humans can have important beneficial effects. Second, conclusions from the study of creatine supplementation in several myopathic conditions are deeply hampered by methodological problems including difficulties in statistical power due to rarity of diseases. Third, there are virtually no observed negative effects of creatine supplementation compared to currently available chemotherapeutic interventions. Together, these three points strongly justify ongoing efforts in establishing basic research, randomized clinical trials, or other experimental designs on testing the benefits of creatine supplementation.

3. L-Carnitine

3.1. The Nutritional Biochemistry of L-Carnitine. L-Carnitine (3-hydroxy-4-N-trimethylaminobutyrate, Figure 2), the bioactive form of carnitine, is an endogenous branched nonessential amino acid derivative. L-carnitine is synthesized in kidney, liver, and testes, starting from L-lysine and L-methionine, having ascorbic acid, ferrous iron, pyroxidine, and niacin as cofactors. L-carnitine can also be consumed with diet, especially with foods of animal origin. Omnivores have a dietary intake of carnitine from 20 and 300 mg/d mostly from red meat (50–150 mg/100 g), fish, and dairy

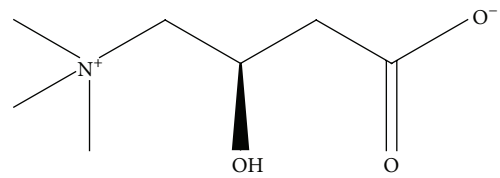

Figure 2: Chemical structure of L-carnitine.

products (up to 10 mg/100 g) consumption, whereas vegetarians have a dietary intake of about 1–3 mg/d. Dietary carnitine is absorbed in the small intestine and enters the bloodstream [66]. Inside the cells, carnitine is involved in lipid metabolism since it allows the transport of fatty acids with more than 14 carbon atoms from the cytoplasm to mitochondria where they undergo β-oxidation [63, 67]. The transition takes place through three steps. The first step is catalysed by carnitine palmitoyl transferase 1 (CPT1) and the transmembrane transport is facilitated by acylcarnitine transferase. Within the mitochondrion, free carnitine is regenerated by the action of carnitine palmitoyl transferase 2 (CPT2) and the released fatty acyl-CoAs enter the β-oxidation pathway. Taking into account that free CoA is involved in the pyruvate dehydrogenase reaction and in the process of β-oxidation, carnitine contributes to the coordinated integration of fat and carbohydrate metabolism. When glucose oxidation increases, acetyl groups can be translocated from acyl-CoA within the mitochondrial matrix to the cytoplasm. The accumulation of cytosolic acetylcarnitine may result in a limitation of CPT-1 activity because of the decrease in availability of free carnitine [26]. Moreover, fatty acid oxidation could occur, considering that skeletal muscle predominantly expresses an isoform of CPT-1 with low affinity for L-carnitine [68]. Thus, the regulation of free fatty acids β-oxidation occurs through the regulation of their mitochondrial content due to leakage of acyl and acetyl moieties leading to a modification of the ratio between esterified carnitine and free carnitine.

Considering its fundamental role in lipid metabolism, L-carnitine is a drug approved by the Food and Drug Administration to treat primary and selected secondary carnitine-deficiency syndromes [69] and is widely used as food supplement for its potential positive effect on health [70] even if the results across available studies remain inconclusive about a real beneficial effect to treat chronic complaints as type 2 diabetes [71] and Alzheimer's neurodegenerative disease [72].

Importantly, lines of evidence suggest positive effects of carnitine supplementation in cardiovascular diseases. A recent meta-analysis revealed that carnitine administration leads to 27% reduction in all-cause mortality, 65% reduction in ventricular arrhythmias, and 40% reduction in anginal symptoms in patients experiencing an acute myocardial infarction. Therefore L-carnitine and propionyl-L-carnitine can be used along with conventional treatment in presence of stable angina, thus contributing to secondary prevention of cardiovascular diseases [73]. Interestingly promising results

have been also obtained in presence of intermittent claudication, the most frequent symptom of mild moderate peripheral vascular disease, as propionyl-L-carnitine supplementation can help reducing symptoms and is associated with significant amelioration of functional impairment [74].

3.2. The Effects of L-Carnitine Supplementation on Muscle Diseases. The skeletal muscle is the most relevant depository of carnitine, and its availability is critical for the physiological bioenergetics of this tissue. Carnitine deficiency greatly affects skeletal muscle function as found in presence of primary and secondary deficiencies. Primary carnitine deficiency (OMIM 212140), which affects between 1 : 37.000–1 : 100.000 newborn individuals, is an autosomal recessive disorder of fatty acid oxidation resulting from defective carnitine transport caused by mutations in carnitine transporter gene SLC22A5 [75] coding for OCTN2 transport protein. The disease, characterized by very low level of free and total carnitine (free carnitine 1–5 μM and normal 20–55 μM), may have a predominant metabolic or cardiac presentation. The metabolic presentation usually before 2 years of age is characterized by frequent gastrointestinal and respiratory infections, lethargia, hepatomegaly, hypoketotic hypoglycemia, hyperammonemia, and serum creatine kinase elevation [76]. Later cardiomyopathy and hypotonia dominate the medical case [77]. Secondary carnitine deficiency is commonly associated with hemodialysis. In chronic renal insufficiency, undialyzed patients total carnitine, free carnitine, and acylcarnitine accumulate in body tissues due to reduced renal clearance [78, 79]. Besides, following regular hemodialysis, a significant creatine loss arises as demonstrated by reduction of creatine content in serum and skeletal muscle during the dialysis session [80] which is not compensated by endogenous synthesis [79]. Considering that the dialysate carnitine content before and after hemodialysis is far below that of control subjects and that the loss of carnitine into the dialysate greatly exceeds that into urine, the net loss of carnitine is mostly attributed to dialysis procedures [81, 82]. Indeed also the carnitine cofactors and precursors, vitamin B6, niacin, vitamin C, lysine, and methionine, may be lost throughout the dialysis procedures [83]. Considering the role of carnitine in the cell, bioenergetics, dyslipidemia, muscle fatigue, cardiomyopathy, and anemia have been considered potential targets for L-carnitine supplementation in several trials conducted in a large cohort of hemodialyzed patients [83]. Meta-analysis of the literature does not allow to draw consensus on whether carnitine supplementation can improve the patient's health status. In particular, although initial systematic reviews of published literature on the topic put forward a promising effect on management of anemia and failed to demonstrate a significant efficacy in controlling dyslipidemia [83], a recent meta-analysis involving more than 1700 participants failed to confirm the previous findings regarding the effects of L-carnitine on hemoglobin but showed that L-carnitine significantly decreases serum low-density lipoprotein (LDL) and C-reactive protein (CRP). In this study the extent of LDL decrease did not appear clinically relevant, whereas a significant and clinically relevant decrease of CRP serum content was observed [84]. Importantly, these effects were not confirmed in another meta-analysis of randomized controlled trials, which failed to demonstrate any improvement of inflammation, oxidative stress, nutrition, anemia, dyslipidemia, hyperparathyroidism status, or quality of life in hemodialyzed patients [85]. Uncertainties also regard the effect of L-carnitine administration on skeletal muscle function of hemodialyzed patients. Long-term administration (12 months) of L-carnitine (2 g/day) to hemodialyzed patients resulted in increased serum and muscle carnitine levels, and selective type 1 fiber hypertrophy [86] and similar results have been obtained in uremic patients following 24 weeks administration of the same dose [87] but the functional significance of such changes remains to be elucidated. Taken together, available inconclusive results put forward that high-quality and long-term randomized trials are still required to fully elucidate the clinical value of L-carnitine administration in these patients.

Other clinically relevant conditions may determine secondary carnitine deficiency, that is, intestinal resection, severe infections, liver disease, and cancer [88] where a negative impact on skeletal muscle is demonstrated by the appearance of pathological manifestations, including fibers accumulation of neutral lipids, structural damages, and subsarcolemmal accumulation of large aggregates of mitochondria. Therefore, carnitine supplementation may represent a useful tool for the management of muscle deterioration and the appearance of fatigue in presence of carnitine loss.

Carnitine deficiency is not the only condition that allows to focus on its central role in muscle energy disposal and handling. Carnitine availability may be the limiting factor for fatty acid oxidation and/or the removal of acyl-CoAs at rest and during low intensity exercise [89], and an increase in skeletal muscle total carnitine content would be expected to increase fatty acid oxidation and decrease pyruvate dehydrogenase complex activation and glycogen use during such exercise tasks. On the other side, during high intensity exercise, carnitine shifts towards acetylcarnitine formation, thus maintaining the pyruvate dehydrogenase complex and and tricarboxylic acid flux [90]. In accordance with a dual role of carnitine in skeletal muscle energetics, beneficial effect of L-carnitine supplementation has been observed at low intensity exercise where its availability increases the rate of oxidation of intramuscular fatty acids and triacylglycerols, thus postponing the appearance of fatigue [91, 92]. Furthermore at high intensity exercise its availability may lead to better matching of glycolytic and mitochondrial flux, thus reducing ATP formation by anaerobic mechanisms [90, 93–97]. Importantly, some studies failed to observe such effects [98–104], and inconsistencies may be laid on the relevance of dietary means to carnitine retention after supplementation, being favored by carbohydrates coingestion through increased insulin level [90].

One of the most promising areas of research on L-carnitine supplementation regards its potential role in ameliorating and accelerating recovery from exercise-induced muscle injury. It has been found that supplemental carnitine is effective in attenuating signs of tissue damage (muscle

soreness and serum CK elevation) induced by lengthening or intense contractions [105–108] also in sarcopenic muscle [109]. The observed benefits of L-carnitine supplementation in preventing load-induced muscle injury have been attributed to its known role as antioxidant. In skeletal muscle, reactive oxygen species (ROS) and nitrogen species are physiologically synthesized at low levels and are required for normal force production [110]. When ROS production overtakes tissue antioxidant capacity, oxidative stress activates pathophysiologic signaling leading to proteolysis and apoptosis within the myofibers. This sequence of events is considered as a major cause of sarcolemmal damage and leakage of cytosolic proteins as CK into the circulation and the origin of reduced muscle strength capacity that contributes to fatigue [111–113]. In exercise-induced muscle damage L-carnitine supplementation has been found to reduce postexercise serum CK [105, 106] and myoglobin concentrations [106], suggesting a quicker muscle recovery from damage. Further evidence demonstrated that L-carnitine has an effective free-radicals scavenging activity at least *in vitro* [114]. In contrast inconclusive results have been obtained *in vivo* at least in humans on the effects of L-carnitine on xanthine oxidase, a marker of metabolic stress that, in presence of high glycolytic rates, mediates the oxidation of AMP to hypoxanthine [115]. Accumulation of xanthine oxidase is the consequence of the activation of calcium-dependent proteases, which cleave a portion of xanthine dehydrogenase and convert it into xanthine oxidase. This response appears to be attenuated by L-carnitine supplementation which reduces intracellular hypoxanthine and xanthine oxidase following resistance exercise bouts [109] whereas other experimental investigations failed to demonstrate such effect [116, 117].

Another mechanism, by which high intensity muscle contractions may exert toxic effect on skeletal muscle, is transient hypoxia. Under hypoxia, an increased concentration of blood ammonia and a lower concentration of free carnitine have been found [118, 119]. In this condition supplementation with L-carnitine may prevent ammonia formation through its antioxidant activity. Besides exercise, L-carnitine is known to protect against muscle mitochondrial dysfunctions associated with oxidative stress caused by a series of conditions such as aging, ischemia reperfusion, inflammation, degenerative diseases, carcinogenesis, and drug toxicity, *in vivo* or *in vitro* [120–134]. For instance, studies suggest that cancer cachexia, which includes anorexia, weight loss, muscle loss, skeletal muscle atrophy, anemia, and alterations in carbohydrate, lipid, and protein metabolism [135], is associated with a decrease in intracellular glutathione concentration in the muscle [136–138], and L-carnitine supplementation increases the tumor-induced decrease in muscular glutamate and glutathione levels at least in animal models [136]. Gramignano et al. [139], studying the efficacy of L-carnitine supplementation (6 g/day for 4 weeks) in a population of advanced cancer patients, found a decreased ROS and increased glutathione peroxidase levels. Promising antioxidant activities have been also found following supplementation in patients with nonalcoholic steatohepatitis [140], renal disease [141], and phenylketonuria [142], as well as in several experimental models of oxidative stress [143–146]. Interestingly, recent evidence suggests that carnitine supplementation may also directly act as radical scavenger, thus contributing to protection against statin-induced oxidative muscle damage [146, 147].

In summary, considering its importance in muscle bioenergetics and its antioxidant potential, L-carnitine supplementation may be considered an aid in presence of carnitine deficiency and in skeletal muscle diseases in which oxidative stress and altered fatty acid oxidation mostly contribute to pathophysiology. Despite this potential, further research is needed to conclusively elucidate the mechanisms underlying its protective effects and to establish whether they may also arise in presence of muscle diseases of different origin.

4. ω3 Long Chain Polyunsaturated Fatty Acids (ω3 LC-PUFAs)

4.1. The Nutritional Biochemistry of ω3 LC-PUFAs. ω3 PUFAsare among the most studied nutrients which show healthy properties [148]. α-Linolenic acid (ALA—C18:3, ω3, Figure 3), which is not synthesized in the human body and therefore must be consumed with the diet, is the precursor of the two most important bioactive long chain polyunsaturated fatty acids (ω3 LC-PUFA, Figure 3): eicosapentaenoic acid (EPA—C20:5 ω3) and docosahexaenoic acid (DHA—C22:6 ω3). In the endoplasmic reticulum, ALA is converted in EPA and DHA through the development of enzymatic elongation and desaturation reactions, in which Δ-6 desaturase and elongase and Δ-5 desaturase enzymes are involved. In peroxisomes, these reactions are followed by β-oxidation to produce DHA. ω3 LC-PUFAs are considered "conditionally essential" because occasionally they are not synthesized in sufficient amounts to meet human needs. This condition occurs when the dietary intake of ALA is too low in comparison with linoleic acid (LA, C18:2-ω6) and the ratio ω6/ω3 is higher than 5/1. Thus, due to the competition of ALA and LA for the same Δ-6 desaturase enzyme, which is shared in the two metabolic pathways, the production of LC-PUFAs shifts towards the synthesis of ω6 fatty acids. It is therefore evident that the intake of both ALA and EPA and DHA should be encouraged. The most important sources of ω3 LC-PUFAs in human nutrition are fatty fish, such as sardines, salmon, and tuna, as well as walnuts, and flaxseed, and canola oils.

Once produced from ALA, EPA and DHA, in turn, are precursors of eicosanoids that mediate the anti-inflammatory effects that underlie the beneficial effects ascribed to ω3 LC-PUFAs in numerous physiological and pathological states. In particular, increasing evidence suggests that ω3 PUFAs improve blood lipid levels (inducing a decrease of triglyceride levels and an increase of HDL cholesterol), reduce arrhythmias and risk of stroke, and help to prevent and treat atherosclerosis [149, 150]. Moreover, ω3 PUFAs were shown to have chemopreventive properties against various types of cancer, including colon and breast cancer [151, 152]. Some recent studies suggest that ω3 PUFAs intake is associated with reduced depressive symptoms, particularly in females, potentiating the effects of antidepressants, and helps to reduce mood swings [153, 154]. The protective effect of

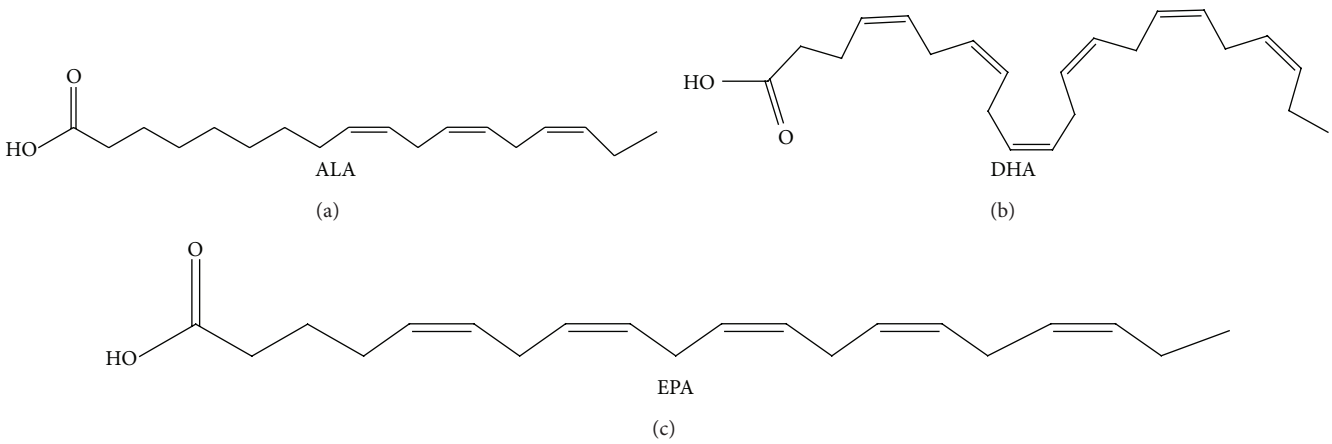

Figure 3: Chemical structure of α-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA).

DHA in amyloid-β peptide-infused rats was associated with increased membrane fluidity which also provided oxidative stress resistance in hippocampal cells [155, 156]. *In vivo*, ω3 LC-PUFAs increased membrane fluidity in rat hippocampus and improved memory formation, whereas their reduction exerted opposite effects [157, 158]. Preclinical studies supported the idea that DHA maintained membrane fluidity, improved synaptic and neurotransmitter functioning, enhanced learning and memory performances, and displayed neuroprotective properties [159]. Moreover, DHA decreased the amount of vascular amyloid-β peptide (Aβ) deposition and reduced Aβ burden [160, 161].

Interesting avenues of research also regard the treatment of chronic inflammatory diseases of the bone as rheumatoid arthritis [162, 163]. Importantly, ω3 PUFAs have shown beneficial effects on bone health in animal studies [164, 165] but current research suggests only a modest increase in bone turnover in humans [166].

4.2. The Effects of ω3 PUFA Supplementation on Muscle Diseases. Research on the role of PUFAs in muscle health and functionality is still incomplete and deserves future in-depth analysis. Promising observations suggest an important role of dietary ω3 PUFA supplementation on protein synthesis and inflammation and its potential efficacy in lean body mass sparing. Initial evidence suggests that fish-oil-derived ω3 PUFAs might be useful in preventing and treating sarcopenia of ageing. This effect appears mediated by increased insulin levels and amino acid mediated activation (i.e. phosphorylation) of signaling proteins of the mTOR/p70S6 K1 pathway, as demonstrated in animal models (i.e. growing steers and burned guinea pigs) [167, 168]. In 2011, Smith et al. in two randomized studies [169–171] demonstrated the effect of ω3 PUFA supplementation (1.86 g EPA and 1.50 g DHA for 8 weeks) on muscle protein metabolism in young/middle-aged (mean age: 37 years) and elderly subjects (mean age: 65 years) of both sexes. Importantly, in all individuals, ω3 PUFA supplementation, although not exerting any effect on the basal rate of protein synthesis, determined the augmentation of the hyperaminoacidemia-hyperinsulinemia-induced rate. This change was accompanied by increased phosphorylation of mTOR in serine 2448 and downstream p70S6 K in threonine 389, whereas no change in the level of Akt (the main effector of insulin activation upstream mTOR) phosphorylation was observed. Interestingly, the effect on protein synthesis was not associated with lower plasma concentration of inflammatory markers and triglycerides, thus suggesting that the anabolic effect of PUFAs may arise independently of their known anti-inflammatory effect [169]. Furthermore the observed anabolic effect did not produce significant amelioration of glycemic control through changes in skeletal muscle insulin sensitivity, which is generally, but not universally [172–175], accepted to be improved by ω3 PUFA supplementation. In fact, Liu et al.[172] showed that in rats fed with a high-ω3 fatty acid diet there is an increase of insulin binding to sarcolemma due to changes of the fatty acyl composition of phospholipids surrounding the insulin receptor. The authors suggested that this might be the mechanism by which dietary fatty acids modify insulin action. More recently, in rats fed with a high-saturated fat diet, Holness et al. [173] reported that hyperinsulinemia can be rapidly reversed via the dietary provision of small amounts of ω3 PUFA. However, the saving of insulin induced by ω3 PUFA supplementation occurs in the absence of an acute improvement of insulin sensitivity. These results are not surprising as the published available information on the effects of PUFA supplementation on insulin sensitivity in humans is inconsistent and often contradictory [176–180].

Considering that maintenance of muscle mass is a fundamental determinant of its capacity to generate force, of interest is the potential correlation between PUFAs consumption and muscle strength in the elderly. So far, inconclusive results are available [180, 181]. In particular, in 2009, a cross-sectional study found no correlation between total ω3 or ω6 PUFA intakes and muscle strength in aged Americans [180], whereas, in 2013, the Tokyo Oldest Old Survey on Total Health showed that higher consumption of EPA and DHA is significantly associated with higher functional mobility in men. This effect was not observed in women [181]. The only available randomized double blind pilot study analyzed

the effects of 2 fish oil (1.2 g EPA and DHA) or 2 placebo (olive oil) capsules per day for 6 months in 126 postmenopausal women [182]. Fatty acid levels, frailty assessment, hand grip strength, 8-foot walk, body composition, and inflammatory biomarkers were taken at baseline and after 6 months of supplementation. Fish oil supplementation resulted in higher red blood cell DHA, compared to baseline and placebo, and improvement in walking speed compared to placebo. In this work a linear regression model including age, vitamin intake, osteoarthritis, frailty phenotype, and tumor necrosis factor alpha (TNF-α) explained that the change in DHA/arachidonic ratio, TNF-α, and selenium intake had the major contribution to the observed change in walking speed [182]. Importantly, new avenues of research highlight that a fundamental booster of the functional effects of fish oil supplementation is physical exercise and, in particular, strength training. In 2012, Rodacki and coworkers [183], in a randomized study enrolling elderly women, demonstrated that the use of fish oil for 90 days in addition to strength training (3 times/week, for 12 weeks, 36 training sessions) was associated with an additional increase of peak torque and rate of torque development, which, *de facto*, defines an improvement in whole body functional capacity as demonstrated by higher chair-rising performance. Notably, the mechanisms underlying these changes remain obscure and the potential role of fish oil in changing the fluidity of the muscle fibers membrane and acetylcholine sensitivity should be taken into consideration [183].

Therefore, available data suggest a potential role of ω3 PUFA supplementation in muscle anabolism and functionality at least in presence of sarcopenia of ageing. Future studies are needed to investigate whether these effects might be quantitatively relevant in other myopathic conditions.

Another fundamental mechanism to be taken into consideration when analyzing the potential benefit of ω3 PUFA supplementation in healthy and diseased skeletal muscle is the largely demonstrated inhibitory effect on inflammation [184]. ω3 PUFAs serve as precursors to prostaglandins such as prostaglandin E3, which are powerful hormone-like substances that reduce inflammation [185], and inhibit arachidonic acid, derived 2-series prostaglandins, and the 4-series leucotrienes, which are known to modulate the production of proinflammatory and immunoregulatory cytokines [186]. *In vivo* and *ex vivo* animal studies have indicated that ω3 PUFAs reduce the production of TNF-α, IL-1, IL-2, and IL-6 [187–189]. In humans contradictory observations still exist, since some lines of evidence suggest that supplementation of the diet with fish oils results in a reduced production of IL-1, IL-6, TNF-α, and IL-2 [190], and an alteration of gene expression profiles to a more antiinflammatory and antiatherogenic status in peripheral blood mononuclear cells *in vitro* [191]; whereas, *in vivo*, dietary supplementation with PUFAs produced either no change [192] or amelioration of hallmarks of muscle damage (i.e., soreness and creatine kinase levels) and inflammatory mediators (i.e. IL6), following physiological proinflammatory events such as strenuous exercise or eccentric contractions in unaccustomed individuals [193, 194]. Finally, the change in lipid composition of the membranes (i.e. the sarcolemmal membranes) might be considered an additional, not enough explored, effect of PUFA supplementation on skeletal muscle. It is well known that changes in lipid composition may influence membrane function by regulating protein and lipid membrane homeostasis [195–197], and PUFAs either as constituents of membrane phospholipids or free molecules contribute to membrane chemophysical features (i.e., membrane organization, ion permeability, elasticity, and microdomain formation). In particular, it has been shown that ω3 PUFA supplementation decreases membrane thickness [198, 199], modulates proton membrane permeability and leaflet thickness enhancing fatty acid flip-flop rate, and increases bilayer propensity to be in a liquid-disordered phase [196]. The anti-inflammatory potential of ω3 PUFAs together with their efficacy in modulating myocyte membrane composition and conformation [197, 200–202] was the rational basis for recent preliminary attempts aiming at identifying new strategies for the treatment of cancer cachexia and muscular dystrophies where inflammation plays a major role in the pathogenesis of muscle wasting [203].

Unfortunately, only few and contradictory studies are available. Inconclusive results are available on whether the anti-inflammatory effects of PUFAs can counteract the action of proinflammatory cytokines involved in the pathogenesis of cancer cachexia [203], and the available randomized controlled clinical trials could not safely demonstrate positive effects of supplementation on muscle wasting [204]. Few and contradictory results have also been obtained in muscular dystrophies. In particular, Fiaccavento et al. have demonstrated that supplementation of dystrophic UM-X7.1 hamsters, carrying a phenotype similar to limb girdle muscular dystrophy 2F (LGMD2F) with ALA, precludes myocyte and muscular tissue damage and modulates cells proliferation promoting myogenic differentiation. Two major factors concurred to determine such effects including the modulation of the lipid membrane composition and configuration which appears altered in such model [205] associated with the preservation of the expression and location of key-role signaling proteins (i.e. β-catenin, caveolin-3, sarcoglycan, and dystroglycan) and the slowing down of the myocyte degeneration/regeneration cycling rate associated with the enhancement of the myogenic differentiation [206]. Further, initial observations suggest that ω3 PUFA supplementation with fish oil or EPA decreases muscle degeneration and inflammation in mdx mouse model mirrored by reduced functional impairment evaluated by grip strength tests [207, 208]. On the contrary, by using a highly controlled diet design, Henderson et al. [209] have recently put forward a detrimental effect of a high intake of ω3 PUFA, unlike high MUFA, in the same animal model as demonstrated by higher serum CK activity and no changes in skeletal muscle histopathology and inflammatory markers (p65) [209]. Congruent with the latter observations, Galvao and coworkers [210] have recently demonstrated that high ω3-PUPA diet enriched with α-linoleic and α-linolenic acid, unlike high long chain saturated fatty acids diet, promotes a negative effect on lifespan in the same animal model with genetic cardiomyopathy. Importantly the harmful effect on survival of the high PUFA diet appeared to be associated with highly significant increase in plasma free fatty acids whose elevation

is correlated with higher risk of ventricular arrhythmias and is considered a strong predictor of sudden cardiac death [211, 212].

Therefore, considering the available research studies, it is not currently possible to draw safe conclusions on the role of $\omega 3$ PUFA in muscle diseases and further studies are first needed to elucidate whether supplementation may be harmful in certain myopathic conditions.

5. Conclusion

Myopathies are chronic degenerative diseases that induce the deterioration of the structure and function of skeletal muscle characterized by progressive strength loss, muscle fatigue, pain, or tenderness, cramps, stiffness, and tightness. So far, curative therapies are not available and the goals of treatment are now finalized to delay the onset of the disease and to relieve symptoms. Therefore, myopathic patients are submitted to interventions, such as rehabilitation, ventilatory assistance, and nutritional approach, which have the main purposes of improving the quality of life and slowing the progression of the disease. In particular an adequate diet, which has sufficient caloric content and balanced nutritional composition, and specific nutritional supplements can help myopathic patients. The presented review of the literature suggests that creatine, L-carnitine, and $\omega 3$-PUFA supplementation may have the potential to exert beneficial effects in selected myopathies. Nevertheless, to date, experimental evidence of short-term and long-term effects of supplementation in myopathies with heterogeneous physiopathology is missing. Indeed, the evidence of potential harmful effects in short-term settings ($\omega 3$ PUFA in animal models of dystrophy and cardiomyopathy and creatine in McArdle disease) and the lack of studies on the potential harmful effects of prolonged supplementations strongly highlight that further rigorous studies are required before these supplementations could be recommended as a treatment in selected muscle diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Variability in Myosteatosis and Insulin Resistance Induced by High-Fat Diet in Mouse Skeletal Muscles

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Nutrient overload leads to impaired muscle oxidative capacity and insulin sensitivity. However, comparative analyses of the effects of dietary manipulation on skeletal muscles with different fiber composition are lacking. This study aimed to investigate the selective adaptations in the soleus and tibialis anterior muscles evoked by administration of high-fat diet for 12 weeks in 10 mice (HFD mice) compared to 10 animals fed with a normal chow diet (control mice). Mice fed with the HFD diet exhibited hyperlipidemia, hyperinsulinemia, hyperglycemia, and lower exercise capacity in comparison to control mice. In control mice, soleus fibers showed higher lipid content than tibialis anterior fibers. In contrast, the lipid content was similar between the two muscles in HFD mice. Significant differences in markers of muscle mitochondrial production and/or activity as well as of lipid synthesis were detected between HFD mice and control mice, especially in the tibialis anterior. Moreover, translocation of GLUT-4 transporter to the plasma membrane and activation of the insulin signaling pathway were markedly inhibited in the tibialis and slightly reduced in the soleus of HFD mice compared to control mice. Overall, these results show that adaptive responses to dietary manipulation occur in a muscle-specific pattern.

1. Introduction

Myosteatosis (also known as ectopic skeletal muscle adiposity) represents the fat infiltration within myocytes (intramyocellular fat) and within the fascia surrounding skeletal muscle (intermuscular fat) [1]. It has been observed that its association with muscle atrophy (also known as "fatty atrophy") may result in impaired muscle oxidative capacity that, in turn, triggers a fast-to-slow transition of muscle fibers to enhance the muscle oxidative potential and/or mitochondrial content [2, 3]. Animal studies performed in diet-induced obesity models have suggested that a prolonged mismatch between intramyocellular lipid accumulation and adaptations in oxidative capacity of muscle fibers may ultimately result in impaired oxidation of lipids and glucose (i.e., mitochondrial dysfunction) that implies decreased ATP production and increased production of reactive oxygen species [2, 3], resulting in insulin resistance [4, 5]. Possible mechanisms underlying mitochondrial dysfunction are the increased saturation of phospholipids at the mitochondrial membranes, downregulation of genes involved in oxidative phosphorylation, and oxidative stress [2–5].

Given the well-known differences in oxidative capacity between different fiber types, it may be hypothesized that muscle-specific adaptations occur in response to high-fat-diet- (HFD-) induced myosteatosis. To our knowledge, so far,

there is only one previous study investigating this hypothesis and showing that triglyceride accumulation, fast-to-slow transition, and impaired oxidative capacity in response to HFD occurred in a muscle-specific pattern in mice [3]. We hypothesized that the myosteatosis-induced changes in oxidative capacity of muscle fibers could also be associated with decreased exercise capacity and changes in muscle contractile properties and that these associations could occur in a muscle-specific manner predicting the development of muscle-specific insulin resistance. Therefore, the aim of this study was to investigate in two animal muscles (characterized by different fiber type composition such as tibialis anterior and soleus) the differences in HFD-induced myosteatosis and changes in muscle oxidative metabolism, contractile properties, and insulin sensitivity. We chose to study the diet-induced adaptations in two muscles characterized by high dependence on either oxidative metabolism, the soleus (which mostly contains type I fibers), or glycolytic metabolism, the tibialis anterior (which mostly contains fast type IIb and type IIa and a minority of type I fibers) [6–8].

2. Materials and Methods

2.1. Animals and Diet. Four-week-old male C57Bl6/J mice (Harlan-Italy, Udine, Italy) were housed in a controlled environment at 25 ± 2°C with alternating 12-h light and dark cycles. All the animals were fed with a normal pellet diet for 1 week prior to the experiment. The animals were then allocated to two dietary regimens: chow diet (control animals: $n = 10$) or high-fat diet (HFD animals: $n = 10$) for 12 weeks. The kinetics of dietary manipulation has been chosen according to previously published papers, showing that mice fed with a high-fat diet for 12 weeks were more susceptible to the development of insulin resistance, with a significant increase in intramuscular triglycerides [9, 10]. The HFD contained 45% fat, 20% protein, and 35% carbohydrate (Research Diets, New Brunswick, NJ). Body mass and intake of water and food were recorded weekly. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/92) and the experiment was approved by the Turin University Ethics Committee.

2.2. Oral Glucose Tolerance Test. One day before the mice were due to be killed, the oral glucose tolerance test (OGTT) was performed after a fasting period of 6 h by administering glucose (2 g/kg) by oral gavage. Once before administration and 15, 30, 60, and 120 min afterward, blood was obtained from the saphenous vein, and glucose concentration was measured with a conventional Glucometer (GlucoGmeter, Menarini Diagnostics, Florence, Italy).

2.3. Blood Biochemical Analysis. After 12 weeks of dietary manipulation, the mice were anaesthetised with i.p. injection (30 mg/kg) of Zoletil 100 (Laboratoires Virbac, France) and killed by aortic exsanguination. Blood samples were collected and plasma was isolated. Glycemia was measured using the GlucoGmeter kit and insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden). The plasma lipid profile was determined by measuring triglycerides and total cholesterol by standard enzymatic procedures using reagent kits (Hospitex Diagnostics, Florence, Italy).

2.4. In Vivo Muscle Function Assessment. Exercise capacity was assessed using an exhaustion incremental treadmill test. This procedure was previously described in detail [11]. Briefly, each animal was placed on the belt of a 6-lane motorized treadmill (Exer 3/6 Treadmill, Columbus Instruments, OH, USA), supplied with shocker plates, which could be individually enabled or disabled for each lane. During each exhaustion test the electrical stimulus was fixed at 200 ms duration, 0.34 mA amplitude, and 1 Hz repetition rate.

After acclimatization, all mice were subjected to initial exhaustion treadmill tests at 0° inclination according to the following protocol: 5 min at 5 m/min and followed by incremental increase of speed of 1 m/min every min until exhaustion. Exhaustion was defined as spending time on the shocker plate without attempting to reengage the treadmill within 20 s. Three tests were performed on each animal, allowing 4 days between each test. Values obtained were averaged, providing a single value per animal. In a second set of experiments, endurance was evaluated as the time spent on the treadmill belt running at 50% of the maximal velocity reached in the previous exhaustion incremental test.

2.5. Ex Vivo Functional Assessment of Tibialis Anterior Muscle Preparations. The method used for mechanical analysis of intact muscles was described previously [12]. Briefly, the tibialis anterior muscle of the right leg was dissected, placed in an organ bath filled with Krebs solution (composition: NaCl 120 mM, KCl 2.4 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, glucose 5.6 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 24.8 mM, and pH 7.4), bubbled with 95% O_2 and 5% CO_2 at a constant temperature of 22°C, and attached to a force transducer (Radnoti Organ Bath System, AD Instruments). Electrical pulses were delivered through platinum electrodes connected to a stimulator (Tumiati, Italy). Tetanic isometric contractions were evoked (110 Hz, 500 ms, supramaximal amplitude) at L_o (the length at which the maximal isometric force is observed) and twich time to peak and maximal tetanic force (Tf) were measured. Specific tetanic force (i.e., maximal tetanic force normalized for muscle volume: g/μL) was considered for the comparison between control mice and HFD mice. Further, the fatigue index was measured (and expressed in %) as the tetanic force drop for different stimulation frequencies (0.03, 0.09, and 0.3 Hz) compared to the maximal tetanic force [8].

2.6. Muscle Extracts. The soleus and tibialis anterior muscles were isolated, weighed, and rapidly freeze-clamped with liquid nitrogen and stored at −80°C, as previously described [13]. Total extracts of soleus and tibialis anterior were obtained from 10% (w/v) homogenates in RIPA buffer containing 20 mmol/L TRIS-HCl pH 7.4, 150 mmol/L NaCl, 2 mmol/L EGTA, 1 mmol/L EDTA, 1% TRITON-X100, and protease inhibitors [1 mM dithiothreitol (DTT), 0.5 mM

Table 1: Body mass, muscle mass, and glycometabolic parameters in control mice and high-fat diet (HFD) mice after 12 weeks of dietary manipulation.

Variable	Control mice ($n = 10$)	HFD mice ($n = 10$)	P value
Body weight increase (g)	8.5 ± 1.6	17.2 ± 4.6	$2 * 10^{-5}$
Tibialis anterior weight (% body weight)	0.55 ± 0.05	0.48 ± 0.06	0.021
Soleus weight (% body weight)	0.067 ± 0.009	0.051 ± 0.007	0.002
Plasma glucose (mg/dL)	73 ± 19	120 ± 21	0.008
Plasma insulin (mg/mL)	85.8 ± 5.3	106.0 ± 12.6	0.016
Plasma total cholesterol (mg/dL)	77.2 ± 5.7	129.8 ± 26.3	0.008
Plasma triglycerides (mg/dL)	32.8 ± 7.8	77.0 ± 33.5	0.017

phenylmethyl sulphonyl fluoride (PMSF), 5 μg/mL aprotinin, and 2.5 μg/mL leupeptin]. After 40 minutes of incubation in ice, samples were sonicated and cleared by centrifugation at 15,000 g at 4°C for 40 min. Supernatants were removed and protein content was determined using the Bradford assay. Protein extracts were stored at −80°C until use.

2.7. Skeletal Muscle Lipid Content. Triglycerides were extracted from skeletal muscle homogenates and assayed using reagent kits according to the manufacturer's instructions (Triglyceride Quantification Kit, Abnova Corporation, Aachen, Germany). Intramyocellular lipid accumulation in the soleus and tibialis anterior was evaluated by Oil Red O staining on 10 μm cryostatic sections. Stained tissues were viewed under an Olympus Bx4I microscope (40x magnification) with an AxioCamMR5 photographic attachment (Zeiss, Gottingen, Germany).

2.8. Western Blot Analysis. About 60 μg of total proteins was loaded for Western blot experiments. Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane, which was then incubated with a primary antibody (rabbit anti-total GSK-3β, dilution 1 : 200; goat anti-pGSK-3β Ser^{9}, dilution 1 : 200; rabbit anti-total Akt, dilution 1 : 1000; mouse anti-pAkt Ser^{473}, dilution 1 : 1000; rabbit anti-total IRS-1, dilution 1 : 200; goat anti-pIRS-1 Ser^{307}, dilution 1 : 200; rabbit anti-GLUT-4, dilution 1 : 2000; rabbit anti-total ACC, dilution 1 : 500; rabbit anti-pACC Ser^{79}, dilution 1 : 1000; rabbit anti-CPT-1l m, dilution 1 : 200; and mouse anti-SDH-A, dilution 1 : 200). Blots were then incubated with a secondary antibody conjugated with horseradish peroxidase (dilution 1 : 10000) and developed using the ECL detection system. The immunoreactive bands were visualised by autoradiography and the density of the bands was evaluated densitometrically using Gel Pro Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA). The membranes were stripped and incubated with alpha-tubulin monoclonal antibody (dilution 1 : 5000) and subsequently with an anti-mouse antibody (dilution 1 : 10000) to assess gel-loading homogeneity.

2.9. Immunohistochemistry. For immunodetection of the glucose transporter type-4 (GLUT-4), immunohistochemical staining was performed on 10 μm acetone fixed cryostatic sections of soleus and tibialis anterior muscles. Endogenous peroxidases were inactivated for 5 minutes with 3% H_2O_2. Sections were blocked for 30 minutes with 3% BSA in PBS. Thus, samples were incubated overnight with rabbit anti-GLUT-4 antibody and then for 1 hour with an anti-rabbit IgG-HRP conjugated secondary antibody. The specific staining was detected with diaminobenzidine and sections were visualized with Olympus-Bx4I microscope connected by a photographic attachment (Carl Zeiss, Oberkochen, Germany). A negative control was included in which the primary antibody was replaced with a nonimmune isotypic control antibody.

2.10. Materials. Unless otherwise stated, all compounds were purchased from the Sigma-Aldrich Company Ltd. (St. Louis, MI, USA). The BCA Protein Assay kit and SuperBlock blocking buffer were from Pierce Biotechnology Inc. (Rockford, IL, USA) and PVDF was from the Millipore Corporation (Bedford, MA, USA). Antibodies were from Cell-Signaling Technology (Beverly, MA, USA) and from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Luminol ECL was from PerkinElmer (Waltham, MA, USA).

2.11. Statistical Analysis. The Mann-Whitney U test was used for comparisons between muscles (tibialis anterior versus soleus) and groups (control mice versus HFD mice). Data were expressed as mean ± standard deviation (SD). Threshold for statistical significance was set to $P = 0.05$. All statistical tests were performed with Statistica 6 (Statsoft Inc., Tulsa, OK, USA) software package.

3. Results

3.1. Effect of HFD on Body Mass, Muscle Mass, and Glycometabolic Balance. After 12 weeks of dietary manipulation, HFD mice had significantly higher body mass increase and lower tibialis and soleus mass in comparison to control mice (Table 1). Moreover, HFD caused significant increases in glucose, insulin, total cholesterol, and triglyceride levels.

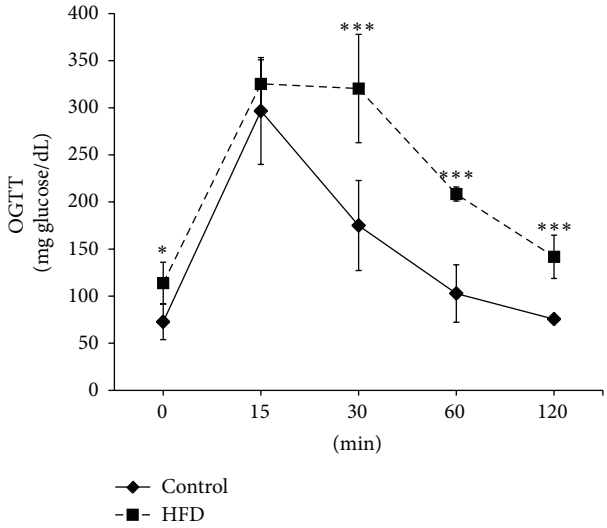

Figure 1: Effect of high-fat diet (HFD) on oral glucose tolerance in mice administered glucose ($2\,g{\cdot}kg^{-1}$) by oral gavage. Values are means ± SD of 10 animals per group.

When compared to control animals, HFD mice showed more than 60% increase in fasting glucose levels, which was associated with almost 30% increase in insulin concentrations (Table 1). Consistently, HFD mice showed a significant impairment in glucose tolerance to exogenously administered glucose (Figure 1).

3.2. Effect of HFD on Muscle Function. Muscle function was assessed *in vivo* by treadmill tests (exhaustion incremental treadmill test and endurance treadmill test): HFD mice showed reduced resistance to fatigue (Figures 2(a)-2(b)) compared to control mice.

To assess *ex vivo* muscle function, time to peak tension and specific tetanic force of the tibialis anterior muscle were considered: HFD mice showed a significantly higher time to peak tension (Figure 2(c)) and lower specific tetanic force (Figure 2(d)) in comparison to control animals. Moreover, the fatigue index was lower in HFD mice compared to control mice (Figure 2(e)).

3.3. Effect of HFD on Muscle Lipid Content. Oil Red O staining sections highlighted low levels of lipid deposition in both soleus and tibialis anterior of control mice (Figure 3(a)). Moreover, soleus fibers contained higher levels of lipid droplets than tibialis anterior fibers in control mice. In contrast, the density of lipid droplets was similar between the two skeletal muscles of HFD mice, thus suggesting a higher increase in lipid content in tibialis anterior than in soleus after 12 weeks of dietary manipulation. As expected, the qualitative differences in the histological evaluation of muscle lipid deposition were confirmed by measurement of skeletal muscle triglyceride content (Figure 3(b)). In control mice, the triglyceride content was doubled in soleus when compared to anterior tibialis. Interestingly, HFD evoked a threefold increase in tibialis triglyceride accumulation and less than 30% increase in soleus triglyceride accumulation (in comparison to control mice), thus resulting in no significant differences in triglyceride content between the two muscles after dietary manipulation.

3.4. Effect of HFD on Muscle Oxidative Metabolism. To elucidate the molecular mechanism(s) underlying the differences in skeletal muscle triglyceride accumulation, selective changes in markers of muscle mitochondrial production and/or activity were evaluated. Specifically, the expression of the succinate dehydrogenase complex subunit-A (SDH-A), a typical marker of mitochondrial density, and carnitine palmitoyl transferase-1 (CPT-1), a key regulatory enzyme in mitochondrial β-oxidation, as well as the phosphorylation/activation of acetyl-CoA carboxylase (ACC), whose central role in the regulation of lipid synthesis is well known, were measured on both soleus and tibialis homogenates by immunoblotting. As reported in Figure 4, HFD evoked a slight increase in protein expression of SDH-A and CPT-1 in soleus homogenates. Notably, a more relevant increase in the same markers was recorded in the tibialis anterior of HFD mice when compared to control mice. When the activation of the ACC pathway was assessed, induction of Ser^{79} phosphorylation of ACC was massive in tibialis anterior of HFD mice compared to control mice, whereas no significant differences between the two groups of animals were observed in soleus homogenates.

3.5. Effect of HFD on GLUT-4 Translocation and Insulin Signaling Pathway. As shown in Figure 5, GLUT-4 was detected in both soleus and tibialis anterior of control mice, with highest levels in soleus. A reduction in GLUT-4 immunostaining was observed in muscle sections of HFD mice. Specifically, translocation of GLUT-4 transporter to the plasma membrane was markedly inhibited in the tibialis and slightly reduced in the soleus of HFD mice compared to control animals.

Changes in the activity of the insulin signal transduction pathway were evaluated by immunoblotting experiments on homogenates from both soleus and tibialis anterior muscles (Figure 6). The HFD did not alter the protein expression of the IRS-1, Akt, or GSK-3β in both muscles. However, when compared with the tibialis anterior of control mice, HFD mice showed a significant increase in Ser^{307} phosphorylation of IRS-1 in parallel with reduced Ser^{473} phosphorylation of Akt. Ser^{9} phosphorylation of GSK-3β, a downstream target of Akt, was also reduced in the tibialis anterior of HFD mice compared to control animals. In contrast, HFD feeding exerted only mild effects on protein phosphorylation in soleus: none of the HFD-induced changes were statistically significant.

4. Discussion

In the present study we showed in mice that HFD feeding for 12 weeks caused body weight increase, dyslipidaemia, hyperinsulinaemia, and hyperglycemia. These effects were

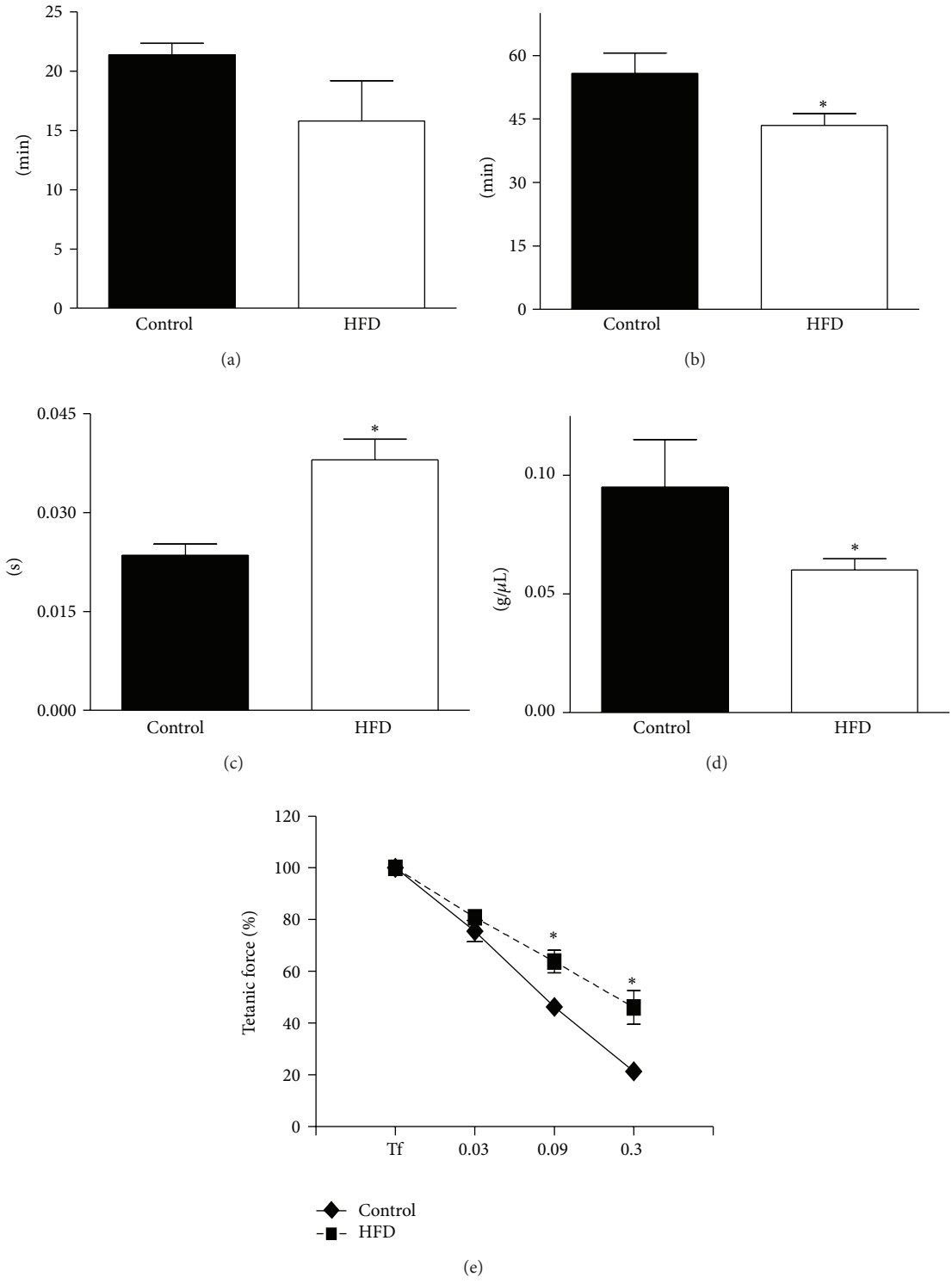

Figure 2: ((a)-(b)) *In vivo* muscle function determined by exhaustion incremental treadmill test and endurance treadmill test. Exhaustion time (a) and endurance time (b) are expressed in min. Control mice: closed bars; HFD mice: white bars. $^{*}P < 0.05$ versus control mice, $n = 5$ experiments. All data are expressed as mean ± SD. ((c)–(e)) *Ex vivo* functional parameters of tibialis intact preparations from control (closed bars or closed diamonds) and HFD mice (white bars or closed squares). (c) Twitch time to peak (s); (d) specific tetanic force (g/μL); (e) fatigue index measured as the tetanic force drop for different stimulation frequencies (from 0.03 to 0.9 Hz) compared to the maximal tetanic force (Tf) and expressed in %. $^{*}P < 0.05$ versus control mice, $n = 5$ experiments. All data are expressed as mean ± SD.

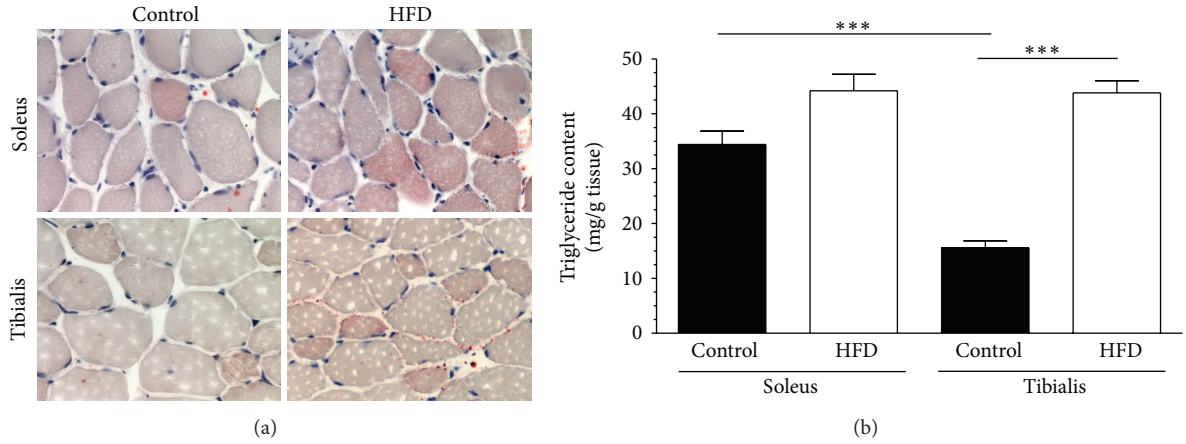

Figure 3: Effect of high-fat diet (HFD) on muscle histology and triglyceride content. Panel (a): representative photomicrographs (magnification 400x) of histological analyses performed in soleus and tibialis anterior sections of control mice and HFD mice with Oil Red O staining (performed on 10 animals per group). Panel (b): triglyceride content assessed in homogenates of soleus and tibialis anterior muscles of control mice and HFD mice. Values are means ± SD of 10 animals per group. $^{***}P < 0.001$.

associated with significant changes in muscle mass, structure, contractile properties, and exercise capacity. Although a number of studies have previously shown that prolonged exposure to elevated levels of fatty acids leads to insulin resistance and impaired muscle glucose and lipid metabolism [4, 14, 15], here we documented that these changes were muscle-specific as the HFD-induced myosteatosis and insulin resistance were more pronounced in the tibialis anterior muscle compared to the soleus muscle.

4.1. Effects of HFD on Muscle Mass, Structure, and Function. Mice exposed to HFD increased body weight, decreased muscle mass, and increased muscle triglyceride content. These changes were associated with the following adaptations in muscle contractility and fatigability: the time to peak tension increased, the specific tetanic force decreased, and the fatigue index was reduced in HFD mice compared to control mice.

As differences in skeletal muscle composition significantly affect muscle function, we chose to study diet-induced adaptations in two muscles characterized by high dependence on either oxidative metabolism, the soleus (which mostly contains slow fibers), or glycolytic metabolism, the tibialis (which mostly contains fast fibers) [6–8]. Although we did not assess the HFD-induced fast-to-slow transition of muscle fibers, changes in muscle composition plausibly occurred, as previously documented [2, 3], thus explaining, at least in part, the adaptations in muscle contractile properties. In fact, the tibialis anterior muscle of HFD mice was slower and weaker than that of control mice and the fatigue index was lower in HFD mice compared to control mice. However, resistance to fatigue (assessed by treadmill tests) was lower in HFD mice compared to control mice possibly because the body composition was significantly impaired by HFD in the former group (that showed increased body mass and decreased muscle mass) compared to the latter group of animals.

Defects in mitochondrial functions have been identified in skeletal muscles of obese and/or type 2 diabetic patients [16–18]. Here we observed that mitochondrial changes (i.e., changes in markers of mitochondrial production and activity) were elicited by dietary manipulation, providing a further indirect evidence that a shift toward a greater contribution of oxidative fibers represents a pathophysiological adaptation to high-fat feeding. Interestingly, this adaptation mainly involves fast muscles such as the tibialis anterior. Although the fast-to-slow muscle fiber type transition has previously been documented (and characterized) in response to several conditions (e.g., increased neuromuscular activity, mechanical loading, and hypothyroidism), the molecular mechanism(s) underlying the changes in muscle composition in response to HFD still need to be clarified.

4.2. Effects of HFD on Muscle Lipid Metabolism. Tibialis anterior of control mice presented lower triglyceride content than the soleus. This is in agreement with previous findings demonstrating that oxidative muscles store higher amount of lipids than glycolytic muscles, being lipid oxidation their preferential source of energy [19, 20]. Interestingly, when animals were exposed to dietary manipulation, the triglyceride content of the two muscles was comparable, thus suggesting that the glycolytic muscles are more susceptible to alteration evoked by chronic high-fat exposure. Similarly, the elevation of plasma free fatty acids in response to starvation has been reported to lead to muscle-specific responses in intramyocellular fatty acid metabolism. Specifically, starvation evoked increases in intramyocellular lipid levels in glycolytic muscles, but not in oxidative muscles [21, 22]. Notably, a similar intermuscle variability in starvation-induced dynamics of intramyocellular lipid levels has also been reported in humans [23]. We also documented a greater activation

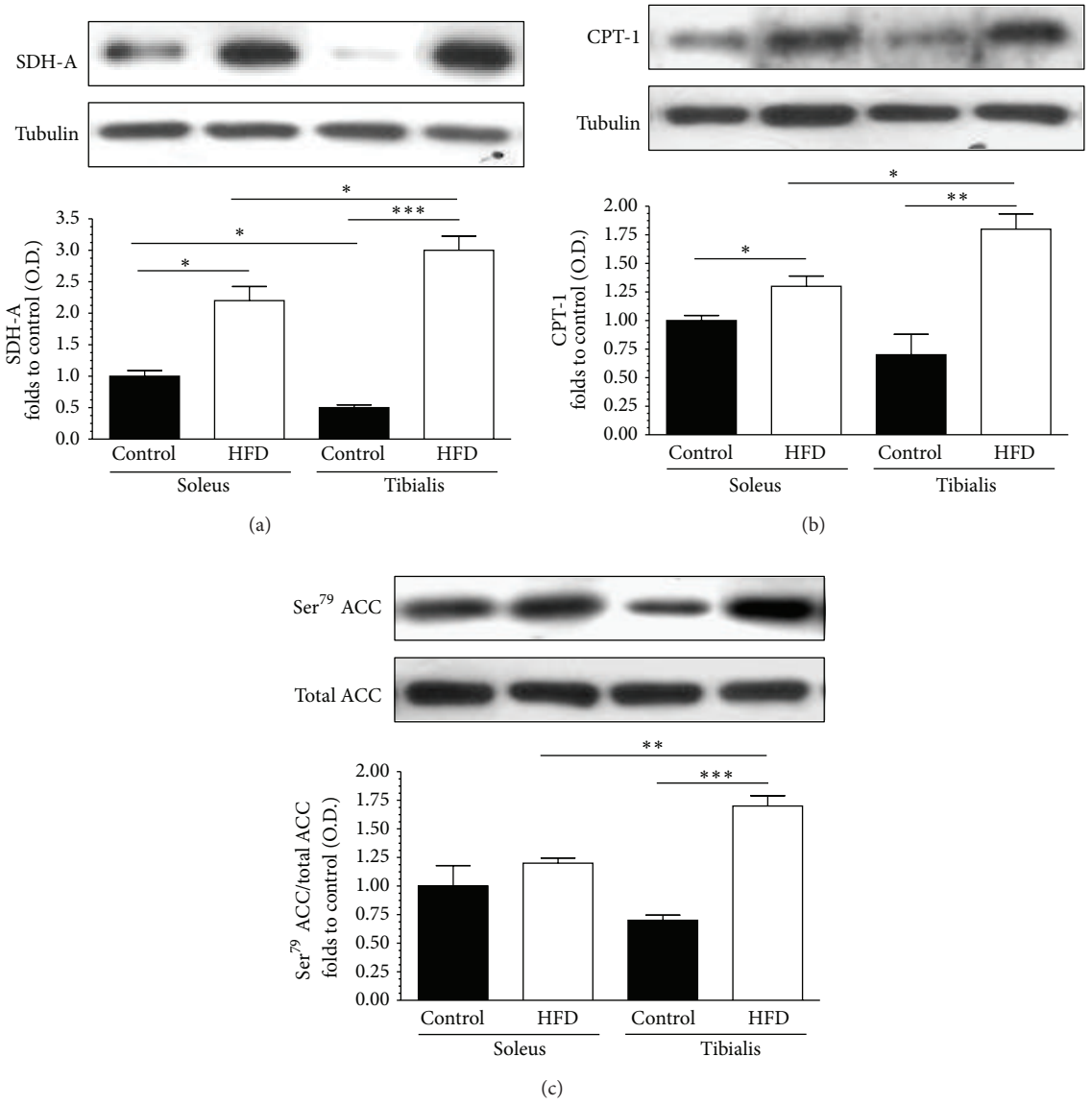

Figure 4: Effect of high-fat diet (HFD) on lipogenesis and lipid oxidation. Succinate dehydrogenase (SDH) activity (panel (a)), citrate transport protein (CTP)-1 expression (panel (b)), and total acetyl-CoA carboxylase (ACC) protein expression and Ser^{79} phosphorylation (panel (c)) were analyzed by Western blot on muscle homogenates. Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding tubulin contents and normalized using the related control band. The data are means ± SD of 10 animals. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of ACC, suggesting increased triglyceride synthesis, in the tibialis compared to the soleus of HFD mice. Although the mechanisms underlying the intermuscle variability in HFD-induced myosteatosis are presently not defined, these findings confirm that oxidative muscles dispose free fatty acids by oxidation when their plasma availability is elevated. In contrast, glycolytic muscles neutralize free fatty acids by reesterification to triglycerides [24, 25]. However, whether myosteatosis represents either a marker of the mitochondrial adaptation/dysfunction or a result of the impaired glucose and lipid oxidation remains to be established. In the latter case, it may be hypothesized that fast muscle fibers are more prone to the intramyocellular triglyceride accumulation due to their lower mitochondrial oxidative phosphorylation reserve compared to slow muscle fibers.

4.3. Effects of HFD on Glycometabolic Balance and Muscle Insulin Sensitivity. The intramyocellular triglyceride accumulation was associated with whole body insulin resistance, as documented (by the glucose tolerance test) in the present investigation as well as in previous studies [3, 25, 26]. A novelty of our study compared to previous findings is the assessment of the profile of insulin sensitivity of individual

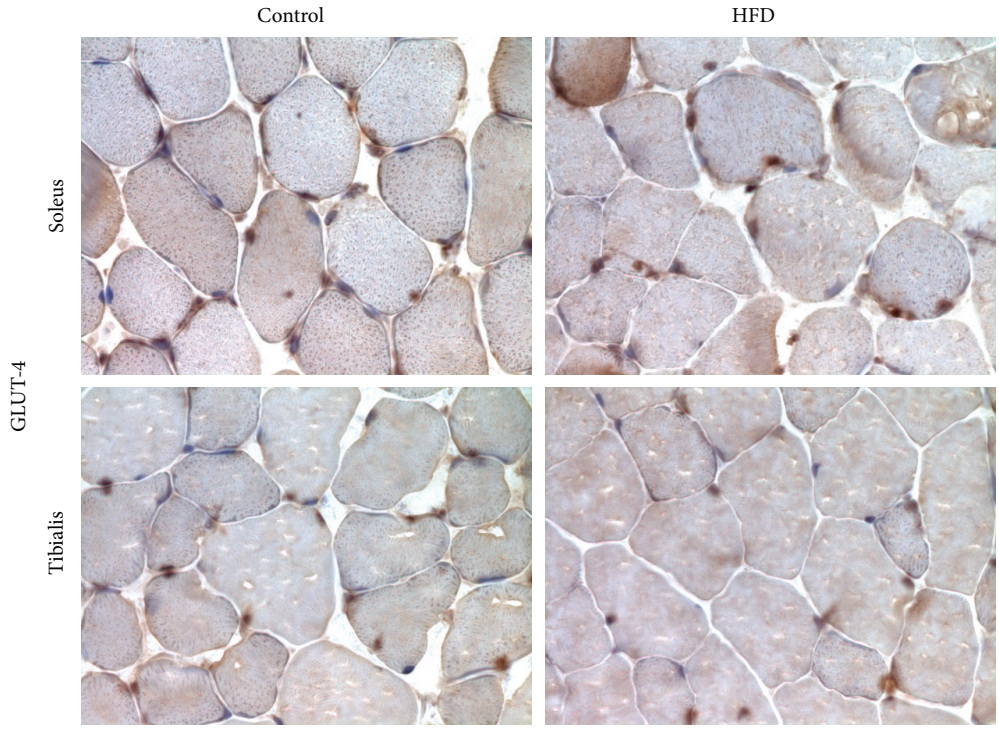

Figure 5: Representative photomicrographs (magnification 400x) showing the effect of high-fat diet (HFD) on GLUT-4 membrane translocation in the soleus and tibialis anterior muscles of control mice and HFD mice (assessed by immunohistochemistry on 10 animals per group).

muscles. Previous studies have shown that impairment in insulin signaling is a major component of skeletal muscle insulin resistance [13, 27, 28]. Here we documented that HFD-induced impairments in activation (i.e., phosphorylation) of IRS-2 and the downstream key insulin signaling kinases, Akt and GSK-3β (an Akt substrate), were more relevant in the tibialis anterior muscle compared to the soleus muscle. In skeletal muscle cells, insulin-stimulated glucose transport has been shown to occur through the translocation of GLUT-4 from the intracellular pool to the plasma membrane. Therefore, a decreased insulin signaling should imply a defect in insulin stimulation of the GLUT-4 translocation. Consistently, we observed that GLUT-4 translocation to the membrane was markedly inhibited in the tibialis and slightly reduced in the soleus of HFD mice compared to control mice. Importantly, the existence of an association between intramyocellular triglyceride accumulation and insulin-resistance does not imply causality. As previously reported, muscle triglycerides are unlikely to directly cause insulin resistance primarily because they are located within lipid droplets. Instead, it has been proposed that in skeletal muscle they protect fibers from the deleterious actions of other lipid metabolites such as diacylglycerol, ceramides, and long chain acyl-CoAs whose levels can be increased through diet or other perturbations (e.g., lipid and heparin infusion and exercise) [26, 29]. However, we may speculate that myosteatosis changes the composition of the low-density microsomal membranes and thus impairs the insulin-stimulated translocation of GLUT-4 from these intracellular membranes to the plasma membrane. Overall, our study substantiates the previous findings by Shortreed et al. [3] that skeletal muscles respond selectively to high-fat diet intervention and extends our understanding of skeletal muscle adaptive response to dietary insults in keeping to their specific oxidative capacity. As the kinetics and composition of dietary manipulation may be critical, further investigation are warranted to determine whether different types and/or duration of dietary manipulation may induce disturbances similar to those found in our study.

4.4. Conclusions. Besides the (causal or protective) effects of myosteatosis on insulin resistance, we found in mice that the soleus muscle stores more triglycerides and is more insulin sensitive than the tibialis anterior muscle in control animals, while the triglyceride content of the two muscles was comparable in HFD mice. A pathophysiological adaptation to lipotoxicity was probably represented by the observed mitochondrial changes that were possibly paralleled by changes in muscle composition and function. These observations deserve future confirmations also in human studies, as to our knowledge no human investigation has previously been performed to assess whether muscle-specific adaptations occur in response to HFD-induced myosteatosis.

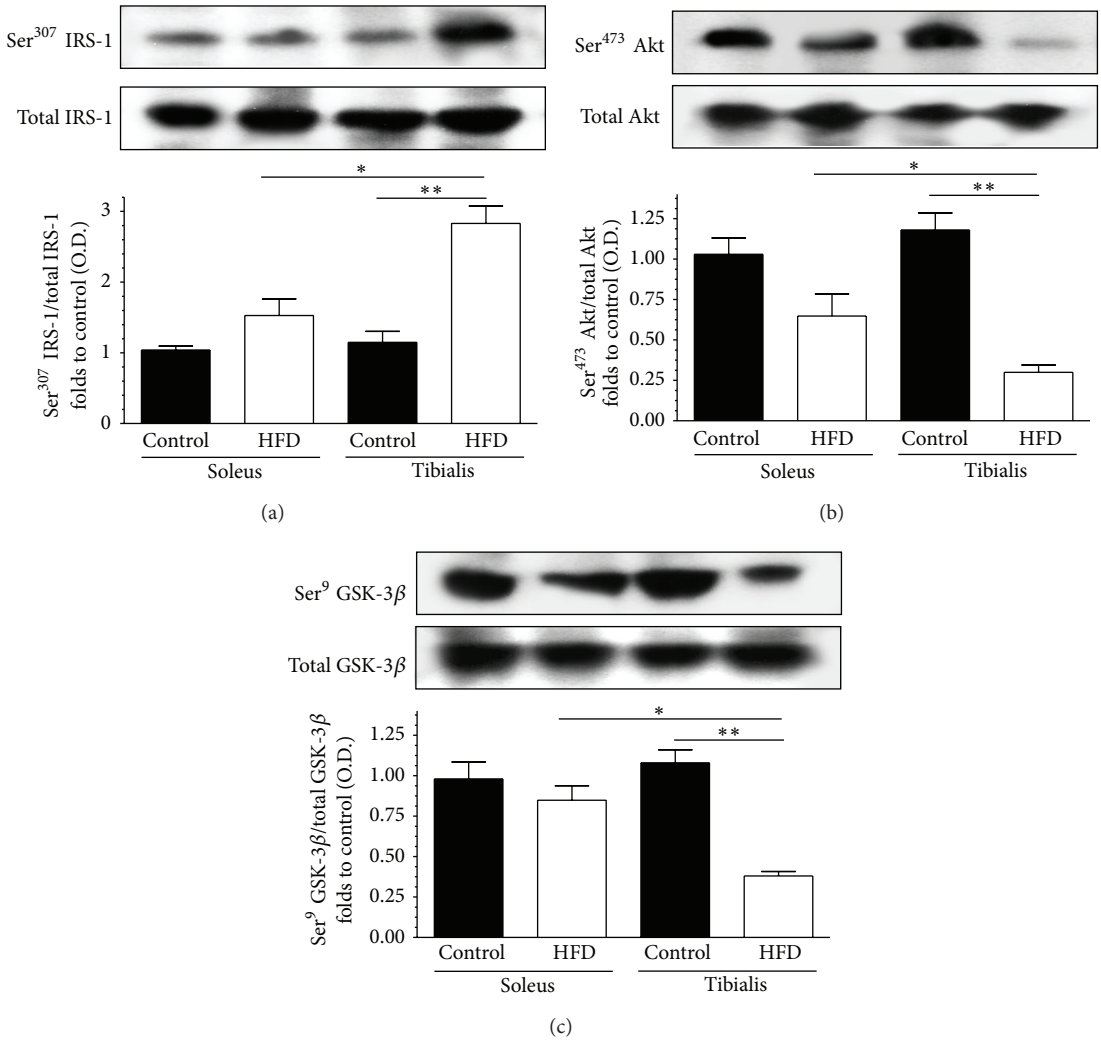

Figure 6: Effect of high-fat diet (HFD) on insulin signal transduction in the soleus and tibialis anterior muscles of control mice and HFD mice. Total IRS-1 protein expression and Ser^{307} phosphorylation (panel (a)), total Akt protein expression and Ser^{473} phosphorylation (panel (b)), and total GSK-3β protein expression and Ser^{9} phosphorylation (panel (c)) were analyzed by Western blot on muscle homogenates. Densitometric analysis of the bands is expressed as relative optical density (O.D.), corrected for the corresponding tubulin contents and normalized using the related control band. The data are means ± SD of 10 animals. $^{*}P < 0.05$; $^{**}P < 0.01$.

Clarifying the cellular and molecular factors modulating the expression and posttranslational control of key proteins in triglyceride metabolism may unmask novel approaches to safely manipulate muscle triglyceride content and the associated insulin resistance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Massimo Collino and Raffaella Mastrocola equally contributed to this work.

Acknowledgments

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Review Article

Ketogenic Diet in Neuromuscular and Neurodegenerative Diseases

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An increasing number of data demonstrate the utility of ketogenic diets in a variety of metabolic diseases as obesity, metabolic syndrome, and diabetes. In regard to neurological disorders, ketogenic diet is recognized as an effective treatment for pharmacoresistant epilepsy but emerging data suggests that ketogenic diet could be also useful in amyotrophic lateral sclerosis, Alzheimer, Parkinson's disease, and some mitochondriopathies. Although these diseases have different pathogenesis and features, there are some common mechanisms that could explain the effects of ketogenic diets. These mechanisms are to provide an efficient source of energy for the treatment of certain types of neurodegenerative diseases characterized by focal brain hypometabolism; to decrease the oxidative damage associated with various kinds of metabolic stress; to increase the mitochondrial biogenesis pathways; and to take advantage of the capacity of ketones to bypass the defect in complex I activity implicated in some neurological diseases. These mechanisms will be discussed in this review.

1. Introduction

It is known that single nutrients may exert positive effects on skeletal muscle health and moreover a combination of nutrients can attenuate signs and symptoms of some neuromuscular diseases. On the other side it is also known that the effects of dieting on health are related to the general ratio of the different macro- and micronutrients rather than each single component. From this point of view great interest has raised in the last years over ketogenic diet (KD).

Since the third decade of the XX century KD is used to treat patients with pharmacological resistance to epilepsy [1–3]. In more recent periods, KD has also been claimed to be useful in other totally different diseases as obesity [4], PCOS [5], cancer [1, 6, 7], diabetes [8], or other pathological conditions [9–11]. Whilst many studies have pointed out potentially positive effects of KDs on many neurological and neuromuscular diseases only few researches have investigated the mechanisms of this promising nutritional approach [12]. The aim of our review is to discuss the role of KDs in selected diseases that affect nervous system with implications in muscular function.

2. Inside the Ketogenic Diet

After a few days of fasting or a drastic reduction in carbohydrate from the diet (below 20 g per day), glucose reserves become insufficient both (1) for normal fat oxidation through the supply of oxaloacetate in the Krebs cycle and (2) for the supply of glucose to the CNS (central nervous system) [13, 14] (Figure 1).

Regarding point (1) oxaloacetate is relatively unstable at body temperature and cannot be accumulated in the mitochondrial matrix; thus, in this "glucose deprivation" condition, it is necessary to supply oxaloacetate for an efficient functioning of the tricarboxylic acid cycle. Oxaloacetate is supplied via the anaplerotic cycle that synthesizes it from glucose through ATP dependent carboxylation of pyruvic acid by pyruvate carboxylase [15].

Regarding point (2), since the CNS cannot use fatty acids (FFA) as an energy source (FFA cannot cross the blood-brain barrier), it normally utilizes glucose. After 3-4 days without any carbohydrate intake the CNS must find alternative energy sources as demonstrated by the classic experiments by Felig et al. [13, 14, 16, 17]. This alternative energy source is the

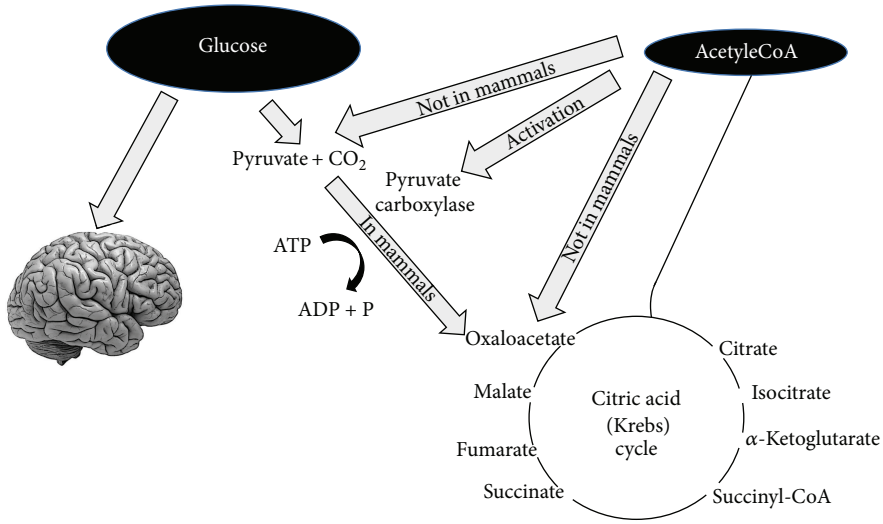

Figure 1: Glucose is necessary not only to supply energy for the central nervous system but also to produce pyruvate that can be transformed into oxaloacetate. Oxaloacetate must be maintained at a level sufficient to allow citric acid cycle function (i.e., the condensation between acetyl-CoA and oxaloacetate). Oxaloacetate is unstable and must be refurnished (this kind of reactions is called anaplerotic). The main way to produce oxaloacetate is from pyruvate that derives from glucose. In mammals pyruvate cannot be produced from acetyl-CoA as shown in the figure.

ketones bodies (KBs): acetoacetate (AcAc), 3-hydroxybutyrate (3HB), and acetone [18], derived from the overproduction of acetyl-CoA without a concomitant production of an adequate amount of oxaloacetic acid. This process is called ketogenesis and principally occurs in the mitochondrial matrix in the liver [19]. It is important to underline that the liver produces KBs but is also unable to use them because of the absence of the succinyl-CoA: 3-CoA transferase (SCOT) enzyme required to convert acetoacetate into acetoacetyl-CoA [18].

The main ketone body produced in the liver is acetoacetate but the primary circulating ketone is 3-hydroxybutyrate. Under normal conditions the production of free acetoacetic acid is negligible and can be metabolised by various tissues such as skeletal muscle and the heart. In conditions of overproduction of acetoacetic acid this accumulates above normal levels and part is converted to the other two ketone bodies. High level of KBs in the blood and their elimination via urine cause ketonemia and ketonuria. Under normal conditions the concentration of KBs is usually very low (<0.3 mmol/L) compared to glucose (approx. 4 mmol/L) [20, 21]. Once KBs reach a concentration of about 4 mmol/L (which is close to the k_M for the monocarboxylate transporter [22]) they start to be utilised as energy source by the CNS [21]. KBs are used by tissues as a source of energy [19, 21, 23] through a pathway that firstly converts 3HB back to AcAc which is then transformed into acetoacetyl-CoA. The latter is finally divided into two molecules of acetyl-CoA, which are subsequently used in the Krebs cycle (Figure 2). It is interesting to note that, compared to glucose, the KBs are able to produce a higher quantity of energy due to the changes in mitochondrial ATP production that they induce [21, 24, 25].

Another point to highlight is, as shown in Table 1, that glycaemia, even though reduced, remains within physiological

Table 1: Blood levels during a normal diet, ketogenic diet (i.e., <20 grams of carbohydrates per day), and diabetic ketoacidosis [10].

Blood levels	Normal diet	Ketogenic diet	Diabetic ketoacidosis
Glucose (mg/dL)	80–120	65–80	>**300**
Insulin (μU/L)	6–23	6.6–9.4	≅**0**
KBs conc. (mmol/L)	0.1	7/8	>**25**
pH	7.4	7.4	<**7.3**

levels [26, 27], due to main two sources: (1) glucogenic amino acids and (2) glycerol liberated via lysis from triglycerides [28, 29]. During physiological ketosis (fast or very low calorie KD) ketonemia reaches maximum levels of 7/8 mmol/L with no changes in pH while in uncontrolled diabetic ketoacidosis this can exceed 20 mmol/L with a concomitant lowering of blood pH [16, 30] (Table 1). Blood levels of KBs in healthy people do not exceed 8 mmol/L because the central nervous system (CNS) efficiently uses these molecules as energy supply in place of glucose [16].

3. Ketogenic Diet Simulates Fasting and Its Molecular Effects

Traditionally, physicians are afraid of ketosis because they associate the severe hyperketonemia that results from insulin deficiency and that can lead to severe acidosis and death in individuals with type 1 diabetes with physiological ketosis resulting from fasting or KD. Hans Krebs was the first who used the term "physiological ketosis" to distinguish the mild (≅8 mmol/L of KBs) ketosis of starving or KD from

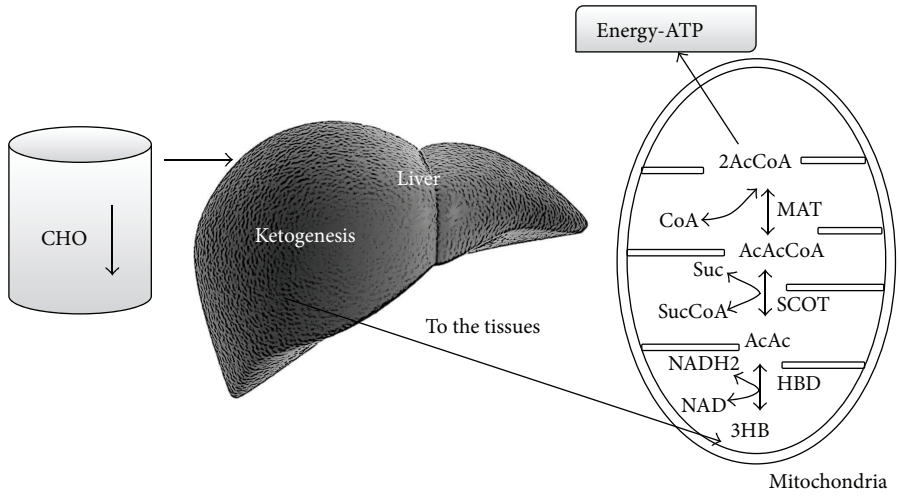

Figure 2: A reduced availability of dietary carbohydrates leads to an increased liver production of KBs. The liver cannot utilize KBs because it lacks the mitochondrial enzyme succinyl-CoA: 3-ketoacid (oxoacid) CoA transferase (SCOT) necessary for activation of acetoacetate to acetoacetyl-CoA. KBs are utilized by tissues, in particular by brain. KBs enter the citric acid cycle after being converted to acetyl-CoA by succinyl-CoA: 3-CoA transferase (SCOT) and methylacetoacetyl-CoA thiolase (MAT).

the "pathological ketoacidosis" of metabolically unbalanced diabetes [31].

Despite the concerns of many physicians, it is known that no other species, except humans, are evolutionarily adapted to chronic undernutrition as a consequence of ecologically dictated dietary restrictions [32]. The periods of fasting or undernutrition are themselves ketogenic [23] during which the concentrations of insulin and glucose decrease while those of glucagon increase with the attempt to maintain normal blood glucose levels. When the body passes from a condition of food abundance to one of deprivation (or else via KD simulated deprivation), there is, with a slight delay, an increase in the concentration of FFA and KBs in the blood. Thus, from this point of view a KD could be compared to a caloric restriction, undernutrition, or fasting. This manipulation of nutrients, both in quantity and quality, seems to act both on blood glucose and KBs and also has the capacity to promote changes in metabolic pathways and cellular processes such as stress resistance and autophagy. KDs can also act in a way similar to caloric restriction (CR) on AMPK and SIRT-1 [33]. Thus, to understand the complex effects of KDs we have to take into account these molecular and intracellular pathways. PGC1α is activated in the phosphorylated state. Once phosphorylated, PGC1α translocates from the cytosol to the nucleus, where it promotes the transcription of genes involved in fatty acid transport, fat oxidation, and oxidative phosphorylation [34]. PGC1α could be phosphorylated through several different pathways, among them AMPK, calcium-calmodulin-dependent protein-kinase, and p38 mitogen-activated protein kinase pathways [35]. PGC1α can also be activated by a SIRT1-mediated deacetylation [36]. AMPK can exert its action both via phosphorylation of PGC1α or in a direct manner. AMPK activation promotes enhanced expression of skeletal muscle oxidative-related enzymes, proteins, and metabolism, which are consistent with the findings that obese skeletal muscles are less oxidative and have lower AMPK activation (during fasting conditions). At the same time, AMPK activation also inhibits mTOR signalling. However, it seems counterintuitive to inhibit an important growth-mediated pathway (i.e., mTOR), regulating muscle mass, so that skeletal muscles can grow [37]. Thus, nutrients manipulation may influence these pathways; for example, relative deficiency in carbohydrate availability has been demonstrated to be a stimulus *in vivo* for activation of AMPK and SIRT-1, increasing phosphorylation of AMPK and deacetylation of PGC1α in skeletal muscle without affecting the total amount of AMPK, PGC1α, or SIRT 1 [38]. These mechanisms appear to be activated just after few hours (5 hours) of starvation in mice [39] whilst no data are available on KD and humans. Once activated, SIRT1 and AMPK produce beneficial effects on glucose homeostasis and insulin action [40].

4. Ketogenic Diet and Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder that affects spinal and cortical motor neurons, leading to progressive weakness and loss of skeletal muscle. Affected subjects die within 2 to 5 years of symptom onset. Death usually occurs from respiratory paralysis. At the moment, there are no effective treatments for ALS, and the only US FDA approved pharmacological therapy is limited to riluzole that causes only a modest decrease of the disease progression and increases survival by only 2 to 3 months [41]. The causes of ALS are complex and multifactorial, embracing genetic and environmental factors: excessive oxidative damage, neurofilament accumulation, excitotoxicity, and mitochondrial membrane dysfunction are some of the supposed causes [42–44].

Due to its multifactorial origins, specific targets of treatment have not been identified yet and, unfortunately, an effective therapy is still missing. As other neurodegenerative disorders the probable mitochondrial involvement makes the KD a promising synergic tool for the treatment of ALS [45]. In about 10% of patients with ALS, this is an inherited disorder (familial amyotrophic sclerosis FALS) and in 20% of these subjects there is a mutation in the gene encoding the enzyme Cu/Zn superoxide dismutase 1 (SOD1) [46]. This mutation is linked to mitochondrial activity; it is in fact the mutant SOD1 that has been localized in the mitochondria binding bcl2 (a cell antiapoptotic protein) [47]. Moreover decreased mitochondrial complex I activity has been measured in skeletal muscle and spinal cord of ALS patients [48]. Results show that KB can act on mitochondrial function, restoring, for example, complex I function, after a pharmacological blocking. Moreover, in cultured neurons treated with pharmacologic agents blocking complex I, an addition of KB restores the function of the complex [49].

Recently, researchers have demonstrated that in an ALS mouse model (a KO mouse for the copper/zinc SOD1-G93A) the administration of KD led to a higher motor neuron survival and an improvement in motor function compared to KO mice without KD [50]. Other interesting studies have reported that a supplementation with a precursor of KB (caprylic acid) improved mitochondrial function and motor neuron count in an ASL mouse model [51]. Authors explained these results through a neuroprotective effect of DHB. Moreover, they suggested that the hyperketonemia might improve the mitochondrial defects by increasing mitochondrial function and ATP production (measured in purified mitochondria from an ASL mouse model) even if in both studies there were no significant increases in survivals. It is important to underline that during the KD the percentage of dietary fat was very high (60%) and this could explain the measured improvements. As a matter of fact, cholesterol and phospholipids are essential for axonal membrane health and for peripheral nerve membrane injury repairs, in particular low-density lipoproteins [52]. Interestingly, there are some epidemiological data demonstrating that hyperlipidaemia is a significant prognostic factor for survivals in patients with ASL [53], but these results were not confirmed by Paganoni et al. [54] who showed a "U-"shaped association between BMI and mortality, with the highest survival in subjects with higher BMI (30–35); in this study dyslipidemia is not an independent predictor of survival in ALS. Wills et al. [55] have recently showed that patients which received a high caloric/high carbohydrate enteral nutrition had a smaller total number of adverse events and deaths than those of the high fat/high calorie group or the control group. These apparently contradictory results depict a complex scenario in ALS. However there are some common features: a higher caloric intake seems to improve survival in ALS patients, even though no conclusive relationships have been found between cholesterol and improved conditions. It is known that endogenous cholesterol production is enhanced by insulin and reduced by exogenous cholesterol [4]; thus a high carbohydrate diet could have been useful to improve cholesterol production. It could be hypothesised that the positive effects of a high caloric high carbohydrate diet could be used in alternating periods with a high fat (high butter [56]) KD in some types of ALS (SOD1) but not in those linked to RNA processing perturbations (TDP43, FUS, and C9orf72). More CRTs are needed to investigate the role of nutrition, and more in detail of KD, in ALS therapy.

5. Mitochondrial Disorders and Ketogenic Diet

In the previous paragraph we have discussed the role of mitochondria in a neurological disease like ALS. There is an increasing amount of evidence that KD can improve mitochondrial functioning and stimulate mitochondriogenesis [57–60]. As stated by Wallace and colleagues, "Ironically, one of the oldest therapeutic approaches—fasting and the ketogenic diet—remains the most promising treatment for mitochondrial defects" [61]. As a matter of fact, even though KD as therapeutic tool is known since the 20's of XX century its effects on mitochondria are a, relatively, recent finding. Some mitochondrial defects may cause seizures with different epileptic phenotypes [62]. There are some encouraging data about the effects of KDs in mitochondriopathies. Kang et al. [63] showed that a KD could be a safe and effective therapy that reduces seizures in children with intractable epilepsy and various respiratory complex defects (complex I, II, IV, or combined).

Ahola-Erkkilä et al. [64] have treated a mouse model for late-onset mitochondrial myopathy that is known to cause in humans autosomal dominant progressive external ophthalmoplegia, with generalized muscle weakness, accumulation of generalized mtDNA deletions, and cytochrome c oxidase negative muscle fibers with a KD. The KD decreased the amount of cytochrome c oxidase negative muscle fibers and prevented the formation of the mitochondrial ultrastructural abnormalities in the muscle. The diet cured most of the metabolic and lipidomic anomalies not through an action on mtDNA but inducing mitochondrial biogenesis. Nevertheless, we have to consider the two sides of the same coin: even though the KD might be a therapeutic tool in many mitochondrial-based diseases it is contraindicated in several metabolic disorders. Patients with fat metabolism disorder might undergo severe catabolic crisis.

Inborn errors in the enzymes involved in lipid metabolism: from mitochondrial membrane long-chain fatty acids transport mechanism to beta-oxidation and Krebs cycle could be potentially fatal during fasting or KDs. Thus, carnitine deficiency, carnitine palmitoyltransferase (CPT) I or II deficiency, carnitine translocase deficiency, b-oxidation defects, or pyruvate carboxylase deficiency should be screened before initiating the KD treatment. Moreover, KD can exacerbate acute intermittent porphyria in affected subjects [65].

6. Alzheimer's Disease and Ketogenic Diet

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease and the leading cause of dementia among the aged population. AD symptoms are in general a cognitive

impairment with progressive memory deficits and personality changes. The causes of such cognitive decline can be attributed to a progressive synaptic dysfunction and the subsequent loss of neurons; this loss seems to be located in many vulnerable regions of the brain: mainly neocortex, limbic system, and the subcortical regions [66]. The hippocampus is a specific target of KD; McDaniel et al. demonstrated that in a kainic acid- (KA-) induced status epilepticus rat model, the KD inhibited mTOR pathway signalling in the brain preventing late hippocampal mTOR activation after KA-induced status epilepticus [59]. It is important to underline that neurons in the hippocampus play critical roles during learning and memorization and are particularly vulnerable to dysfunction and degeneration in AD. AD has been categorized into two major forms: familial AD (FAD) and sporadic AD (SAD) or late onset age-related AD (LOAD); the latter is the leading cause of dementia, accounting for more than half of all cases. Whilst quite all FAD cases can be attributed to mutations in three genes (amyloid precursor protein APP, presenilin 1 PSEN1, and presenilin 2 PSEN2 [67]), the exact etiology of SAD is not still completely understood. It is well known that age is the greatest risk factor (AD increases exponentially with age in people aged 65 or older) [68], together with some other factors as (1) allelic variations in apolipoprotein E (Apo E), (2) degeneration of anatomical pathways, (3) mitochondrial dysfunction, (4) compromised blood-brain barrier, (5) immune system dysfunction, (6) infectious agents and other environmental factors such as exposure to aluminium, (7) repeated head injury, and (8) malnutrition [69].

As in other chronic diseases, treatments for AD can be divided into two categories: (A) symptomatic treatments (that offer temporary amelioration of symptoms without modifying disease progression over years) and (B) treatments that are potentially able to modify disease's history (slowing or halting the decline of cognitive functions over years). Despite some FDA approved drugs like acetyl cholinesterase inhibitors and memantine (a glutamate antagonist used to ameliorate behavioural symptoms, in the moderate phase of the disease) currently no effective treatment exists to prevent, modify, or stop AD. Most of the approved drugs for treatments only offer a moderate symptomatic effect [70, 71]. As for other diseases the development of effective treatments is hindered by the not complete knowledge of AD etiology [71] even now that the "amyloid cascade" hypothesis has been extensively studied. This pathogenetic hypothesis is based on the neurotoxic characteristics of β-amyloid (Aβ) and on its accumulation related initiation of a cascade of neurotoxic events, including not only the formation of well-known neurofibrillary tangles (NFT) but also chronic inflammatory responses, an increase in oxidative stress, and in conclusion a mitochondrial dysfunction [71]. The two major lesions in AD are caused by distinct proteins, tau in the case of the neurofibrillary tangles and amyloid β-protein in the case of amyloid plaques.

Nevertheless, as aforementioned, there is no unified etiopathogenic mechanism for both FAD and SAD. In the latter there are data suggesting that the deposition of amyloid β-protein and NFTs act together inducing a decline in mitochondrial function and alter brain metabolic activity, all relate aging processes. In consideration of the strong link between aging process and AD and of the positive effects of KD in ageing brains [72], the multifaceted nature of AD that includes mitochondrial and metabolic dysfunctions suggests that there could be a rationale for the use of KD in these patients [73, 74]. For example, an *in vitro* study has demonstrated that addition of KB (beta-hydroxybutyrate) protects the hippocampal neurons from Aβ toxicity; this suggests possible therapeutic roles for KD on mitochondrial defects related to AD [75]. Animal studies though showed contrasting results: Van der Auwera et al. [76] demonstrated a decrease of Aβ in the brain of young transgenic AD mice overexpressing the London APP mutation fed with KD for 1.5 months whilst on aged canines the effect of KD on Aβ seemed to be limited to the parietal lobe of the brain [77]. It was also demonstrated that long-term (8 months) feeding of a ketone ester in middle-aged mice (8.5 months old) improved cognition and ameliorated Aβ and tau pathology [75]. Beckett et al. [78] demonstrated that AD mice model fed with a high-fat, low-carbohydrate ketogenic diet showed an improved motor function without changes in Aβ. The contradictory results between animal studies could be due to the age of the animals: mice are in most cases young or middle aged but there are recurrent metabolic alterations mainly expressing in the elderly. For example, AD is also associated with metabolic dysregulation and insulin resistance [79]. Many researchers have demonstrated that KD could significantly improve glucose homeostasis, reducing metabolic dysregulation and insulin resistance [80–82]. There is another pathophysiological mechanism ascribed to an altered mitochondrial function and glucose metabolism in AD: the accumulation of advanced glycation end products (AGEs) [83]. Despite the fact that the accumulation of AGEs in cells and tissues is a normal feature of aging, this process is accelerated in AD. AGEs can be also found in amyloid plaques and neurofibrillary tangles. Increases of AGEs can clearly explain many neuropathological aspects of AD (protein crosslinking, glial induction of oxidative stress, and neuronal cell death). We can speculate that the neuroprotective effects of KDs and the KDs' related reduction of glycaemia could also improve these features in AD. Another intriguing hypothesis, in our opinion, is the putative effects of a KD on mitochondriogenesis together with the improvement of mitochondria machinery [61, 72, 74, 84–86].

As previously stated, mitochondrial dysfunction has been hypothesised to be implicated in the aetiology of AD [72]. A reduction in neuronal and glial mitochondrial metabolism was shown in elderly compared to healthy young subjects [87]. This dysfunction, related to diminished energy production from mitochondrial glucose/pyruvate oxidation, could enhance the pathologic deposition of Aβ and tau. This impaired mitochondrial function could be represented by an increased superoxide production with oxidative damage, decrease in oxidative phosphorylation, and, consequently, impairment of the mitochondrial electron transport chain. Other glucose metabolic impairments in specific zones of the brain, which are characteristic of AD, are related to mitochondrial dysfunction [88]. It is interesting to note that

earlier reduced glucose utilization could be detected by FDG-PET in cognition-related brain sites in subjects with familiar history of AD [89]. It is possible that a reduced level of brain glucose utilization may contribute to the development of AD neuropathology. As suggested by Vanitallie and coworkers an early impairment in brain glucose metabolism can be detected before any measurable cognitive decline [90]. Other evidence supports this theory, such as a reduced concentration of glucose transporters (GLUT 1 and 2 but also the neuronal glucose transporter GLUT 3). It is shown that in the brain there is abnormal hyperphosphorylation of tau in AD related to this phenomenon [91]. Considering the shift in the brain's metabolism from glucose to ketones, during a KD [17], shift that is useful in glucose transporter type I deficiency syndrome [92], KD might be a therapy for neuronal degeneration related to GLUT deficiency in AD [73]. The brain in the AD also seems to be able to use KB as a fuel, when glucose utilization is impaired. A study has demonstrated that a supplementation with medium chain triglycerides that induce an increase of KB improved performance in the AD Assessment Cognitive Scale with a direct correlation between ketone concentration and cognitive improvement [93].

Finally, even though there are no direct or strong evidence of the usefulness of KD in humans, this nutritional approach appears promising and so deserves further clinical extensive trials.

7. Parkinson Disease and Ketogenic Diet

The pathogenesis of sporadic Parkinson disease (PD) remains unresolved, but numerous studies suggest that the primary cause is excitotoxic degeneration of dopaminergic neurons in the substantia nigra, leading to abnormalities of movement, and to an increasing extent in cognition and other cortical function disorders. It has been suggested that an impairment of mitochondrial function involving the substantia nigra plays an important contributory role in PD beginning and progression [94]. For example, Kashiwaya et al. used a heroin analogue 1-methyl-4-phenylpyridinium, MPP(+), that produces death of dopaminergic substantia nigra cells by inhibiting the mitochondrial NADH dehydrogenase multienzyme complex, producing a syndrome similar to Parkinson's disease in cultured mesencephalic neurons. β-Hydroxybutyrate protected these neurons from MPP(+) toxicity neurodegeneration [74]. In animal models, 1-methyl-4-phenol-1,2,5,6-tetrahydropyridine (MPTP) is used to produce selective destruction of dopaminergic neurons in the substantia nigra that mimics human Parkinson's disease-like syndrome. As for other, abovementioned, diseases the positive effects of KD on mitochondrial function could be a key factor in the utilization of such diet as ketones may bypass the defect in complex I activity implicated in PD. Infusion of β-hydroxybutyric acid in mouse protects from ageing the dopaminergic neurodegeneration and motor deficits induced by MPTP [49]. Moreover KD protected dopaminergic neurons of the substantia nigra against 6-hydroxydopamine neurotoxicity in a rat model of Parkinson disease [95]. VanItaille et al. [96] demonstrated that in humans, able to prepare a "hyperketogenic" diet at home and adhere to it for 28 days, the high level of KB was related to an improvement in the Unified Parkinson's Disease Rating Scale scores.

8. Glycogenoses and Ketogenic Diet

Glycogenoses (glycogen storage diseases, GSD) are a group of inherited disorders due to enzyme defects, affecting glycogen metabolism and leading to intracellular accumulation of glycogen of normal or abnormal structure in a variety of cell types. Classically, GSD were numbered I to VIII, according to the chronology of their discovery and the specific enzyme defect [97]. In recent years, other primary glycogenoses (GSD 0, GSD IX to XV) were identified [98]. GSD are transmitted as autosomal recessive, with the exception of GSD VIII (also classified as IXa), that is X-linked. From a functional point of view, GSD I, III, IV, VI, and VIII/IXa can be grouped as hepatic GSD [99], since defective enzymes are mostly expressed in liver cells. Given the central role of liver in regulation of glycaemia through glycogenolysis, it is not surprising that hypoglycemia is the main manifestation of hepatic GSD [97, 100]. This, in turn, causes neurological symptoms ranging from convulsion to seizure, particularly in the early years of life. In the long term, recurrent severe hypoglycemia may cause brain damage, particularly in GSD I (von Gierke disease, deficiency of G-6-P phosphatase), the most frequent hepatic GSD. Up to date, treatment of hepatic GSD is based on dietary therapy to prevent hypoglycemia, feeding patients with foods rich in starches during the night and the day [100, 101]. The scientific rationale for a potential use of a ketogenic diet (KD) stems from the early observation that symptoms related to hypoglycemia improved with age in GSD [102], as well as GSD III [100] patients. It is well known that this adaptation occurs in the brain during starvation as well as during fever [102]. This observation was classically interpreted as a consequence of adaptations taking place in the brain and allowing an increased use of ketone bodies as fuel substrates alternative to glucose. The same mechanism has been put forward for explaining the effect of calorie restriction [100] that also results in low blood glucose levels. The mechanistic interpretation would be that KD would increase the utilization in the brain of pathways in energy metabolism independent of glycogen breakdown. Based on these considerations, KD has been effectively used in the treatment of muscular GSD V (McArdle disease) [103, 104]. The anticonvulsing effects of the KD are well established, even though the mechanisms are not fully clarified yet [105]. The potential use of KD in pathological conditions characterized by chronic hypoglycemia is further strengthened by the fact that KD is the gold standard in the treatment of GLUT1 deficiency syndrome [106], which can be considered a metabolic phenocopy of hepatic GSD, since blood glucose cannot be transported into neuron. Finally, a recent study [107] suggested that KD might be successfully used in treatment of severe cardiomyopathy complicating the muscular form of GSD III. Altogether, these observations might encourage

further studies on the use of KD in treatment of selected forms of GSD.

9. Conclusions

The peculiar metabolic state induced by a KD has been widely investigated in the last years. The increase of KBs concentration, the reduction of blood glucose together with the involvement of many important pathways (e.g., IGF-1/AKT/mTor, AMPK/PGC1α) has shown to be a potential therapeutic weapon against many neurological and neuromuscular diseases.

Although these studies provide a theoretical basis for the effect of KDs on a number of neuromuscular diseases, several important hurdles remain before these findings can be applied widely to clinical practice or public health efforts. First, little is known about the precise mechanism of KD action on neuromuscular diseases and, second, long term effects of this kind of diet should be investigated in these patients.

Despite the fact that we only have preliminary evidence based mostly on animal models, most available data sets indicate that the putative mechanism of KDs on some neurological and neuromuscular diseases could be as follows.

(1) Provide an efficient source of energy for the treatment of certain types of neurodegenerative diseases characterized by focal brain hypometabolism such as Parkinson and Alzheimer diseases. Neuronal cells are capable of metabolizing KBs even in the presence of a deficiency of glucose. Ketones can increase the ΔG of ATP hydrolysis and provide a source of cytoplasmic acetyl-CoA that can blunt the lowering of acetyl choline characteristic of Alzheimer's brains.

(2) Decrease the oxidative damage associated with various kinds of metabolic stress. If compared with glucose metabolism, ketones generate lower levels of oxidative stress in the brain together with a greater cellular energy output and antioxidant capacity. Moreover, ketosis can increase glutathione peroxidase in hippocampal cells and in general decreases mitochondrial ROS production.

(3) Increase the mitochondrial biogenesis pathways (through activation of AMPK and PGC 1 α pathway). The improvement of mitochondrial pathways can help to improve brain and neuronal metabolism.

(4) Allow ketones to bypass the defect in mitochondrial complex I activity founded in skeletal muscle and spinal cord of ALS. In cultured neurons treated with pharmacologic agents blocking complex I, an addition of KB restores the function of the complex.

(5) Decrease the amount of cytochrome-c oxidase negative muscle fibers in some mitochondrial myopathy and prevent the formation of the mitochondrial ultrastructural abnormalities in the muscle.

In conclusion, we believe that KD should be studied in more depth for its encouraging prospective as a therapy for many neuromuscular and neurodegenerative diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Vitamin D Signaling in Myogenesis: Potential for Treatment of Sarcopenia

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Muscle mass and strength progressively decrease with age, which results in a condition known as sarcopenia. Sarcopenia would lead to physical disability, poor quality of life, and death. Therefore, much is expected of an effective intervention for sarcopenia. Epidemiologic, clinical, and laboratory evidence suggest an effect of vitamin D on muscle function. However, the precise molecular and cellular mechanisms remain to be elucidated. Recent studies suggest that vitamin D receptor (VDR) might be expressed in muscle fibers and vitamin D signaling via VDR plays a role in the regulation of myoblast proliferation and differentiation. Understanding how vitamin D signaling contributes to myogenesis will provide a valuable insight into an effective nutritional strategy to moderate sarcopenia. Here we will summarize the current knowledge about the effect of vitamin D on skeletal muscle and myogenic cells and discuss the potential for treatment of sarcopenia.

1. Introduction

Muscle wasting is observed in various disease states, in conditions of reduced neuromuscular activity, with ageing. Age-related muscle wasting is referred to as "sarcopenia" coined by Irwin H. Rosenberg from the Greek words sarx (meaning flesh) and penia (meaning loss) [1, 2]. There has been no consensus about definition of sarcopenia suitable for use in research and clinical practice [3]. Therefore, some studies [4, 5] suggest a working definition of sarcopenia: sarcopenia is a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life, and death. Sarcopenia is characterized by the fact that it progresses very slowly throughout several decades. Muscle mass fairly consistently decreases at a rate of approximately 0.5–1%/year beginning at 40 years of age [6, 7] and the rate dramatically accelerates after the age of 65 years [8]. Muscle strength appears to decline more rapidly than muscle mass. Muscle strength declines at a rate of 3-4% per year in men and 2.5–3% per year in women aged 75 years [9]. Although the precise molecular and cellular mechanisms underlying age-related loss of muscle mass and strength have remained unknown [10, 11], multiple contributing factors have been proposed. The development and progress of sarcopenia have been thought to be mediated by the combination of these contributing factors.

Based on large-scale studies [12–16], on average, it is estimated that the prevalence of sarcopenia reaches 5–13% in those aged 60–70 years and ranges from 11 to 50% in those aged over 80 years [17]. In USA in 2000, it was estimated that direct healthcare costs related to sarcopenia were $18.5 billion ($10.8 billion in men, $7.7 billion in women), which represented approximately 1.5% of total healthcare expenditures for that year [18]. Globally, the number of people aged over 60 years is 600 million in the year 2000 [19]. It is predicted that people aged over 65 years will double by 2020 and will triple by 2050 [20]. Therefore, sarcopenia is being recognized as not only a serious healthcare problem but also a social problem. Much is expected of an effective intervention for sarcopenia. Nutritional interventions would be a promising candidate in combating sarcopenia.

Epidemiologic, clinical, and laboratory evidence provide an effect of vitamin D on muscle function. Numerous studies have investigated the effect of vitamin D supplementation on muscle strength and physical performance in elderly people. However, the precise molecular and cellular mechanisms remain to be elucidated. Immunohistochemical studies have demonstrated that vitamin D receptor (VDR) might be localized in human muscle fibers [21–23] with some contradictions [24, 25]. In addition, recent studies have reported that vitamin D signaling via VDR plays a role in the regulation of myoblast proliferation and differentiation [26–32]. Understanding how vitamin D signaling contributes to myogenesis will provide a valuable insight into an effective nutritional strategy to moderate sarcopenia. Here we will summarize the current knowledge about effect of vitamin D on skeletal muscle and myogenic cells and discuss the potential for treatment of sarcopenia.

2. Vitamin D Signaling Pathways: Genomic and Nongenomic Pathways

Vitamin D signaling has been extensively investigated in a variety of cell types. During the past two decades, considerable progress has been made in understanding the action of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] on myogenic cells. The biological effect of 1α,25$(OH)_2D_3$ is exerted through genomic or nongenomic mechanisms (for reviews see [33–35]). Better understanding of the molecular and cellular mechanisms of vitamin D action on skeletal muscle will enable us to develop an effective intervention for sarcopenia. We will focus on 1α,25$(OH)_2D_3$ signaling via VDR in genomic and nongenomic mechanism related to myogenic cells, although rapid alteration in intracellular calcium, which is nongenomically regulated by 1α,25$(OH)_2D_3$, has been well demonstrated both *in vivo* and *in vitro*; for details, excellent review article is already available on this subject [36].

An active form, 1α,25$(OH)_2D_3$, acts by binding to VDR [33]. The binding affinity of 25$(OH)D_3$ for human vitamin D receptor (VDR) is approximately 500 times less than that of 1α,25$(OH)_2D_3$ but the circulating level of 25$(OH)D_3$ is approximately 1000 times higher than that of 1α,25$(OH)_2D_3$ [37, 38]. In genomic mechanism, 1α,25$(OH)_2D_3$ binds to VDR and is transported to the nucleus [35]. VDR is heterodimerized with 9-*cis*-retinoic acid receptor (RXR) and VDR:RXR complex modulates gene expression via binding to specific target gene promoter regions, known as vitamin D response elements (VDREs), to activate or suppress their expression [35]. In general, VDREs possess either a direct repeat of two hexanucleotide half-elements with a spacer of three nucleotides (DR3) or an everted repeat of two half-elements with a spacer of six nucleotides (ER6) motif, with DR3s being the most common [39]. Wang et al. [40] investigated direct 1α,25$(OH)_2D_3$-target genes on a large scale by using a combined approach of microarray analysis and in silico genome-wide screens for DR3 and ER6-type VDREs. Microarray analyses, performed with RNA from human SCC25 cells treated with 1α,25$(OH)_2D_3$ and cycloheximide, an inhibitor of protein synthesis, revealed 913 regulated genes [40]. Of the 913 genes, 734 genes were induced and 179 genes were repressed by treatment of 1α,25$(OH)_2D_3$ [40]. In addition, a screening of the mouse genome identified more than 3000 conserved VDREs, and 158 human genes containing conserved elements were 1α,25$(OH)_2D_3$-regulated on microarrays [40]. These results support their broad physiological actions of 1α,25$(OH)_2D_3$ in a variety of cell types.

With respect to several genes related to myogenesis, we will describe them in more detail. For example, 1α,25$(OH)_2D_3$ induced expression of the gene encoding *Foxo1* [40], which is a member of the FOXO subfamily of forkhead/winged helix family of transcription factors, governs muscle growth, metabolism, and myoblast differentiation. When transfected C2C12 cells with adenoviral vector encoded a constitutively active Foxo1 mutant, they effectively blocked myoblast differentiation [47]. This was partly rescued by inhibition of Notch signaling [47], which inhibits myoblast differentiation [48]. In addition, loss of Foxo1 function precluded Notch signaling-mediated inhibition of myoblast differentiation [47]. To elucidate the possible role of Notch signaling in Foxo1-mediated inhibition of myoblast differentiation, by combining coculture system, transfection assay, chromatin immunoprecipitation assay, and short interfering RNA (siRNA) technology, authors showed that Foxo1 physically and functionally interacted with Notch by promoting corepressor clearance from DNA binding protein, CSL [CBF1/RBPjk/Su(H)/Lag-1], leading to inhibition of myoblast differentiation through activation of Notch target genes [47]. Another gene, Id (inhibitor of differentiation) gene, is also known target of 1α,25$(OH)_2D_3$ [49]. Id mRNA was constitutively expressed in rat osteoblastic osteosarcoma ROS17/2.8 cells and its level was transcriptionally suppressed by 1α,25$(OH)_2D_3$ [50]. 1α,25$(OH)_2D_3$ exerted its negative effect on Id1 gene transcription via the 57 bp upstream response sequence (−1146/−1090) [49]. Id proteins (Id1, Id2, Id3, and Id4) dimerize and neutralize the transcriptional activity of basic helix-loop-helix (bHLH) proteins [51]. It has been shown that Id inhibits MyoD activity either by forming transcriptionally inactive complexes of MyoD-Id or by forming heterodimers with E-proteins and effectively blocking the formation of active MyoD/E-protein complexes [52]. At this time, there are only limited data available on Id expression and vitamin D during muscle development. For example, in VDR knockout mice with abnormal muscle development, there were no differences in expression levels of Id1 and Id2 [46]. Therefore, we cannot conclude whether vitamin D regulates myogenesis by modulating Id expression.

A nongenomic response to 1α,25$(OH)_2D_3$ is characterized by a rapid (the seconds to minutes range) activation of signaling cascades and an insensitivity to inhibitors of transcription and protein synthesis [34]. The rapid response to 1α,25$(OH)_2D_3$ has been hypothesized to elicit the classic VDR translocation to the plasma membrane. When treating chick myoblasts with 1α,25$(OH)_2D_3$, translocation of VDR from the nucleus to the plasma membrane rapidly occurred within 5 min after the addition of 1α,25$(OH)_2D_3$ [53]. This translocation was blocked by colchicine, suggesting the possible role of the intracellular microtubular transport system

in the distribution of VDR [53]. The VDR translocation appears to depend on intact caveolae that are specialized plasmalemmal microdomains originally studied in numerous cell types for their involvement in the transcytosis of macromolecules [54]. Confocal microscopy revealed that $1\alpha,25(OH)_2D_3$-induced VDR translocation to the plasma membrane was abolished by methyl-beta-cyclodextrin, a reagent to disrupt the caveolae structure [55]. Both disruption of caveolae and siRNA-mediated silencing of caveolin-1 suppressed $1\alpha,25(OH)_2D_3$-dependent activation of protooncogene c-Src (cellular Src) with tyrosine-specific protein kinase activity [55]. Immunocytochemical analysis provided evidence that caveolin-1 colocalized with c-Src near the plasma membrane under basal conditions [55]. When treated with $1\alpha,25(OH)_2D_3$, the colocalization of caveolin-1 and c-Src was disrupted and they were redistributed into cytoplasm and nucleus [55]. On the basis of these results, it can be hypothesized that (1) interaction caveolin-1/c-Src inactivates the kinase under basal conditions and (2) when $1\alpha,25(OH)_2D_3$ stimulates VDR translocation to the plasma membrane, it dissociates the caveolin-1/c-Src complex allowing c-Src activation [55]. Non-genomic action of $1\alpha,25(OH)_2D_3$ might be required for a reciprocal interaction between c-Src and caveoline-1. Besides the classical VDR, it has been identified as a potential candidate as an alternate membrane-associated receptor for $1\alpha,25(OH)_2D_3$: $1,25D_3$-MARRS (membrane-associated, rapid response steroid binding) also known as ERp57, GRp58, ERp60, and Pdia3 [56]. Since $1,25D_3$-MARRS has been shown to function in various cell types [57], it also may potentially mediate vitamin D signaling in myogenic cells.

The c-Src tyrosine kinase induced by $1\alpha,25(OH)_2D_3$ is required for activation of mitogen-activated protein kinases (MAPKs), ERK1/2 (extracellular signal-regulated kinase 1/2) [58], and p38 [59]. $1\alpha,25(OH)_2D_3$ rapidly promoted phosphorylation of ERK1/2 through c-Src activation [58], Raf-1/Ras/MEK (MAPK/ERK kinase), and PKCα (protein kinase C alpha) [60]. In addition to ERK1/2 activation, $1\alpha,25(OH)_2D_3$ rapidly stimulated MKK3/MKK6 (mitogen-activated protein kinase kinases 3/6)/p38 MAPK through c-Src activation [59]. Although another MAPK family member, JNK1/2 (c-Jun NH2-terminal kinase 1/2), was also activated by $1\alpha,25(OH)_2D_3$ [59], an upstream mediator of $1\alpha,25(OH)_2D_3$-dependent JNK1/2 activation was characterized less than that of ERK/1/2 and p38. The molecular links between JNK and c-Src have been shown in *Drosophila melanogaster*. The JNK homolog Basket (Bsk) is required for epidermal closure [61]. Src42A, a *Drosophila* c-Src protooncogene homolog functions in epidermal closure during both embryogenesis and metamorphosis [61]. The severity of the epidermal closure defect in the Src42A mutant depended on the Bsk activity. These results suggest the possibility that JNK activation in mammals may also be required for Src tyrosine kinase activity. These MAPK signaling pathways have been shown to contribute to myogenesis [62–65]. For example, inactivation of the Raf-1/MEK1/2/ERK1/2 pathway in MM14 cells through the overexpression of dominant negative mutants of Raf-1 blocked ERK1/2 activity and prevented myoblast proliferation [62]. Pharmacological blockade of p38α/β kinases by SB203580 inhibited myoblast differentiation [63–65]. JNK was involved in regulating myostatin signaling [66], which is known as a member of tumor growth factor β family and functions as a negative regulator of muscle growth [67]. MAPK signaling pathways function at different stages of myogenesis.

Apart from MAPKs, PI3K (phosphatidyl inositol 3-kinase)/Akt signaling pathway, which is essential for initiation of myoblast differentiation [68], also seems to be activated by $1\alpha,25(OH)_2D_3$. After exposure to $1\alpha,25(OH)_2D_3$, Akt phosphorylation was enhanced through PI3K in C2C12 cells [68]. Intriguingly, suppression of c-Src activity by PP2, a specific inhibitor for all members of the Src family, and knockdown of c-Src expression by siRNA decreased Akt phosphorylation in $1\alpha,25(OH)_2D_3$-treated C2C12 cells [28]. In addition, when treating C2C12 cells with $1\alpha,25(OH)_2D_3$ in the presence of U0126 or SB203580 to inhibit ERK1/2 and p38 MAPK, respectively, SB203580 but not U0126 markedly blocked both basal and $1\alpha,25(OH)_2D_3$-induced Akt phosphorylation. These results suggest that $1\alpha,25(OH)_2D_3$-induced Akt phosphorylation may occur through c-Src and p38 MAPK [28]. Taken together, $1\alpha,25(OH)_2D_3$ can simultaneously activate multiple signaling pathways in myogenic cells but their relative contribution to myogenesis remains to be established.

3. Effects of Ageing on Serum Concentration of Vitamin D, Muscle Morphology, and Muscle Fiber Type

Vitamin D status varies with age [69]. Serum levels of $25(OH)D_3$ are qualitatively categorized as deficiency (<20 ng/L or <50 nM), insufficiency (21–29 ng/L or 50–75 nM), and normal (30 ng/L or >75 nM) [70]. van der Wielen et al. [69] measured wintertime serum $25(OH)D_3$ concentrations in 824 elderly people from 11 European countries [69]. They reported that 36% of men and 47% of women had $25(OH)D_3$ concentrations below 30 nM [69]. Vitamin D deficiency in elderly is thought to occur mainly due to restricted sunlight exposure, reduced dietary vitamin D intake, and decreased capacity of the skin to produce vitamin D [69]. MacLaughlin and Holick [71] examined the effects of ageing on the capacity of the skin to produce previtamin D3 in the skin by comparing young subjects (8 and 18 years old) with aged subjects (77 and 82 years old). They showed that ageing decreased the capacity less than half of young subjects [71], suggesting that elderly people are potentially at risk for vitamin D insufficiency/deficiency.

Vitamin D deficiency appears to be associated with changes in muscle morphology. For example, patients with osteomalacic myopathy associated with vitamin D deficiency show degenerative changes such as opaque fibers, ghost-like necrotic fibers, regenerating fibers, enlarged interfibrillar spaces, infiltration of fat, fibrosis, glycogen granules, and type II muscle fiber atrophy [72]. As is the case with vitamin D-deficient patients, it is well known that elderly people show aberrant muscle morphology. Scelsi et al. [73] performed histochemical and ultrastructure analysis using

biopsies taken from the vastus lateralis of healthy sedentary men and women aged 65–89 years. They observed myofibrillar disorganization, streaming of Z-line, rod formation, intracellular lipid droplets, lysosomes, and type II muscle fiber atrophy [73]. The very elderly people had "flattened" or "crushed" shaped muscle fibers, whereas the young people had mature-appearing polygonal muscle fibers [74]. These aberrant changes were much more pronounced in the type II muscle fibers than in type I muscle fibers [74]. Although the precise mechanisms remain to be elucidated, it can be speculated that specific type II muscle fiber atrophy with ageing may be associated with a muscle fiber type-specific reduction in satellite cell content. Satellite cells are essential for normal muscle growth [75]. Verdijk et al. [76] examined whether satellite cells could specifically decrease in type II muscle fibers in the elderly people. Biopsies were taken from the vastus lateralis of elderly (average age: 76 years) and young (average age: 20 years) healthy males [76]. They found significant reduction in the proportion and mean cross-sectional area of the type II muscle fibers and the number of satellite cells per type II muscle fiber in elderly subjects compared to young subjects [76]. This study is the first to show type II muscle fiber atrophy in elderly people to be associated with a muscle fiber type-specific decline in satellite cell content. It remains unknown whether vitamin D supplementation specifically attenuates atrophy of type II muscle fibers with recruitment of satellite cell. Whether vitamin D has positive effects on myoblast proliferation and differentiation is currently under debate. Recent studies [27] suggest that vitamin D treatment enhances fast type (type IIa) MyHC expression in fully differentiated C2C12 myotubes. Type II muscle fibers contain type IIa MyHC [77]. Therefore, vitamin D could potentially contribute to the changes in phenotype of existing muscle fibers and/or the maintenance of type II muscle fibers.

4. Effects of Ageing on Expression of VDR

Bischoff-Ferrari et al. [22] investigated the effect of ageing on VDR expression in human skeletal muscle. Biopsies were taken from the gluteus medius of 20 female patients undergoing total hip arthroplasty (average age: 71.6 years) and from the transversospinalis muscle of 12 female patients with spinal operations (average age: 55.2 years). Immunohistochemical analysis revealed that the number of VDR-positive myonuclei decreased with ageing [22]. Importantly, VDR expression was not affected by $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ levels [22]. Buitrago et al. [78] showed that silencing of VDR expression in C2C12 myoblasts suppressed p38 MAPK phosphorylation and decreased ERK1/2 activation induced by $1\alpha,25(OH)_2D_3$. Tanaka et al. [31] demonstrated that knockdown of VDR expression resulted in downregulation of MyHC mRNA in differentiating C2C12 myoblasts when treated with $1\alpha,25(OH)_2D_3$. Therefore, it is possible that decreased expression of VDR observed in elderly people might reduce the functional response of the muscle fibers to $1\alpha,25(OH)_2D_3$.

5. Effects of Vitamin D Supplementation on Muscle Injury

The regenerative potential of skeletal muscle decreases with age [79–81]. Satellite cells are absolutely required for muscle regeneration [82]. Satellite cells are defined anatomically by their position beneath the basal lamina and adhered to muscle fibers [83]. They, traditionally considered as a population of skeletal muscle-specific committed progenitors, play a crucial role in the postnatal maintenance, repair, and regeneration [75]. Under normal physiological conditions, they remain in a quiescent and undifferentiated state [75, 84]. However, when skeletal muscle is damaged by unaccustomed exercise or mechanical trauma, they are activated to proliferate, differentiate, and fuse with the already existing muscle fibers or fuse to form new muscle fibers [75, 84]. Few studies have examined the effects of vitamin D treatment on muscle injury. Stratos et al. [85] investigated whether systemically applied vitamin D could restore muscle function and morphology after trauma. Rats were injected subcutaneously with 7-dehydrocholesterol (332,000 IU/kg) immediately after crush injury and muscle samples were collected at days 1, 4, 14, and 42 after injury [85]. Vitamin D treatment increased cell proliferation and inhibited occurrence of apoptosis at day 4 compared to control rats [85]. In addition, a faster recovery of contraction forces was observed at day 42 in vitamin D-treatment group compared to control group [85]. Notably, the number of satellite cells was not influenced by vitamin D [85], suggesting the possibility that vitamin D supplementation has relatively little effect on satellite cell function *in vivo*. It is necessary to scrutinize thoroughly efficacy, duration, optimal dose, and side effects in relation to vitamin D treatment. Srikuea et al. [29] demonstrated that VDR was highly expressed in the nuclei of regenerating muscle fibers, indicating a potential role for vitamin D in muscle regeneration following injury. Relationship of vitamin D signaling and myogenesis will be discussed below in Section 10.

6. Vitamin D and Type 2 Diabetes Mellitus

Although the incidence of type 2 diabetes mellitus increases with age [86], the precise underlying mechanisms are still not fully understood. Skeletal muscle is the primary target for insulin action and glucose disposal. Therefore, elderly people with excessive loss of muscle mass are at risk for development of type 2 diabetes mellitus [87]. Meta-analysis reveals that vitamin D supplementation has beneficial effects among patients with glucose intolerance or insulin resistance at baseline [88]. However, an explanation for the beneficial role of vitamin D supplementation in the lowering of glycemia in diabetes mellitus remains to be determined. Skeletal muscle can increase glucose uptake through insulin-dependent and muscle contraction-dependent mechanisms [89]. Insulin and muscle contractions stimulate glucose transport in skeletal muscle via translocation of intracellular glucose transporter type 4 (GLUT4) to the cell surface. Manna and Jain [90] examined the mechanism by which vitamin D supplementation regulates glucose metabolism in 3T3L1 adipocytes.

When 3T3L1 adipocytes were treated with high glucose in the presence of $1\alpha,25(OH)_2D_3$, it increased expression of GLUT4 and its translocation to cell surface, glucose uptake, and glucose utilization [90]. $1\alpha,25(OH)_2D_3$ also enhanced cystathionine-γ-lyase (CSE) activation and H_2S formation [90], which is an important signaling molecule produced mainly by CSE in the cardiovascular system [91]. Furthermore, the effect of $1\alpha,25(OH)_2D_3$ on GLUT4 translocation and glucose utilization was prevented by chemical inhibition or silencing of CSE [90]. In muscle cells, it is currently not known whether CSE may be associated with $1\alpha,25(OH)_2D_3$-induced glucose metabolism. Therefore, further studies are required to elucidate the physiological role of CSE in regulation of glucose metabolism in skeletal muscle. Tamilselvan et al. [92] examined the effect of calcitriol (1,25, dihydroxycholecalciferol) on the expression of VDR, insulin receptor (IR), and GLUT4 in L6 cells when exposed to high glucose and high insulin which mimics type 2 diabetic model [92]. Calcitriol partially restored VDR, IR, and GLUT4 expression in type 2 diabetic model [92], raising the possibility that vitamin D could contribute to improving insulin signaling in type 2 diabetes mellitus. In a recent study, the effect of $1\alpha,25(OH)_2D_3$ on glucose uptake in rat skeletal muscle is investigated [93]. $1\alpha,25(OH)_2D_3$ stimulated glucose uptake with increased expression of GLUT4 protein and enhanced translocation of GLUT4 to the plasma membrane not through PI3K-signaling pathway [93], which is essential for insulin-stimulated GLUT4 translocation and glucose transport [94]. In addition, $1\alpha,25(OH)_2D_3$-stimulated glucose uptake was suppressed concomitantly with downregulation of GLUT4 protein by treatment with cycloheximide [93], suggesting that it may be mediated by genomic signaling of vitamin D. Taken together, vitamin D may improve glucose metabolism in skeletal muscle by modulating GLUT4 expression and translocation through insulin-dependent and/or insulin-independent mechanisms.

7. Vitamin D Receptor Expression in Skeletal Muscle and Myogenic Cells

VDR is known to be expressed in a wide variety of tissues including bone, bronchus, intestine, kidney, mammary gland, pancreas, parathyroid, pituitary gland, prostate gland, spleen, testis, and thymus [25]. However, there has been still some controversy as to whether VDR is expressed in skeletal muscle [24, 29, 32]. For example, some studies have failed to detect VDR in skeletal muscle [24, 25, 95–97]; other studies have shown that VDR protein and/or mRNA are detectable in skeletal muscle [21–23, 29, 46, 98] and myogenic cells [26, 27, 29, 31, 32, 42, 45, 46, 53, 55, 98–104]. In brief, Wang et al. [105] call into question the specificity of various commercially available VDR antibodies. They systematically characterized these antibodies in terms of their specificity and immunosensitivity using negative control samples from VDR knockout mice [105]. They demonstrated that the mouse monoclonal VDR antibody against the C-terminus of human VDR, D-6 (Santa Cruz Biotechnology), possesses high specificity, high sensitivity, and versatility [105]. They showed that VDR protein was not detected in skeletal muscle by immunohistochemical analysis using this antibody and that VDR mRNA was detectable only at extremely low levels by quantitative RT-PCR assay [24]. By contrast, Kislinger et al. [106] used large scale gel-free tandem mass spectrometry to monitor global proteome alterations throughout the myogenic differentiation program in C2C12 cells. They observed upregulation of VDR protein during early stage of myoblast differentiation [106]. Srikuea et al. [29] provided strong evidence for the presence of VDR in myogenic cells, by combining immunoblot assay, immunocytochemical analysis, PCR-based cloning, and DNA sequencing to validate the expression of VDR in C2C12 cells. They showed that the full-length VDR mRNA transcript could be isolated from myoblasts and myotubes and VDR protein was primarily localized in the nucleus of myoblasts and in the cytoplasm of myotubes [29]. In addition, they examined the localization of VDR protein using a model of myogenesis *in vivo*. $BaCl_2$ treatment was used to induce regeneration and immunohistochemical analysis was performed on sections from control and regenerating muscle. In control muscle, VDR was detected in muscle fibers but levels were very low, whereas in regenerating muscle, VDR expression was detected in the central nuclei of newly regenerating muscle fibers [29]. More recently, Girgis et al. [32] demonstrated that VDR protein was detectable in C2C12 myoblasts by immunoblot assay using VDR antibody (D-6). The discrepancy among studies may be explained, at least in part, by the difference in the expression of VDR during the stages of muscle development. For example, Endo et al. [46] reported that VDR mRNA was detected in skeletal muscle from 3-week-old wild-type mice but not 8-week-old wild-type mice. Wang and DeLuca [24] showed that VDR protein was undetectable in skeletal muscle from 6- to 7-week-old C57BL/6 mice. A similar result was also reported by Srikuea et al. [29] using 12-week-old C57BL/6 mice. Therefore, VDR expression may be dependent on the context of muscle development. It requires further clarification whether VDR is expressed in muscle fibers.

8. The Conversion of $25\ (OH)D_3$ to $1\alpha,25(OH)_2D_3$ Might Occur in Myogenic Cells

Vitamin D, in the form of vitamin D_3, is synthesized from 7-dehydrocholesterol in the skin through the action of ultraviolet irradiation [33]. Alternatively, vitamin D, in the form of either vitamin D_2 or vitamin D_3, can also be taken in the diet [33]. An active form, $1\alpha,25(OH)_2D_3$, is synthesized from vitamin D_3 through two hydroxylation steps [33]. Vitamin D_3 is converted to 25-hydroxyvitamin D_3 [$25(OH)D_3$] in the liver by 25-hydroxylases (encoded by the gene *CYP27A1*) [33]. The generated $25(OH)D_3$ is further hydroxylated to $1\alpha,25(OH)_2D_3$ by 25-hydroxyvitamin D_3 1α-hydroxylase (encoded by the gene *CYP27B1*) in the kidney [33]. However, CYP27B1 has been detected in various extrarenal tissues [107, 108], raising the possibility that $1\alpha,25(OH)_2D_3$ might be locally synthesized and activate VDR in myogenic cells [29, 32]. Inactive form of vitamin D_3, $25(OH)D_3$, could inhibit cell

proliferation in a similar manner to $1\alpha,25(OH)_2D_3$ [29, 32], indicating that the conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ by CYP27B1 occurs in myogenic cells. Girgis et al. [32] confirmed this possibility using luciferase reporter assay system that luciferase activity results from $1\alpha,25(OH)_2D_3$ binding to GAL-4-VDR and subsequent activation of the UASTK luciferase gene via its GAL4 promoter. They transfected C2C12 cells with GAL4-VDR (switch) and UASTK luciferase reporter with treatment of $25(OH)D_3$ and showed that luciferase activity increased in a dose-dependent manner, suggesting the conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ by CYP27B1 and the subsequent activation of luciferase expression via $1,25(OH)_2D$-bound GAL4-VDR [32]. Srikuea et al. [29] confirmed that C2C12 cells express the full-length *CYP27B1* mRNA transcript and CYP27B1 protein could be detected in the cytoplasm of myoblasts, exhibiting partially overlapping with the mitochondria to which CYP27B1 has been reported to be typically localized [109]. Furthermore, they showed that siRNA-mediated knockdown of CYP27B1 could alleviate inhibitory effects of $25(OH)D_3$ on cell proliferation [29]. These observations provide direct evidence that CYP27B1 is biologically active in myogenic cells and mediates to convert $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$. However, it should be noted that the agonistic action of $25(OH)D_3$ has been demonstrated in cells derived from CYP27B1 knockout mice [110]. Although further studies are needed to elucidate the basic mechanisms, locally synthesized $1\alpha,25(OH)_2D_3$ in myogenic cells might act through autocrine/paracrine mechanisms via VDR.

9. The Role of VDR in Muscle Development

Since the process of myogenesis has been extensively studied both *in vivo* and *in vitro*, substantial progress has been made in understanding the molecular and cellular mechanisms. The myogenic regulatory factors, a group of basic helix-loop-helix transcription factors, consisting of MyoD, Myf5, myogenin, and MRF4, play critical roles in myogenesis [111]. MyoD and Myf5 have redundant functions in myoblast specification [112, 113], whereas myogenin [114, 115] and either MyoD or MRF4 [116] are required for differentiation. These myogenic factors can form heterodimers in combination with less specific factors such as members of E12/E47 [117], which are generated by alternative splicing of the E2A gene [118], leading to activation of muscle-specific gene transcription [117].

VDR knockout mice model has provided insight into the possible physiological roles of vitamin D signaling via its receptor in muscle development [46]. VDR null mice recapitulate a human disease of vitamin D resistance, vitamin D-dependent rickets type II [119]. VDR null mice grow normally until weaning and thereafter develop various metabolic abnormalities including hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and bone deformity [46, 119]. Muscle fiber diameter of VDR null mice was approximately 20% smaller and fiber size was more variable than that of the wild-type mice at 3 weeks of age (before weaning). By 8 weeks of age, these morphological changes were more prominent in the VDR null mice compared to the wild-type mice, suggesting either a progressive nature of the abnormalities caused by the absence of VDR or additive effects of systemic metabolic changes already present at this age [46]. Although there are neither degenerative nor necrotic changes in VDR null mice, the aberrant myofibers were observed diffusely without any preference to type I or type II fibers [46]. Based on these results, they suggest that the absence of VDR induces these abnormalities probably in late stages of fiber maturation and/or in metabolism of mature muscle fibers. Tanaka et al. [31] showed that siRNA-mediated knockdown of VDR inhibited myotube formation concomitantly with downregulation of MyoD and myogenin using C2C12 and G8 cells. These results demonstrate that a substantial level of signaling via VDR is required for normal muscle development and myogenesis *in vitro*.

Furthermore, Myf5, myogenin, and E2A but not MyoD and MRF4 were aberrantly and persistently upregulated at the protein and/or mRNA levels in VDR null mice at 3 weeks of age [46]. Consistent with the deregulated expression of MRFs that control muscle phenotype, VDR null mice showed aberrantly increased expression of embryonic and neonatal MyHC isoforms but not type II (adult fast twitch) MyHC isoform [46]. These findings observed in VDR null mice may reflect compensatory response to a reduction in muscle fiber size. For example, it can be hypothesized that, in VDR null mice, satellite cells may be anomalously activated, proliferate, and differentiate to form new myonuclei that fuse with existing fibers to restore normal fiber size. Finally, they examined whether $1\alpha,25(OH)_2D_3$ could directly downregulate MRFs and neonatal MyHC gene expression in C2C12 myoblasts. $1\alpha,25(OH)_2D_3$ (10 nM) decreased the steady-state expression levels of these genes [46]. Overall, these results support a role of VDR in the regulation of muscle development, but the precise mechanisms remain to be elucidated and the interpretation is further complicated since negative vitamin D response elements [120–122] in the promoter region of genes encoding Myf5 and myogenin have not been identified.

10. Effects of $1\alpha,25(OH)_2D_3$ on Myoblast Proliferation and Differentiation

As referred to above, decline of intrinsic regenerative potential of skeletal muscle is a hallmark of ageing [79–81] and may be due to age-related changes in satellite cell function. If vitamin D treatment does lead to improvements in muscle function in elderly people, more attention should be directed to the effect of vitamin D_3 on myoblast proliferation and differentiation. Research on effect of $1\alpha,25(OH)_2D_3$ on myogenesis has been performed using an *in vitro* cell culture system. The effects of $1\alpha,25(OH)_2D_3$ on myoblast proliferation and differentiation are summarized in Table 1. Early studies [41, 43] have reported that $1\alpha,25(OH)_2D_3$ stimulates proliferation of myogenic cells. Giuliani and Boland [41] reported that $1\alpha,25(OH)_2D_3$ (0.13 nM) increased cell density of chick myoblasts. Drittanti et al. [43] showed that $1\alpha,25(OH)_2D_3$ (0.1 nM) had biphasic effects on DNA synthesis. $1\alpha,25(OH)_2D_3$ exhibited a mitogenic effect in proliferating chick myoblasts followed by an inhibitory effect during

Table 1: Effects of $1\alpha,25(OH)_2D_3$ on proliferation and differentiation in myogenic cells.

Muscle cell type	Concentration $[1\alpha,25(OH)_2D_3]$	Proliferation	Differentiation	Method of VDR detection	Reference
Myoblast (chick)	0.13 nM	↑	↑	NI	Giuliani and Boland 1984 [41]
G8	3–300 nM	↓	NI	Equilibrium binding assay, chromatography	Simpson et al., 1985 [42]
Myoblast (chick)	0.1 nM	↑	↑	NI	Drittanti et al., 1989 [43]
Myoblast (chick)	1 nM	↑	↑	NI	Capiati et al., 1999 [44]
C2C12	1 nM	ND	NI	Immunoblot	Stio et al., 2002 [45]
C2C12	10 nM	NI	ND	RT-PCR	Endo et al., 2003 [46]
C2C12	100 nM	↓	↑	RT-PCR, immunoblot, and immunocytochemistry	Garcia et al., 2011 [26]
C2C12	1–100 nM	↓	↓	RT-PCR	Okuno et al., 2012 [27]
C2C12	1 nM	↑	↑	NI	Buitrago et al., 2012 [28]
C2C12	20 nM 2 μM $[25(OH)D_3]$	↓	↓	RT-PCR, PCR cloning, DNA sequencing, immunocytochemistry, and immunoblot	Srikuea et al., 2012 [29]
C2C12	0.1 pM–10 μM	NI	↓	NI	Ryan et al., 2013 [30]
C2C12, G8	1–100 nM	NI	↓	RT-PCR	Tanaka et al., 2013 [31]
C2C12	1–100 nM 1–100 nM $[25(OH)D_3]$	↓	↓	RT-PCR, immunoblot	Girgis et al., 2014 [32]

Promote (↑); inhibit (↓); no difference between vehicle and treatment (ND); not investigated (NI).

the subsequent stage of myoblast differentiation. Capiati et al. [44] showed that $1\alpha,25(OH)_2D_3$ (1 nM) increases the rate of $[^3H]$ thymidine incorporation into DNA in chick myoblasts. In addition, they investigated the role of PKC in mediating the effect of $1\alpha,25(OH)_2D_3$ using a PKC inhibitor. PKC activity increased after treatment of $1\alpha,25(OH)_2D_3$ [44]. The specific PKC inhibitor, calphostin, suppressed $1\alpha,25(OH)_2D_3$ stimulation of DNA synthesis in proliferating myoblasts [44]. Finally, they examined $1\alpha,25(OH)_2D_3$-dependent changes in the expression of PKC isoforms α, β, δ, ε, and ζ [44]. They identified PKCα as main isoform correlated with the early stimulation of myoblast proliferation by $1\alpha,25(OH)_2D_3$ [44]. By contrast, several studies suggest that, overall, $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ appears to have antiproliferative effect on myogenic cells [26, 27, 29, 32, 42]. $1\alpha,25(OH)_2D_3$ (1–100 nM) inhibited proliferation of C2C12 myoblasts in a dose-dependent manner [27, 32] without inducing necrotic and apoptotic cell death [32]. Okuno et al. [27] showed that $1\alpha,25(OH)_2D_3$ arrested the cells in the G0/G1 phase concomitantly with induction of cyclin-dependent kinase (CDK) inhibitors, $p21^{WAF1/CIP1}$ that facilitates cell cycle withdrawal [123] and $p27^{Kip1}$ that inhibits a wide range of CDKs essential for cell cycle progression [124]. Girgis et al. [32] also reported the increased expression of genes involved in G0/G1 arrest including Rb (retinoblastoma protein) and ATM (ataxia telangiectasia mutated) and decreased expression of genes involved in G1/S transition, including c-myc (cellular myc) and cyclin-D1. In addition, they found reduced c-myc protein and hypophosphorylated Rb protein [32]. The active form, hypophosphorylated Rb, blocks entry into S-phase by inhibiting the E2F transcriptional program [125, 126]. In summary, the effects of $1\alpha,25(OH)_2D_3$ on myoblast proliferation remain inconclusive. The discrepancy may be due to the differences in the experimental settings. For example, different cell type (primary cells or immortalized cell lines): $1\alpha,25(OH)_2D_3$ concentration, serum concentration, duration of cell culture, and duration of treatment are employed. Further studies are needed to clarify the role of $1\alpha,25(OH)_2D_3$ on myoblast proliferation.

Some studies [43, 44] reported that $1\alpha,25(OH)_2D_3$ (0.1 or 1 nM) had inhibitory effects on DNA synthesis in differentiating chick myoblasts, with an increased MyHC expression, increased myofibrillar and microsomal protein synthesis, and an elevation of creatine kinase activity. Garcia et al. [26] reported that prolonged treatment of C2C12 myoblasts with $1\alpha,25(OH)_2D_3$ (100 nM) enhanced myoblast differentiation

by inhibiting cell proliferation and modulating the expression of promyogenic and antimyogenic growth factors using a culture system without reducing serum concentration to initiate cell differentiation. They showed that 1α,25$(OH)_2D_3$ downregulated insulin-like growth factor-I (IGF-I) and myostatin expression and upregulates IGF-II and follistatin expression [26]. Follistatin antagonizes myostatin-mediated inhibition of myogenesis [127]. Intriguingly, inhibition of myostatin is characterized by increased expression of IGF-1 and IGF-II [128–133], which are known to be potent stimulus of myogenesis [134, 135]. Therefore, it can be hypothesized that 1α,25$(OH)_2D_3$ may contribute to myogenesis by inducing IGF-II expression through modulation of myostatin-follistatin system. It should be noted, however, that in these culture conditions, only small thin myotubes with few nuclei were observed on day 10 [26]. This may not recapitulate normal C2C12 myoblast differentiation as previously reported [136].

In general, C2C12 myoblasts normally proliferate and are mononucleated when kept subconfluently in high-mitogen medium (e.g., 10–20% fetal bovine serum). To initiate cell cycle exit and myogenic differentiation, by switching from high-mitogen medium to low-mitogen medium (e.g., 2% horse serum), they fuse and differentiate into postmitotic, elongated, and multinucleated myotubes. Using this C2C12 myoblast differentiation system, Buitrago et al. [28] showed that 1α,25$(OH)_2D_3$ (1 nM) enhanced the expression of MyHC and myogenin at 72 h after treatment. By contrast, Okuno et al. [27] investigated the effects of 1α,25$(OH)_2D_3$ (1–100 nM) on differentiating and differentiated stage of C2C12 myoblasts. In differentiating phase, 1α,25$(OH)_2D_3$ treatment downregulated the expression of neonatal myosin heavy chain and myogenin and inhibited myotube formation in a dose-dependent manner (1–100 nM) [27]. They showed that the expression of fast MyHC isoform increased when fully differentiated myotubes were treated with 1 and 10 nM 1α,25$(OH)_2D_3$ [27]. Girgis et al. [32] investigated the prolonged treatment of 1α,25$(OH)_2D_3$ (100 nM) on C2C12 myoblast differentiation. When myoblast was treated with 1α,25$(OH)_2D_3$ throughout myogenesis including proliferative, differentiating, and differentiated stages, myotube formation was delayed by day 10 concomitantly with downregulation of Myf5 and myogenin [32]. However, intriguingly, myotubes treated with 1α,25$(OH)_2D_3$ exhibit larger cell size than nontreated myotubes [32]. These results suggest that 1α,25$(OH)_2D_3$ may biphasically act in the process of early and late myoblast differentiation. Furthermore, they showed that the hypertrophic effect of 1α,25$(OH)_2D_3$ on myotubes is accompanied with downregulation of myostatin [32]. Several studies have provided evidence that myostatin acts as a negative regulator of the Akt/mammalian target of rapamycin (mTOR) signaling pathway [137–140], which plays a key role in the regulation of protein synthesis [141]. For example, Trendelenburg et al. [139] show that myostatin reduces Akt/mTOR signaling complex 1 (TORC1)/p70 S6 kinase (p70S6K) signaling, inhibiting myoblast differentiation and reducing myotube size. In addition, 1α,25$(OH)_2D_3$ induced Akt phosphorylation in differentiating C2C12 cells [28]. Intriguingly, 1α,25$(OH)_2D_3$ sensitizes the Akt/mTOR signaling pathway to the stimulating effect of leucine and insulin, resulting in a further activation of protein synthesis in C2C12 myotubes [104]. Taken together, 1α,25$(OH)_2D_3$ may have an anabolic effect on myotubes by modulating Akt/mTOR signaling probably through genomic and nongenomic mechanisms.

11. Conclusions

The randomized-controlled studies and meta-analysis support a role of vitamin D in improving the age-related decline in muscle function. However, the effect remains inconclusive. Girgis et al. [32] emphasize that large studies employing standardized, reproducible assessments of muscle strength and double-blinded treatment regimens are required to identify the effect of vitamin D supplementation on muscle function and guide the recommended level of vitamin D intake. Although it remains intensely debated whether VDR is expressed in skeletal muscle, research on VDR null mice provides insight into the physiological roles of vitamin D in muscle development and suggests that a substantial level of signaling via VDR is required for normal muscle growth. VDR expression seems to be affected by ageing, suggesting that this might reduce the functional response of the muscle fibers to vitamin D. Vitamin D appears to function in primary myoblasts and established myoblast cell lines. Despite limited evidence available at the time, vitamin D might have an anabolic effect on myotubes by modulating multiple intracellular signaling pathways probably through genomic and nongenomic mechanisms. However, not all studies support this result. Further studies on the potential impact of vitamin D on muscle morphology and function are required to develop the effective intervention for sarcopenia.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Essential Amino Acids and Exercise Tolerance in Elderly Muscle-Depleted Subjects with Chronic Diseases: A Rehabilitation without Rehabilitation?

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Exercise intolerance remains problematic in subjects with chronic heart failure (CHF) and/or chronic obstructive pulmonary disease (COPD). Recent studies show that supplemented essential amino acids (EAAs) may exert beneficial effects on CHF/COPD physical capacity. The results from 3 investigations (2 conducted on CHF and 1 on COPD subjects) served as the basis for this paper. The 3 studies consistently showed that elderly CHF and COPD improved exercise intolerance after 1–3 months of EAA supplementation (8 g/d). In CHF exercise capacity increased 18.7% to 23% (watts; bicycle test), and 12% to 22% (meters) in 6 min walking test. Moreover, patients reduced their resting plasma lactate levels (by 25%) and improved tissue insulin sensitivity by 16% (HOMA index). COPD subjects enjoyed similar benefits as CHF ones. They increased physical autonomy by 78.6% steps/day and decreased resting plasma lactate concentrations by 23%. EAA mechanisms explaining improved exercise intolerance could be increases in muscle aerobic metabolism, mass and function, and improvement of tissue insulin sensitivity (the latter only for the CHF population). These mechanisms could be accounted for by EAA's intrinsic physiological activity which increases myofibrils and mitochondria genesis in skeletal muscle and myocardium and glucose control. Supplemented EAAs can improve the physical autonomy of subjects with CHF/COPD.

1. Introduction

Patients with chronic heart failure (CHF) and/or chronic obstructive pulmonary disease (COPD) often have reduced exercise tolerance, limiting participation in daily activities. Early onsets of fatigue and/or dyspnea are the symptoms responsible for this exercise intolerance. These symptoms are caused by peripheral mechanisms including abnormalities

in skeletal muscle histology, metabolism, and function [1]. They are not caused by altered central cardiac function such as left ventricular ejection (CHF) [2] or the rate of airway obstruction (COPD) [3].

Exercise intolerance, accentuated in the elderly because of aging body changes, negatively impacts both functional and life prognosis. Indeed, it increases the risk of physical dependence, poor quality of life [4], and increased mortality rate both in CHF [5] and COPD [3]. In CHF, it is exercise intolerance and not heart function which is the most important prognostic factor [5], which furthermore is more accurate than hemodynamic/ventilator profiles at predicting outcomes [5]. Therefore, improved exercise intolerance of CHF/COPD patients is a key for maintaining the subjects' autonomy and quality of life as well as for increasing survival.

At present, treatments to improve exercise intolerance in CHF/COPD include specific pharmacological therapy and exercise training (ET). However, despite advanced pharmacological therapy, exercise intolerance still remains problematic in CHF/COPD individuals [4]. Although ET is the corner stone of cardiac and pulmonary rehabilitation (Rehab), it has not become an integral part of clinical management of patients with CHF/COPD because only very few of these patients enter a Rehab programme [6] (in Europe less than 20%). The prevalence of COPD patients who undergo any Rehab protocol is not known. Moreover, not all subjects, including less elderly patients, can sustain ET because of compromised CHF/COPD and/or of more frequent comorbidities.

Recently, a nutritional approach has emerged as a potentially useful tool to improve exercise intolerance in CHF/COPD. Some studies [7–10] have found that chronic oral supplementation of essential amino acids (EAAs) (8 g/d over 1–3 months) significantly improves exercise intolerance of elderly patients with severe CHF/COPD, living at home or admitted to a Rehab setting.

In this paper we summarize the main findings of these studies and show that the EAA mechanisms leading to improved exercise intolerance rely on the intrinsic biochemical physiology of EAAs and on changes in skeletal muscle when EAAs are provided. We believe a better understanding of more comprehensive EAA activity can contribute to future clinical research. If confirmed by larger trials, EAA supplementation could allow elderly subjects with CHF/COPD, who are not on ET programmes, to achieve a good prognostic Rehab outcome.

2. EAA-Induced Exercise Improvements in CHF/COPD and Their Mechanisms

There is a huge literature on the effects of acute amino acid administration on exercise performance in healthy subjects and in athletes. However, only a few studies have been carried out on the chronic use of EAAs to improve exercise intolerance in elderly subjects suffering from severe CHF/COPD and altered body composition. Preliminary results show positive effects of EAAs on exercise intolerance. In Tables 1 and 2, the amino acid mixture used in these studies and their respectively changes in certain exercise variables after chronic amino acid supplementation are reported, after evaluation from the original investigations [8–10].

Table 1: Nutritional composition of an individual packet of supplementation, containing 4 g of an amino acid mixture, used in the clinical studies* reported in the current investigation [7–14].

Kcal	35.3
KJ	149.9
Total amino acids, of which	4 g
L-Leucine	1250 mg
L-Lysine	650 mg
L-Isoleucine	625 mg
L-Valine	625 mg
L-Threonine	350 mg
L-Cysteine	150 mg
L-Histidine	150 mg
L-Phenylalanine	100 mg
L-Methionine	50 mg
L-Tyrosine	30 mg
L-Tryptophan	20 mg

*Treated patients were given 2 packets daily (8 g essential amino acids).

2.1. EAAs and Exercise in CHF Subjects (Table 2). Three investigations reported that it is possible to increase exercise capacity of patients with CHF on maximal standard therapy. In one study [8] conducted on muscle-depleted patients (arm muscle area < 5° percentile) ($n = 44$), two-month EAA treatment (8 g/d) was associated with significant improvements in work performance. Six-minute walk distance (meters) increased by 22%, being significantly more than their placebo counterparts (+4%). The improvement in physical capacity was confirmed with the bicycle exercise test (+18.7% watts versus +3.5% watts in placebo). In this test, muscle aerobic metabolism, indicated by peak oxygen consumption (mL/min/kg; peak VO_2), increased by 10.4% in EAA subjects and 0.08% in the placebo group. At rest, lactate concentration (micromole/L) decreased by 25% in EAAs whereas it worsened in placebo CHF (+15%) indicating a reduction for the EAA group but an increase in the placebo group of muscle anaerobic processes. Insulin resistance (HOMA index) diminished by 16% in treated CHF, while it increased by 6.5% in the placebo group.

EAAs also improved nutritional status. Body weight increased in 80% of EAA subjects (+3 Kg; +4.1% baseline) and in 30% of placebo subjects (+0.4 Kg; +0.6%). This difference in distribution between EAA and control groups was significant ($P < 0.05$). Skeletal muscle mass, indicated by arm muscle area, increased by 11.8% in EAA subjects and 8.4% in control (n.s.). Given that, before the protocol started, the criterium chosen for considering EAA efficacy was the combination of an increase in body weight > 1 Kg and an increase of arm muscle area; the EAA associated changes in nutritional status were significantly higher in the treated compared to the placebo group.

Table 2: Changes in exercise variables observed after EAA supplementation in subjects with chronic heart failure or chronic obstructive pulmonary disease.

Exercise variables	Disease	Treatment duration	Changes (% pretreatment)	
			Placebo	EAAs
Mechanical work				
6 min WT (meters)	CHF	12 weeks	n.d.	+12 [9]
	CHF	8 weeks	+4	+22 [8]
Cycle ergometer (watts)	CHF	8 weeks	+3.5	+18.7 [8]
	CHF	4 weeks	+4	+23 [7]
Steps (number/day)	COPD	12 weeks	−7.8	+78.6 [10]
Metabolic variables during cycling				
(a) Aerobic metabolism:	CHF	8 weeks		
VO_2 peak (mL/Kg/min)			+0.08	+10.4 [7, 8]
(b) Anaerobic metabolism:	CHF	4 weeks		
VO_2 recovery time (mL/Kg/min)				
(i) At 30% postpeak decline			−14	−58 [7]
(ii) At 50% postpeak decline			−1	−49 [7]
Resting plasma lactate (μmol/L)	CHF	8 weeks	+15	−25 [8]
	COPD	12 weeks	+13	−23 [10]
Resting insulin resistance (HOMA index)	CHF	8 weeks	+6.5	−16 [8]

WT: walking test; VO_2: oxygen uptake; CHF: chronic heart failure; COPD: chronic obstructive pulmonary disease; n.d.: not determined.

The benefit of EAAs for exercise tolerance was confirmed by two investigations performed in ambulatory CHF patients. In the first study [9], elderly CHF ($n = 15$) on 3-month EAA treatment (8 g/d) improved their physical capacity at 6 min walk distance by 12% (the study had no placebo controls). In the second study [7], elderly CHF ($n = 95$) increased their exercise tolerance (bicycle exercise test) by 23% watts following 1-month EAA supplementation. In placebo subjects exercise capacity improved by 4%. The postpeak VO_2 recovery time, the length of which is an index of anaerobic metabolism, calculated at 30% postpeak VO_2 decline decreased by 58% in EAA treated CHF and by 14% in placebo controls. At 50% postpeak VO_2 decline, the VO_2 recovery time decreased by 49% in EAA subjects and by only 1% in the placebo group.

To sum up, the available studies conducted on elderly subjects with CHF consistently reported significant improvements of exercise intolerance following chronic EAA supplementation. The plausible mechanisms included improved muscle aerobic metabolism energy-producing and nutritional status and reduced insulin resistance.

2.2. EAAs and Physical Capacity in COPD Subjects (Table 2). Similar results were also observed in ambulatory COPD population ($n = 60$) [10]. These patients were sarcopenic (bioimpedance analysis measure) and were on long-term oxygen therapy. Three-month EAA supplementation (8 g/d) improved their physical capacity in terms of number of steps/day by 78.6% whereas subjects on placebo tended to have step diminution (−7.8%). EAA treatment improved muscle efficiency in aerobic energy production as suggested by the 23% reduction in resting plasma lactate levels compared with the pre-EAA treatment period. In contrast, plasma lactate increased by 13% in the placebo group. It is interesting to note that both the time courses and the changes of plasma lactate concentrations were similar for muscle-depleted CHF [8] and sarcopenic COPD. This indirectly confirms the fact that the myopathy of CHF and COPD patients shares several metabolic alterations [1].

Again, similar to the CHF study, EAAs also improved patient nutritional status. Body weight increased by an average of 5.5 kg (+10.3% baseline) of which 3.66 kg was fat free mass (FFM). In controls, body weight decreased by 3.5% but FFM increased by 0.6% (+3.8 kg). Probably in this latter group, the overtime energy intake was inadequate and/or there was an increase of body water. Indeed, in treated subjects but not controls, physical capacity and FFM improvements were associated with increases in muscle strength (handgrip) (+1.6 kg; +7.4%), serum albumin concentrations (+4.28 g/L), cognitive function (+1.62 scores at Minimental Test Examination), and quality of life perception (St. George's Respiratory Questionnaire, −2.7 scores). Like the CHF, improved muscle aerobic metabolism and nutritional status accounted for the improved exercise capacity of patients.

In addition, EAA treatment induced pluridistrict extramuscular anabolic activity in COPD subjects including that in the cerebral region, which is very sensitive to amino acid activity [27].

2.3. Other Interventional Studies Indirectly Suggest Improvement in Exercise Intolerance after EAA Supplementation. Given that muscle mass and, above all, muscle strength are good predictors of physical capacity, their increase after EAA supplementation (8 g/d) indirectly suggests improved physical performance.

Nutritional status significantly improved following EAA treatment (3 months, 8 g/d) in cachectic COPD (dual X-ray absorption measure) [11]. Patients' weight increased by 3.8 ± 2.6 kg in the EAA group and −0.1 ± 1.1 kg in the placebo one. FFM increased in 69% and 15% of EAA and control patients, respectively. FFM average increase was 1.5 ± 2.6 kg in EAA subjects and −0.1 ± 2.3 kg in controls.

In another study, EAA supplementation positively affected muscle strength in healthy elderly people [12] and in institutionalized old individuals [13]. In this study, EAAs subjects also significantly improved daily activity, mininutritional assessment score, depressive symptoms, and quality of life.

The effect of EAAs on insulin resistance [8] was confirmed in a cohort of elderly type 2 diabetics who showed long-term (60 weeks) better glucose control [14]. Interestingly, in another population of elderly diabetics, EAA supplementation improved cardiac performance as indicated by increased left ventricular ejection fraction [9]. This was also reported in healthy elderly individuals after 3 months of EAA treatment [12].

3. EAA Mechanisms Leading to Improved Exercise Intolerance Rely on the Intrinsic Biochemical EAA Activities and Skeletal (Cardiac) Muscle Changes following Chronic EAA Treatment (Table 3)

Improved muscle aerobic metabolism, prevalence of muscle anabolic processes, and reduction of insulin resistance (the latter in CHF) are the main EAA mechanisms explaining increased exercise tolerance in both CHF and COPD subjects on maximal standard therapy [7–10]. These mechanisms mutually influence each other and, in turn, rely on the intrinsic biochemical EAA properties and profound skeletal (and cardiac) muscle changes following chronic EAA supplementation documented by a number of experimental studies. Here the mechanisms will be discussed separately for more clarity.

3.1. Improvements in Muscle Aerobic Energy Production (Table 3). Both CHF and CODP patients at rest are biochemically characterized by impaired formation of muscle energy availability (adenosine three phosphate-ATP and creatine phosphate-CP compounds) [1]. Adequate cell energy availability is essential to develop muscle strength and protein turnover (protein synthesis and proteolysis).

EAAs are the substrates which can enhance muscle aerobic metabolism because they act as fuel for Krebs cycle (tricarboxylic cycle acid: TCA), promoting mitochondria biogenesis and tackling the negative effects of insulin resistance on the TCA cycle. Indeed, EAAs can enter the TCA cycle at various levels and can be used as an alternative fuel for producing energy [28], as their activities are independent of insulin [21]. EAA supplements normalized the ATP content and production rate in aged rat *gastrocnemius* muscle similar to those of adult rats [15].

Furthermore, EAAs upregulate mitochondria synthesis and increase their volume and cellular density [18, 20]. Interestingly, mitochondria biogenesis by EAAs is also upregulated in cardiac muscle [18]. In middle-aged mice, the essential branched-chain amino acid (BCAA) supplement increased mitochondrial biogenesis and sirtuin-1 expression (a member of sirtuin family linked to the life span extension), in cardiac and skeletal muscle, accompanied by enhanced physical performance [19].

All of these experimental studies suggest that chronic EAA supplementation both directly and indirectly increases muscle cell availability in high energy compounds indispensable for improving work performance and muscle strength in elderly individuals. This is independent of whether they are healthy [12] or affected by COPD [10] or CHF [9].

3.2. Improvements in Muscle Insulin Resistance (Table 3). EAA supplementation can make muscle aerobic metabolism more efficient by reducing insulin resistance [14]. EAAs directly upregulate insulin-receptor synthesis and its autophosphorylation [14]. Less insulin resistance diminishes the block of the cell pyruvate dehydrogenase complex caused by insulin resistance [8] and by circulating inflammatory cytokines in both CHF and COPD. Better activity of this enzymatic complex improves energy formation from glucose oxidation. Consequently, the lactate $\rightleftarrows$ pyruvate reaction shifts towards the right [8].

In induced diabetic rat hearts, long-term oral EAA supplementation increased mitochondrial cytochrome c oxidase and NADH-H activities and significantly shifted the ventricular myosin heavy chain pattern towards a faster phenotype [16]. Both in diabetic and in healthy mice, EAA supplements modulate the skeletal muscle redox state by improving the antioxidant defense system as shown by increased superoxide dismutase (SOD) expression and simultaneous decrease in heat shock proteins [17].

EAAs may reduce insulin resistance by lowering the circulating cytokine tumor necrosis factor alpha. This has been demonstrated in sarcopenic elderly subjects [14].

3.3. Improvements in Skeletal (and Cardiac) Muscle Anabolism. Improved nutritional status observed in muscle-depleted patients [8, 10, 11, 13] clearly indicates that even subjects with severely reduced FFM and on maximal standard therapy can improve their nutritional status when supplemented with physiological amounts of EAAs. This is not surprising given that the physiological role of EAAs is both to increase protein synthesis and to decrease protein breakdown.

Chronic supplementation of essential BCAA leucine upregulates protein synthesis in skeletal muscle, adipose tissue, and liver [21] by enhancing activity synthesis of proteins involved in mRNA translation. Particularly relevant to elderly CHF/COPD patients, leucine (and other EAAs) acts as a nutritional signaling molecule, quite independent of insulin [21]. However, amino acids regulate insulin signaling via mTOR nutrient signaling [29] and their adequate availability is indispensable for insulin to exert its anabolic activity [29]. On the other hand, reduced amino acid availability lowers

Table 3: EAA physiological activities and histological-biochemical findings from *in vivo* and human studies following chronic EAA supplementation, explaining the EAA mechanisms in improving exercise intolerance in CHF/COPD.

Mechanisms and measures in CHF/COPD	Physiological activities	Findings from experimental and human studies	
		Biochemistry	Histology
Increased aerobic metabolism WT Steps/day VO_2 peak Time VO_2 peak to baseline Resting plasma lactate levels	EAAs used as fuel for TCA cycle	↑ ATP production and ↑ cell ATP availability [15] Shift of ventricular MHC from β to α type [16] ↑ COX and $NADH^+$ activities [16] ↑ SOD [17]	↑ Mitochondria number: +310% skeletal muscle +40% myocardium [18] ↑ 28% increased mitochondria volume [18] ↑ Mitochondrial biogenesis and sirtuin 1 expression in cardiac and skeletal muscle [19] ↑ Vsar/Vtot fiber ratio [20]
Improved nutritional status FFM Body weight Muscle strength Serum albumin levels	↑ Protein synthesis [21–23] ↓ Proteolysis [24] ↑ IGF-1 expression [25]	↓ TNF alpha/IGF 1 ratio [14]	↑ 40% myofibrils of quadriceps muscle [18] ↓ Muscle fibrosis [18] ↑ Type II A fibers [20] ↑ Cross-sectional area of skeletal muscle fibers [20]
Reduced insulin resistance	Upregulated insulin-receptor synthesis and its autophosphorylation [14]	↓ HOMA index [14] ↓ Fasting insulin levels [14] ↓ Fasting blood glucose [14] ↓ TNF alpha/IGF 1 ratio [14]	

WT: walking test; VO_2: oxygen consumption; FFM: fat free mass; TCA: tricarboxylic cycle acid; ATP: adenosine triphosphate; COX: cytochrome oxidase; NADH: nicotinamide adenine dehydrogenase; SOD: superoxide dismutase; IGF-1: insulin-like growth factor-1; TNF: tumor necrosis factor; Vsar/Vtot: volume of sarcomeres/total volume ratio.

Table 4: Some similarities between the effects of exercise therapy and those following EAA supplementation.

Measures	Exercise therapy [1, 5]	EAA supplementation
Exercise capacity		
Maximal oxygen uptake	Increased	Increased
Six-minute walk distance	Increased	Increased
Anaerobic threshold	Increased	Increased*
Maximal incremental exercise duration	Increased	Increased
Resting ejection fraction	Unchanged or slight increase	Slight, significant increase
Resting lactate production	n.d.	Reduced
Muscle structure and function		
Muscle cross-sectional area	Increased	Increased^
Muscle fiber size	Increased	Increased^
Number of type I muscle fibers	Increased	Increased^
Mitochondrial numbers	Increased	Increased^
Mitochondrial *cristae* density	Increased	Increased^
Muscle dynamic strength	Increased	Increased^
Muscle fatigability	Reduced	Reduced^

*Inferred from improved recovery time of maximal oxygen uptake to baseline value [26]; ^in animals [15–20].
n.d.: not determined.

mTOR activation even with improved insulin signaling. This was documented in healthy and in insulin resistant human skeletal muscle [29]. Even small doses of exogenous EAAs can stimulate muscle protein metabolism [22].

Another pathway by which EAAs induce protein synthesis is by upregulating the hepatic production of the anabolic hormone insulin-like growth factor 1 (IGF-1). This hormone is dependent on EAA availability in the blood and acts only when there is adequate EAA availability. A study reported that 7.5 g EAAs (an amount very close to that used in the studies on EAAs and exercise intolerance) increased IGF-1 levels [25].

The fact that EAAs stimulate muscle protein metabolism in healthy elderly subjects [23] is of practical importance for elderly CHF/COPD individuals needing nutritional supplementation. This is because pharmaceutical formula reflecting the composition of a standard meal or high protein diet fails to increase muscle mass and strength as well as protein synthesis in the elderly [22]. EAA can induce net protein synthesis by reducing muscle protein breakdown. In human studies, the efficiency of protein use is due to the reduced sensitivity of proteolysis rather than any changes in protein synthesis [24].

Particularly interesting is the effect of leucine on protein metabolism in the myocardium. Leucine is the only amino acid that can inhibit protein degradation in the myocardium [30] and its inhibitory effect is mediated by extracellular leucine [30]. On the other hand, BCAA transamination in the heart is 3 times higher than in peripheral skeletal muscles [30]. The possible effect of EAAs on myocardium could explain the improved left ventricular dysfunction in healthy elderly individuals [9] and the faster exercise VO_2 recovery time in elderly subjects with CHF [7, 26].

In synthesis, experimental and human investigations have shown that chronic EAAs supplementation may increase and make the muscle aerobic energy production more efficient and increase skeletal muscle mass/strength and mitochondria number and function. It has been reported [31] that 75% of the body's nitrogen requirement is supplied by leucine, isoleucine, valine, threonine, and lysine, all amino acids contained in the nutritional mixture used here. It is interesting to note that the mixture's amino acids were formulated in reciprocal stoichiometric ratios, specifically both to match metabolism energy needs and to maintain protein synthesis [31].

Table 5: Amount of some types of high quality protein foods containing the same amount of essential amino acids (8 g) as the pharmaceutical formula used in the studies on exercise intolerance.

Food	g
Lean beef meat	97
Chicken (breast)	74
Mortadella	131
Ham	79
Cheese (average of 6 types)	105
Canned tuna fish	74
Trout	153
Codfish	97
Eggs	138
Whole milk	480

The histological and biochemical changes associated with EAA supplementation overlap those induced by ET Rehab (Table 4). It is also interesting to note that the EAA dose (8 g/d) is the amount contained in higher protein quality foods usually consumed by both healthy and ill subjects (Table 5) [32].

To understand the importance of the reciprocal stoichiometric ratios of the amino acids in the mixture [31], very recently an experimental study [33] reported that excessive

neurotransmitter serotonin derived from tryptophan and noradrenergic activity from tyrosine may negatively impact the physical activity status particularly in sedentary animals. Moreover excessive tryptophan consumption can reduce physical activity by inducing central fatigue [34]. Interestingly, excessive tryptophan may also limit the blood-brain-barrier passage of leucine, isoleucine, valine, and tyrosine, therefore reducing their availability within the brain structure [35] and contributing to further limiting physical capacity.

4. Conclusions

Elderly patients with CHF/COPD when supplemented with EAAs not only may improve exercise intolerance but also can achieve some prognostic outcomes typical of ET Rehab. Obviously, EAAs does not substitute comprehensive Rehab but may be very useful for elderly subjects with CHF/COPD who, for various reasons, cannot undergo ET Rehab.

Conflict of Interests

The authors have no conflict of interests regarding the publication of this paper.

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