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# EFFECT OF AMINO ACID SUPPLEMENTATION ON SKELETAL MUSCLE DURING ACUTE IMMOBILIZATION IN HOSPITALIZED ELDERLY SUBJECTS: POSSIBLE IMPACT ON MITOCHONDRIA


PhD student:
**Dr. Aurelio Lo Buglio**

Tutor:
**Prof. Gianluigi Vendemiale**

VENDEMIALE
GIANLUIGI
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10:46:33 UTC

PhD Course coordinator:
**Prof. Alessandro Matteo Del Nobile**


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*A mia moglie*
*con me sempre e incondizionatamente*

# Sommario

# *Abstract*

Objective: Older patients are frequently subjected to prolonged hospitalization and extended bed rest, with a negative effect on physical activity and caloric intake. This results in a consistent loss of muscle mass and function, which is associated with functional decline and high mortality. Furthermore, acute muscle disuse can precipitate sarcopenia, defined as the age-dependent loss of muscle mass and function. The aim of this study was to investigate the effect of oral amino acid (AA) supplementation in acute immobilization. We also aimed to characterize the effect and mechanism of the AA mixture in a rodent model of skeletal muscle atrophy, focusing on mitochondrial function. Methods: In the human study, hospitalized older patients (69-87) were included in the control group (n = 50) or were administered 25 g of AA mixture (n = 44) twice daily throughout 7 d of low mobility. We collected data related to length of stay as primary outcome measure. In-hospital mortality, 90-d post-discharge mortality, 90-d post-discharge rehospitalization, and falls also were considered. Moreover, variations of anthropometric measures, body composition and muscle architecture/strength, circulating interleukins, and oxidative stress markers between the beginning and the end of the supplementation period were analyzed as secondary outcomes. In the animal study, C57/Bl6 mice underwent immobilization of one hindlimb by stapling the foot exploiting normal dorsotibial flexion. Age-matched mice that never had their hindlimbs immobilized were used as controls. Sub-groups of mice subjected to the immobilization procedure were administered the AA mixture in drinking water (I+A) and were compared to a placebo group (I+P). After 10 days, muscle function was studied by both endurance running and grip strength tests. Tibialis anterior (TA) muscles were excised and used for mitochondrial isolation.

Results: Similar values were reported between the two groups regarding age, body weight, and body mass index. Although no difference in terms of in-hospital, 90-d post-discharge, or overall mortality rate was observed between the two groups, a reduction in length of stay, 90-d post-discharge hospitalization, and falls was observed in the AA supplementation group rather than in controls. Furthermore, the AA mixture limited muscle architecture/strength impairment and circulating oxidative stress, which occurred during hospitalization-related bed rest. The latter data was associated with increased circulating levels of anti-inflammatory cytokines interleukin-4 and -10. In animals, hindlimb immobilization reduced maximal running times and distances, along with limb grip strength; however, the extent of reduction was lower in I+A than I+P mice. Immobilization resulted in TA atrophy, characterized by a reduction in both wet weight and TA/body weight ratio. Interestingly, these alterations were slightly observed in mice treated with the AA mixture. The mitochondrial yield from TA of I+P mice was lower than controls; of note, the mitochondrial yield from TA of I+A animals was similar to controls. AA mixture administration also preserved mitochondrial bioenergetics and oxidative damage in TA muscle, which was disrupted in I+P mice with respect to controls. Conclusions: These results suggest that the AA mixture limits several alterations associated with low mobility in older hospitalized patients, such as length of stay, 90-d post-discharge hospitalization, and falls, preventing the loss of muscle function, as well as the increase of circulating interleukins and oxidative stress markers. Furthermore, this study demonstrates that the AA mixture prevents loss of muscle mass and function in skeletal muscle atrophy by protecting mitochondria. Other than providing a further link between mitochondria and proteostatic maintenance to muscle atrophy, these results encourage further research aimed at targeting mitochondria to treat sarcopenia.

**Abbreviation:**

AA: amino acid

AEs: adverse events

CRP: C-reactive protein

CSA: cross-sectional area

FL: fascicle length

IL: interleukin

IL-6: interleukin 6

LOS: length of stay

MNA: Mini Nutritional Assessment

MPTP: mitochondrial membrane permeability transition pore (MPTP)

MT: muscle thickness

PA: pennation angle

PASE: Physical Activity Scale for the Elderly

SD: standard deviation

SM: skeletal muscle

SMM: skeletal muscle mass

SS: subsarcolemmal

# *1. Introduction*

Skeletal muscle mass (SMM) plays a central role in maintaining whole-body homeostasis and global health [1]. In fact, skeletal muscle (SM) exerts important effects on body metabolism, thermoregulation, prevention of osteoporosis and bone fractures, as well as regulation of immune system [2,3]. Also, several studies highlighted the endocrine and paracrine action of SM on systems like insulin receptor pathway, with impact on several organs such as the liver, adipose tissue, pancreas, bone tissue, and the cardiovascular system [4,5]. SM plays a key role on regulation of inflammation status through the release of various cytokines. Particularly, conditions such as sarcopenia, defined by the age-related reduction of both strength and muscle performance, are associated with increased levels of inflammatory mediators including C-reactive protein (CRP) and interleukin 6 (IL-6), which induce a state of chronic systemic inflammation [5,6]. Furthermore, SM represents a vital reserve of glucose and amino acids particularly during acute conditions as sepsis or prolonged bed rest [7].

Both aging and malnutrition exert an important effect on muscle homeostasis, inducing several changes in microstructure and metabolism [8–10].

During aging, the progressive loss of function in skeletal muscle leads to an impairment in physical performance, with higher risk of poor clinical outcomes such as falls, disability, hospitalization and mortality [1]. Likewise, malnutrition exerts several structural and metabolic changes related to higher risk of low-grade chronic inflammation, disability, hospitalization and mortality [11]. In elderly hospitalized patients, the association between low muscle mass and malnutrition leads to a negative impact on treatment response, functional recovery, falls, hospital length-of-stay and costs, and quality of life [12–14]. In this scenario, it is essential to study muscle metabolism alterations in order to plan specific interventions aimed at preventing and treating muscle mass loss, especially in elderly patients [15].

## 1.1 Muscle architecture and physical performance

The sarcomere is the functional unit of skeletal muscle, able to generate both force and rapid movements [16]. Force generation depends on the size and type of fiber which composes the skeletal muscle [16]. Particularly, two main types of muscle fibres are recognized: slow-twitch (Type I) and fast-twitch (Type II). Taking into account the expression pattern of myosin heavy chains isoforms, type II fiber can be further classified in Type IIA, IIB and IIX [16,17].

Muscle performance is strictly dependent by muscle architecture, which in turn can be modified by training, diet, or oral supplement regimen, with positive effects on physical performance [18–20]. Muscle architecture can be easily assessed by using *in vivo* real time ultrasonography [21,22].

### 1.1.1 Pennation angle, fascicle length and muscle thickness

Muscle architecture is represented by muscle thickness (MT), fascicle length (FL) and pennation angle (PA) [23,24]. MT is defined by distance between the superficial and deep aponeuroses (Figure 1). FL is measured considering the length of the fascicle across the deep and superficial aponeurosis while PA is represented by the angle existing between fascicle and deep aponeurosis (Figure 1) [25]. Also, one other parameter can be assessed using extended field of view ultrasound (EFOV) is the cross-sectional area (CSA) can be defined as either anatomical CSA (ASCA) or physiological CSA (PCSA) [24,26]. Studies demonstrated how the maximum force generated by pennate muscle (as skeletal muscle) depends on its PCSA rather than its ACSA [27].

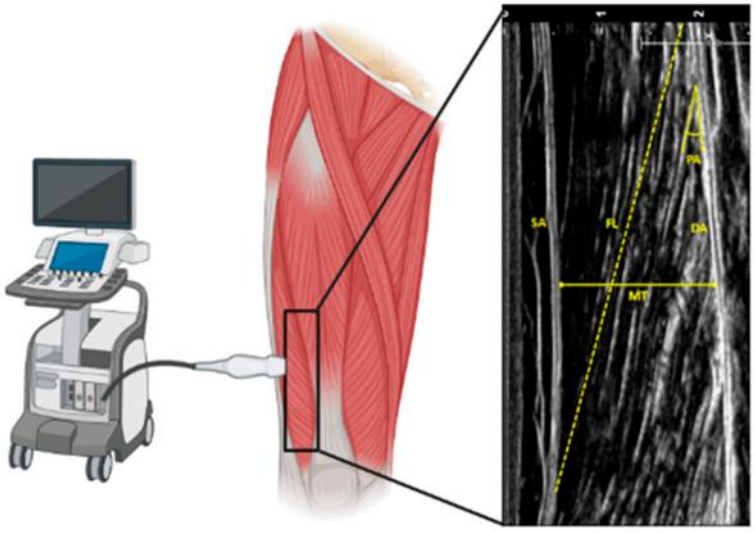


*Figure 1. Schematic representation of architecture of vastus lateralis muscle through ultrasonography approach. SA: superficial aponeurosis; DA: deep aponeurosis; MT: muscle thickness; FL: fascicle length; PA: pennation angle (From Bellanti et al., Biology 2021).*

### 1.1.2 Role of muscle architecture on physical performance

PA is one of the most important parameters of muscle architecture [28]. PA derives from the arrangement pattern of muscle fibers with respect to the force generation axis by the muscle itself, which is an important factor in determining muscle performance [28].

Greater pennation angle is associated with greater muscle ability to produce force. In fact, pennation angle size correlates with more contractile tissue to attach to a given area of tendon, or aponeurosis, increasing the PCSA [19]. Also, PA was found correlates with muscle thickness [29].

Zhu et al. (2019) found positive association between gastrocnemius medialis PA with physical performance including both gait speed and SPPB in women but not in men [21].

FL affect importantly force-velocity and force-length relationship [24].

## 1.2 Muscle Aging

### 1.2.1 Effect of aging on muscle metabolism

Age-dependent decline of SM quality is due to the reprogramming of tissue metabolism leading to impaired glucose, fat and protein uptake and utilization, as well as energy production [25,30]. Alterations in SM metabolism in aging show differences between males and females, and are affected by sex hormones [25,31]. During aging, SM loss and increase in visceral adipose tissue are higher in males, while females present with lower capillarization of type II glycolytic myofibers [25,32].

Skeletal muscle loss is mainly due to the disruption in the regulation of skeletal muscle protein turnover, leading to a negative balance between muscle protein synthesis and muscle protein breakdown [33]. A blunted protein synthetic response to anabolic stimuli in elderly is named anabolic resistance [34]. This evidence is supported by a study of Cuthbertson et al. (2005), who compared the myofibrillar and sarcoplasmic protein synthesis rates in the VL muscles of young and older men, in response to a bolus of amino acids, reporting about 1.5-fold higher synthesis rates in the young [33,35].

Wall et al. (2015) reported a 16% lower skeletal muscle protein response to dietary protein intake in the VL of older adults as compared with younger subjects [33,36]. Also, an increase in protein breakdown with age was reported. Raue et al. (2007) showed higher expression of atrogin-1 mRNA (a ubiquitin proteasome-related gene) after resistance exercise up to 2.5-fold in older women compared to young women [33,37]. The ubiquitin-proteasome pathway is accountable for the breakdown of muscle protein synthesis and probably contributes to muscle protein breakdown during ageing [33,38,39].

### 1.2.2 Effect of aging on muscle architecture

Advancing age is associated with a progressive reduction in muscle mass, mostly prevalence over the seventh decade and beyond [8]. 0.5% to 1.0% annual loss of muscle mass after 70 years of age has been reported [40]. Recently, the median decline in muscle mass during lifespan was reported as 0.37% per year in women and 0.47% per year in men [33]. Aging significantly affects skeletal muscle architecture and performance [27]. These changes involve both quantity (volume) and quality (architecture) of skeletal muscle tissue [41]. Narici et al. demonstrated several changes in muscle architecture in old compared to young subjects (Table 1) [27]. Indeed, old patients PA, FL, muscle volume, $ACSA_{max}$ and PCSA were all significantly reduced with respect to young group [27]. Interestingly, this architectural modification occurs in young and old subjects with similar active lifestyle and daily energy expenditures, so that these changes are mostly attributable to the effect of aging *per se* rather than disuse [27]. CSA of quadriceps muscle was found reduced by 25-35% in old people compared to young, with particular involvement of the vastus lateralis (VL) and lumbar multifidus (LM) muscles [42]. The muscle mass reduction with aging is strongly associated with reduced number and size of muscle fibers [41]. In post-mortem studies, Lexell et al. found a 39% fiber loss in VL associated with old age, with selective atrophy of type II fibers and maintenance of type I fibers [42,43]. Also, Lexell et al. reported a smaller VL muscle size in the elderly, with a 25% reduction in the total number of muscle fibres, suggesting that muscle atrophy with advanced age could be largely due to the loss of muscle fibres [44]. Kubo K. et al. found significant lower mean values of PA and MT in VL of old subjects as compared to young ones. However, no significant differences were found in medial gastrocnemius (MG) and long head of triceps brachii (TB) [45].

Muscle atrophy is characterized by decrease in fiber size, while sarcopenia causes a reduction in both size and number of fibers [46]. The loss of muscle mass observed in sarcopenia as well as during disuse atrophy is associated to the decrease in FL and PA [46,47]. These changes justify about half of the loss in maximum force and shortening velocity [46,48].

*Table 1. Architectural changes in gastrocnemius medialis according to age. Values are means ± SD; n = 14 for the young (27–42 years) group and n = 16 for the elderly (70–81 years) group. $ACSA_{max}$, maximum anatomic cross-sectional area; Vol, muscle volume; FL, fascicle length; PA, pennation angle; PCSA, physiological cross-sectional area.*

| | $ACSA_{max}$, $cm^2$ | Vol, $cm^3$ | FL,cm | PA, ° | PCSA (Vol/FL) , $cm^2$ |
|---|---|---|---|---|---|
| Elderly | 14.0 ± 3.6 | 208.7 ± 48.5 | 4.29 ± 0.67 | 23.6 ± 3.0 | 50.1 ± 12.6 |
| Young | 17.4 ± 2.8 | 279.3 ± 59.3 | 4.78 ± 0.55 | 27.2 ± 4.3 | 59.1 ± 14.4 |
| Difference | 19.1% | 25.3% | 10.2% | 13.2% | 15.2% |
| *P* values (*t*-test) | 0.005 | 0.001 | 0.01 | 0.01 | 0.05 |

### 1.2.3 Effect of aging on muscle performance

Aging negatively affects muscle performance such as power, strength and endurance [49]. After the age of 40, a more rapid loss of muscle power than strength is observed both in men than in women [49]. This impairment in muscle power leads to loss of short-term muscle performance, a common feature among older people that directly correlates with risk of falls [49]. Also, the maximal isometric forces was found 25% lower in old men compared to young [50]. Decrease in muscle mass partially accounts for loss of muscle power in old age [51]. Several other changes were observed, such as fewer fast-twitch motor units and lower maximal shortening velocity, in aged with respect to young fibers [49]. By the age of 80, about 30–40% reduction in skeletal muscle fibers and 40% decrease in strength are reported [42]. Several studies showed reduced force generation in senescent muscle both in men than in women [49]. Particularly, Auyeung TW et al. demonstrated in an old Chinese population a faster loss in max grip strength than in muscle mass (Figure 2) [52]. A rapid rate of strength loss is evident beyond the age of 60 years, with a decrease

at a rate of nearly 15% per decade [49]. After a 12-year period of follow-up, Frontera et al. found a decrease of 2.5% per year in the leg strength of older men [49,53]. In contrast to strength and power, where the evidence overwhelmingly confirms a significant aging effect, the literature regarding senescence-induced alterations in muscle endurance is unclear. While some studies showed decreased muscle endurance among aged people, others reported no difference in the fatigability of both aged and young muscle [49].

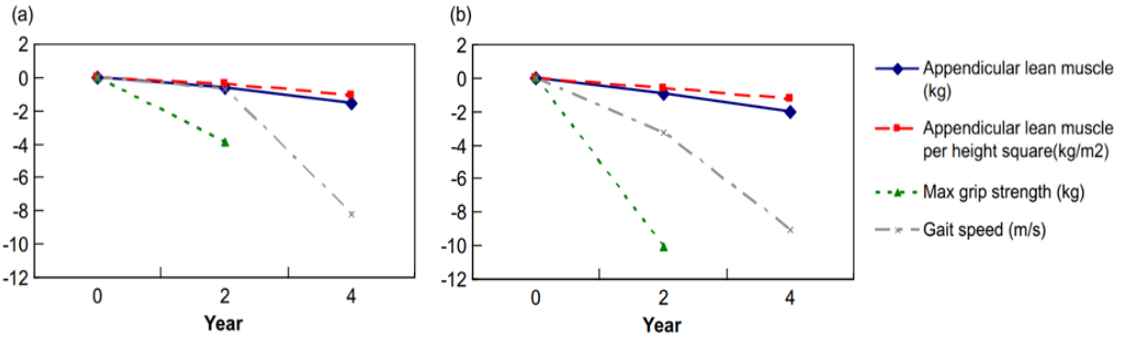


*Figure 2. Muscle performance reduction according to follow up years in a) men and b) women (modified from Auyeung TW et al., 2014).*

## 1.2.4 Role of mitochondria in muscle aging

Mitochondria are important cellular organelles involved in the energy production by both aerobic and anaerobic respiration and oxidative phosphorylation. Cross-sectional evidence from 74 healthy men and women aged 18–90 years showed that age is inversely related with VL mitochondrial DNA (- 0.62, $P < 0.001$) and with mRNA transcription ($r = - 0.48/- 0.54$, $P < 0.001$) [33,54]. This decline leads to lower mitochondrial muscle protein synthesis rates in older adults [33,55]. Mitochondrial dysfunction plays a determinant role in age-related loss of SM mass and strength. Maintenance of mitochondrial integrity is a determinant factor to preserve proteostasis in skeletal muscle. Several animal and human studies showed mitochondrial impairment in sarcopenia [25].

Dysfunctional mitochondria are associated with both ATP depletion and reactive oxygen and nitrogen species (ROS/RNS) excess, with the resultant activation of harmful cellular pathways. A decrease in mitochondrial mass, activity of tricarboxylic acid cycle enzymes, as well as $O_2$ consumption and ATP synthesis is observed in aged SM tissue [25,56]. Changes in function, dynamics, and biogenesis/mitophagy could explain in part changes in oxidative capacity and content of SM mitochondria. Moreover, mitochondrial dysfunction induces the activation of apoptosis, potentially worsening skeletal muscle quality [25,57].

Several mitochondrial functions are compromised in old in comparison to young SM, including the activity of metabolic enzymes and oxidative phosphorylation (OXPHOS) complexes (i.e., citrate synthase and cytochrome c oxidase), respiration, protein synthesis, and ATP production rate (mostly dependent on an increase in mitochondrial uncoupling) [25,54,58–62]. However, the type of physical activity may play an important role for the preservation of mitochondrial function in old SM [25,63–65].

Comparison analysis of transcriptome between young and old SM from animal models and humans reveals a decrease in mitochondrial gene expression as an effect of age, even though proteomic studies concluded with controversial results, suggesting the need for further research [25,66]. Genes related to mitochondrial structure and function are downregulated in older women compared to men, indicating that females may be more predisposed to SM impairment with age [25,67].

The reduced mitochondrial content in aged SM may be also related to lower PGC-1α gene and protein expression, which is reported both in slow- and in fast-twitch fibers [25,62,68,69]. However, further investigation is needed to understand the molecular mechanisms that underpin age-related modifications in SM. Apart from PGC-1α, many studies show different results in the levels of its downstream transcription factor Tfam in

old SM [25,70–72]. Changes related to mitochondrial content and function in old SM may also be linked to reduced amount, increased mutations, deletions, and rearrangements of mitochondrial DNA (mtDNA) [25,73,74]. The protein level of skeletal muscle PIM is similar between young and old animals, suggesting that molecular chaperones and translocases are not involved in the mitochondrial impairment of aged SM [75,76]. An age-dependent increase in SM fibers presenting with alterations of mitochondrial enzymes due to mtDNA deletion mutations is reported both in rhesus monkeys presenting with early-stage sarcopenia and in humans [25,77,78].

The sedentary lifestyle of aging is associated with mitochondrial dysfunction and oxidative damage in human skeletal muscle, but physical activity in old age may prevent mitochondrial-dependent sarcopenia [25,79,80]. In a transgenic mouse model, mtDNA mutations were determinant for ETC assembly and function, with consequent impaired mitochondrial bioenergetics and loss of ATP homeostasis, enhancing SM apoptosis and sarcopenia [25,81]. Defective mitochondrial ETC was also described in spinal motor neurons from older humans, contributing to fiber denervation and loss of skeletal muscle quality [25,82]. Of note, denervation of single skeletal muscle fibers induces the overproduction of mitochondrial ROS/RNS even in neighbouring innervated fibers, suggesting a complementary mechanism in the pathogenesis of sarcopenia [25,83].

Morphological studies in aged skeletal muscle show giant mitochondria with disrupted cristae [25,69]. Moreover, subsarcolemmal (SS) mitochondria appear fragmented and disposed in a thin layer, while IMF mitochondria are less reticular compared with young muscle [25,84]. Of note, a reduction in IMF size was described in older adults; this was particularly evident in women rather than men, even though sex did not affect the difference in whole muscle size [25,85]. Altered morphology in old skeletal muscle mitochondria may be the consequence of impaired mitochondrial dynamics, with a

disbalance in favour of fission rather than fusion [25,68]. Mutations in mtDNA may lead to dysregulation of mitochondrial dynamics in sarcopenia, as suggested by results from old mice expressing a defective mtDNA polymerase gamma, which showed higher mitochondrial fission in skeletal muscle [25,86].

However, a comparison between young versus old mice revealed a higher mitochondrial fusion index (Mfn2-to-Drp1 ratio) in aged skeletal muscle [25,87]. A shift toward mitochondrial fusion rather than fission was also reported in skeletal muscle of very old hip-fractured patients [25,88]. A knock-out of fusion-related Mfn1/2 in skeletal muscle showed higher mtDNA mutations and tissue atrophy [25,89]. Nevertheless, skeletal muscle atrophy and degeneration were also reported from the genetic deletion of fission-related Drp1 [25,90]. Thus, the actual changes of mitochondrial dynamics in skeletal muscle and their involvement in sarcopenia need to be clarified as well as the potential impact of age-associated alteration in mitochondrial dynamics of motor neurons.

Impaired mitochondrial biogenesis is crucial to determine the loss of skeletal muscle quality and sarcopenia. Both mitochondrial homeostasis and OXPHOS in skeletal muscle are regulated by PGC-1α, the master regulator of mitochondrial biogenesis, which is stimulated by contractile activity and induces fiber-type switching from glycolytic toward oxidative fibers [25,91]. Nevertheless, age-related reduction in mitochondrial biogenesis may be supported by the impaired response of PGC-1α to exercise training [25,92]. The potential role of PGC-1α-induced mitochondrial biogenesis as a therapeutic target for sarcopenia is suggested by several preclinical studies, which used agents such as ghrelin, trimetazidine, exerkine, and 5,7-dimethoxyflavone to reverse sarcopenia [25,93–96]. Such

positive effects on metabolism and proteostasis pave the way for future clinical trials. PGC-1α overexpression in skeletal muscle inhibits mitophagy, which is enhanced during

aging [25,97]. A cross-sectional study performed in physically inactive frail older women described the downregulation of genes related to mitophagy [25,98].

The reduced capacity of skeletal muscle cells to remove damaged organelles could be another cause of mitochondrial alteration in aging. Studies performed on rodent models describe controversial results on mitophagy modulators in aged skeletal muscle [25,77,97,98]. A further investigation reported data indicative of increased mitophagy but lysosomal dysfunction in skeletal muscle from old mice, suggesting that lysosomal dysfunction may cause accumulation of disrupted mitochondria [25,99]. Nevertheless, further investigation on the role of mitophagy in old skeletal muscle is needed in humans. Mitophagy and its related modulatory proteins are enhanced in rodent models of sarcopenia [25,100,101]. The deletion of the mitofusin 2 gene in skeletal muscle impairs autophagy and activates an adaptive mitochondrial quality control pathway in mice [25,102]. A mechanism complementary to mitophagy includes the delivery of mtDNA and mitochondrial components through extracellular vesicles (EVs), named mitochondrial-derived vesicles (MEVs) [25,103]. Of note, older adults affected by physical frailty and sarcopenia presented with higher circulating EVs with respect to age-matched controls, but mitochondrial components were lower, suggesting an alteration in the trafficking of MEVs in old skeletal muscle [25,104]. Divergent reports on mitophagy in sarcopenia suggest further investigations on this topic since this aspect could be an interesting therapeutic target. Indeed, the overexpression of the mitophagy regulator Parkin in mouse skeletal muscle attenuates sarcopenia by increasing mitochondrial content and enzymatic activities [25,105].

## 1.3 Role of immobilization on muscle architecture and performance

Muscle architecture undergoes several change during hypomobility as prolonged bed rest (

Figure 3) [46]. In a recent work, we investigated the relationship between immobilization, nutritional status, and muscle architecture in hospitalized elderly patients [10]. Figure 4 represents the changes in body composition (fat mass, free-fat mass, muscle mass), muscle architecture (muscle thickness and pennation angle), and muscle strength (hand-grip) parameters during the first 7 days of hospitalization, stratifying patients according to the nutritional status. Of note, a significant impact on early hospitalization was observed for the pennation angle ($F(1, 130) = 4.693$, $p = 0.0321$), irrespective of the nutritional status. No other changes dependent on the hospitalization, the nutritional status, or the interaction of these factors were observed for the remaining parameters [10].

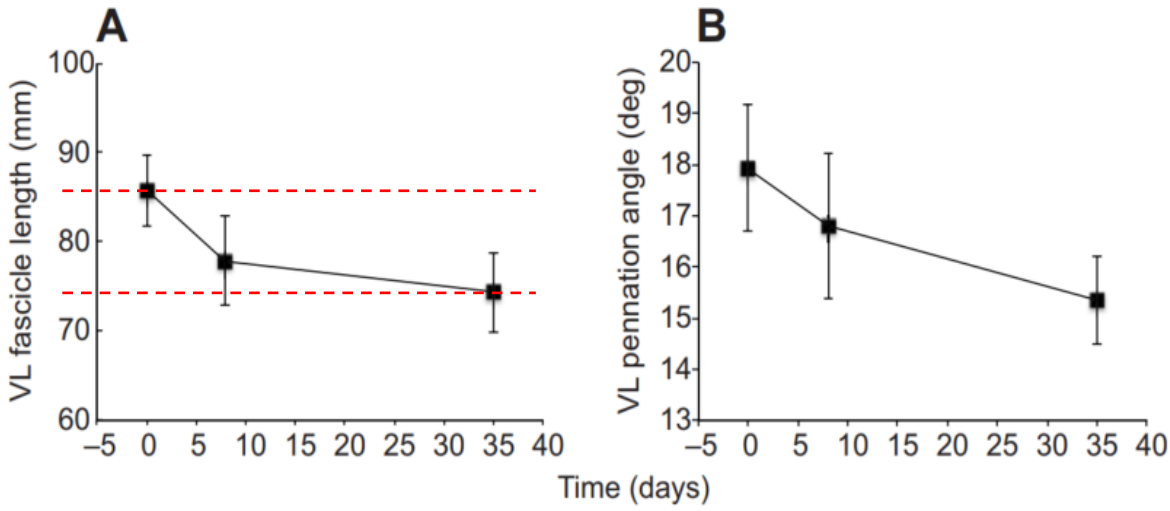


*Figure 3. The effect of 35-day of bed immobilization in healthy men on (A) fascicle length and (B) pennation angle. (Modified from Narici et al., Journal of Experimental Biology 2016)*

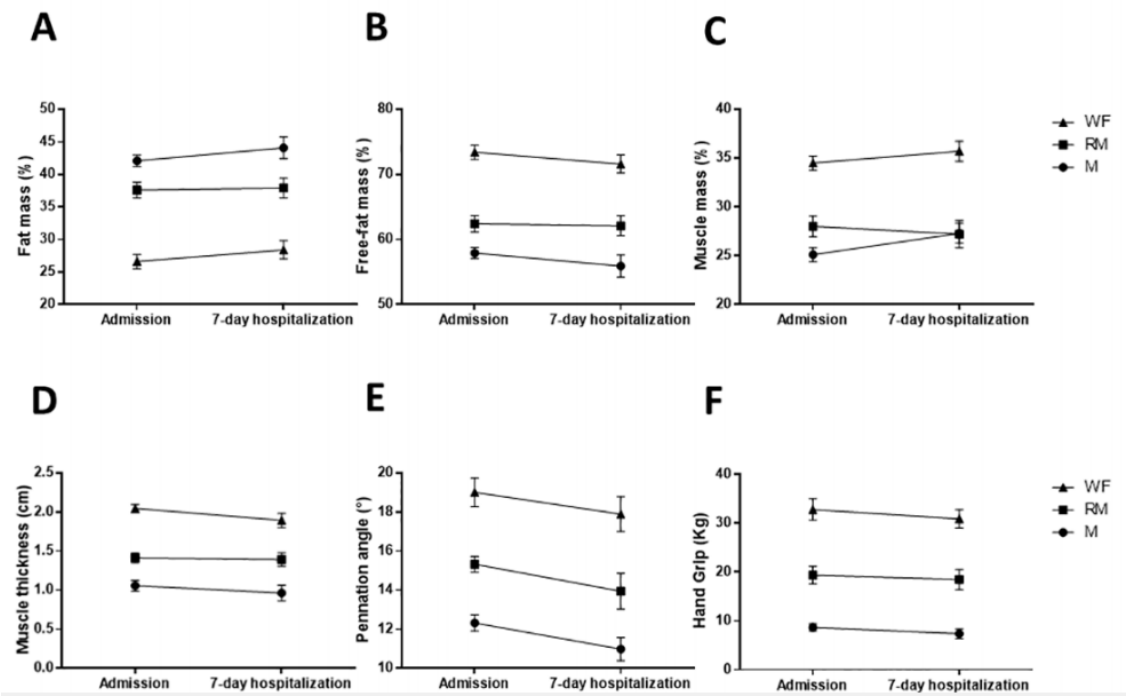


*Figure 4. Variations of bioimpedentiometry (panels A-C), muscle architecture (muscle thickness – panel D – and pennation angle – panel E), and muscle strength (hand-grip, panel F) in patients stratified according to the nutritional status, between in-hospital admission and 7-day hospitalization. Statistical differences were assessed by two-way ANOVA (Modified from Lo Buglio A. et al., J Nutr Heal Aging 2020)*

# 1.4 Role of malnutrition on muscle architecture and performance

In a recent work , we analyzed the relationship among muscle architecture and malnutrition according to the nutritional status (evaluated trough the Mini Nutritional Assessment, MNA) [10]. Data are represented in Figure 5.

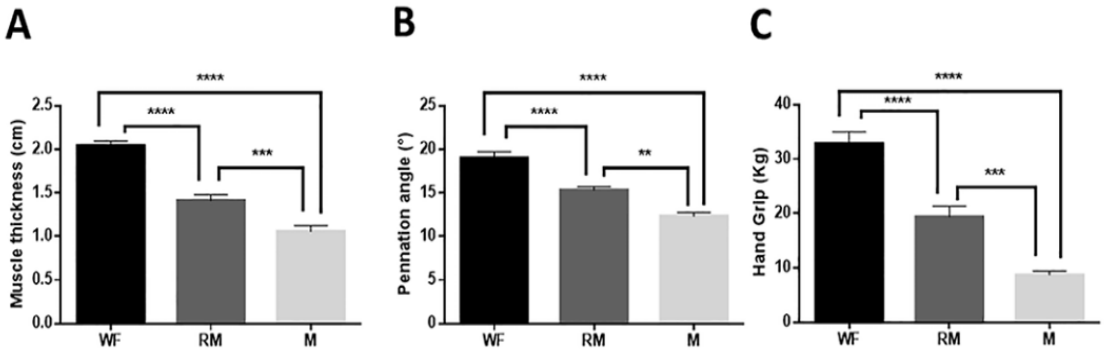


*Figure 5. Muscle architecture (thickness – panel A – and pennation angle – panel B) and strength (hand-grip – panel C) in patients at baseline, according to study groups. WF: well fed; RM: risk of malnutrition; M: malnourished. Statistical differences were assessed by one-way analysis of variance (modified from Lo Buglio A. et al., 2020).*

Compared to well-fed patients, MT and PA were significantly lower in patients with risk of malnutrition, and further in malnourished rather than risk of malnutrition. A similar trend was observed for muscle strength [10]. Also, the analysis of the relationship between the nutritional status and muscle architecture/strength parameters revealed a significant positive correlation between the MNA score and pennation angle ($\rho = 0.716$, $p < 0.001$; Figure 6A), and handgrip strength ($\rho = 0.769$, $p < 0.001$; Figure 6B)[10].

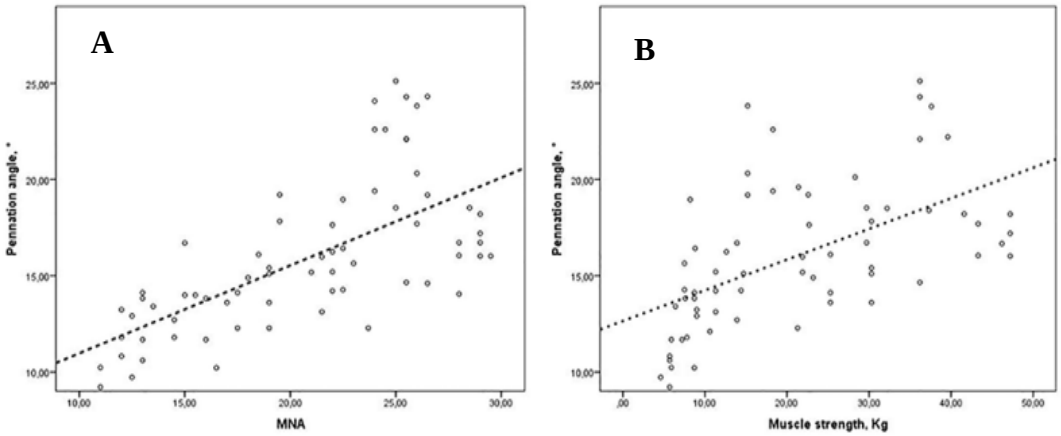


*Figure 6. Correlation between the mini-nutritional analysis (MNA) score and pennation angle (panel A) or muscle strength (panel B). (Modified from Lo Buglio A. et Al., 2020)*

A multivariate analysis was performed to verify the most important factors associated with pennation angle and showed that the presence of malnutrition was the only independent predictor (OR: 1.42, $p < 0.001$) [10].

## 1.5 Effect of oral supplementation on muscle aging process

Several studies showed that protein intake decreases in frail elders, and that even healthy older adults may need additional protein than what was recommended by the most recent protein Dietary Recommended Intakes (DRI) for persons 55 and older [106]. Previous studies have demonstrated that amino acid availability is crucial in the regulation of muscle protein metabolism [106]. Protein and BCAA intake are associated with variations in circulating BCAAs and influence metabolic health in humans and rodents [107]. Specifically, high plasmatic amino acid concentration acutely stimulates muscle protein synthesis by increasing the amino acid transport into muscle cells [106].

Solerte SB et al., showed a significant increase in whole-body lean mass sarcopenic patients after 8 and 16 months of AA supplements, reaching the normal values found in age-matched non-sarcopenic healthy controls at the end of the study (Figure 7) [108].

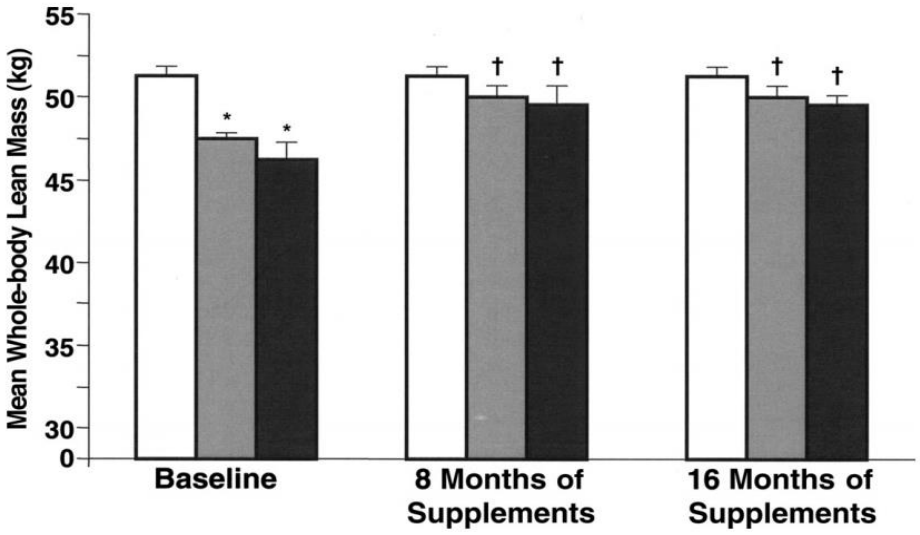


*Figure 7. Mean values of whole-body lean mass evaluated by dual x-ray absorptiometry (DEXA) in elderly subjects with sarcopenia in group A (light gray bars) and group B (dark gray bars) during supplementation with amino acids (AAs) compared with nonsarcopenic matched controls (white bars). *p <0.01 vs nonsarcopenic subjects; †p <0.01 vs baseline (Modified from Solerte SB et al., 2008).*

Previous data showed that aging skeletal muscle may progressively lose its ability to respond to anabolic stimuli, including insulin, and to a lesser extent amino acids, especially within the lower physiological concentration range [106]. However, old muscle can activate an anabolic response to the intake of protein/amino acid; thus, it is conceivable to establish targeted nutritional interventions to restore muscle mass in the elderly [106]. At rest, BCAAs, in particular leucine, exert an anabolic effect by increasing protein synthesis and/or a decreasing the rate of protein degradation, inducing a positive net muscle protein balance [109]. In particular, leucine is involved in the direct phosphorylation and activation of mTOR in SM, further enhancing the protein synthetic response [109].

# *2. Aims*

Older patients are frequently subjected to prolonged hospitalization and extended bed rest, with a negative effect on physical activity and caloric intake. This results in a consistent loss of muscle mass and function, which is associated with functional decline and high mortality. Furthermore, acute muscle disuse can precipitate sarcopenia, defined as the age-dependent loss of muscle mass and function.

The aim of this study was to investigate the effect of oral amino acid (AA) supplementation in acute immobilization. We also aimed to characterize the effect and mechanism of the AA mixture in a rodent model of skeletal muscle atrophy, focusing on mitochondrial function.

# *3. Methods*

# 3.1 Human Study

### *3.1.1 Study design and participants*

We consecutively recruited older people (aged ≥ 65 years) admitted to the University Internal Medicine clinic of the "Policlinico Riuniti" in Foggia (Italy), and subjected to a minimum of 7 day low mobility during hospitalization. This investigation was designed as a pilot study considering the difficult setting, the limited scale, and the multiple outcome parameters. Patients who were moderately active during the previous 7 days before hospitalization, according to the Physical Activity Scale for the Elderly (PASE), were enrolled. PASE is a physical activity questionnaire widely used to assess physical activity [110]. Low mobility was defined as lying in bed during the observation period [60]. Exclusion criteria were:

- age < 65 years
- active cancer
- chronic bedridden conditions
- physical disability
- sever neuromuscular disease
- dysphagia
- cognitive decline (MMSE < 9)
- use of drugs affecting body composition (such as glucocorticoids, statins, active vitamin D metabolites, anabolic steroids, selective estrogen receptor modulators) or redox balance and use of other vitamins
- daily alcohol intake > 40 g

### *3.1.2 Amino acid oral supplementation*

The amino acid (AA) supplementation (Aminoglutam, provided by Errekappa Euroterapici S.P.A., Milano, Italy), was administered within 48h of admission at a dosage of 25 g, delivered in 100 mL drinking water twice daily throughout 7 days of low mobility. The composition and nutritional values of AA drink are reported in Table 2 and 3, respectively.

Compliance and adverse events (AEs) were assessed daily by verbal questionnaire; we used the Italian versions of the Morisky Medication Adherence Scale and the Generic Assessment of Side Effects, respectively [111,112].

The study was approved by the Institutional Review Board at the Policlinico Riuniti in Foggia and performed according to the Declaration of Helsinki. All patients provided written informed consent.

*Table 2. Composition of the amino acid mixture (Modified from Bellanti et al., 2020)*

| Amino Acid | Amount (mg) | Proportion of total (%) |
|---|---|---|
| L-Glutamine | 7000.00 | 28.000 |
| L-Leucine | 1562.50 | 6.2500 |
| L-Lysine | 812.00 | 3.2500 |
| L-Isoleucine | 781.25 | 3.1200 |
| L-Valine | 781.25 | 3.1200 |
| L-Threonine | 437.50 | 1.7500 |
| L-Cystine | 187.50 | 0.7500 |
| L-Histidine | 187.50 | 0.7500 |
| L-Phenylalanine | 125.00 | 0.5000 |
| L-Methionine | 62.50 | 0.2500 |
| L-Tyrosine | 37.50 | 0.1250 |
| L-Tryptophan | 25.00 | 0.1000 |
| Vitamin C | 15.00 | 0.0600 |
| Vitamin B1 | 0.15 | 0.0006 |
| Vitamin B6 | 0.15 | 0.0006 |

*Table 3. Nutritional values of the amino acid mixture. All protein is from AA (Modified from Bellanti et al., 2020)*

| Nutritional Values | Per Dose (25 g) |
|---|---|
| Kcal | 102 |
| Kj | 426 |
| | |
| Proteins | - |
| Carbohydrates | 11.6 g |
| Fat | 0.18 g |

### *3.1.3 Experimental protocol*

Three days dietary intake record were collected for each patient, calculating ingested calories and macro- and micronutrients. We used the food frequency questionnaire and the Winfood software 2.0 package (Medimatica S.R.L., Martinsicuro, Italy), according to the indication of food consumption of the Italian National Institute of Nutrition [113]. From baseline (T0) to the end of treatment (T1), patients were fed a lacto-ovo vegetarian diet providing the recommended daily allowance for protein (0.8 g/kg, according to the World Health Organization and the European Food Safety Authority) during diet stabilization and bed rest. The non-protein component of the diet was modified to provide about 60% energy from carbohydrate and 40% from fat. The Harris-Benedict equation was used to design a 3 day rotation diet to maintain body weight throughout the study [114,115]. Water was provided ad libitum. The AA group was given a supplemental 50 g (25 g twice daily) of AAs in addition to the dietary intake. The AA supplementation also contained vitamins C, B1, and B6. Supplements were ingested during meals, at 08.00 and 20.00. The experimental protocol is presented in Figure 8.

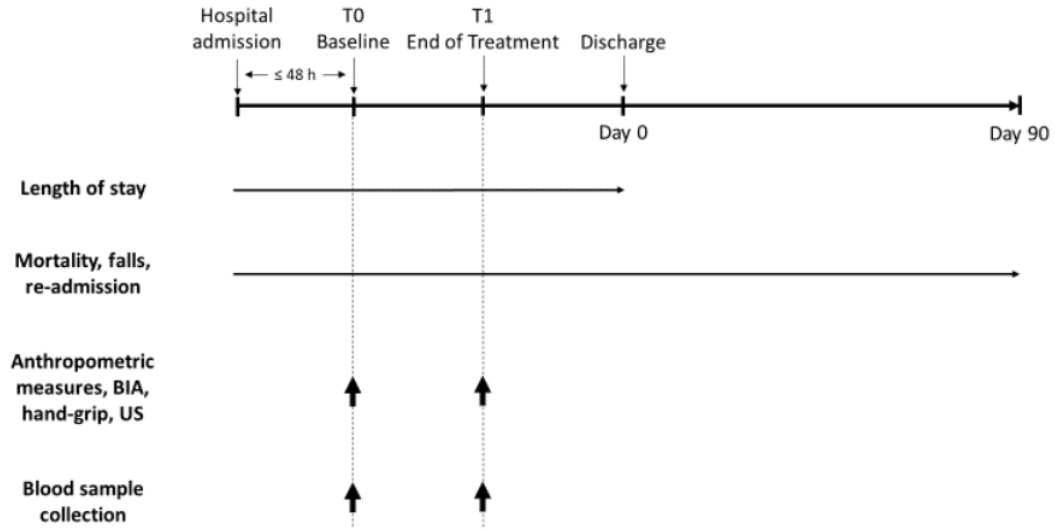


*Figure 8. Study design, timeline, and procedures. (Modified from Bellanti et al., 2020)*

### *3.1.4 Primary outcome*

Data related to hospital length of stay (LOS) were recorded.

### *3.1.5 Post-discharge outcomes*

Data related to in-hospital mortality, death within 90 days post-discharge, 90 days rates of post-discharge hospitalization or falls were analyzed. The 90-d data were collected by telephone interview using a structured questionnaire that was validated by the caregivers.

### *3.1.6 Baseline, anthropometric, and body composition measurements*

Baseline evaluation included patients' demographic characteristics, comorbidities, and socioeconomic factors. The nutritional risk was evaluated through the Mini Nutritional Assessment (MNA) [116]. Height; body weight; and waist, arm, calf, and leg circumferences were measured according to the Anthropometry Procedures Manual of the National Health and Nutrition Examination Survey (2007). While on bed rest, body weight was measured using a LikoScale350 system (LikoAB, Luleå, Sweden). Body mass index (BMI) was calculated as the ratio between weight in kilograms and the square of height in meters ($kg/m^2$). Body composition was assessed by bioelectrical impedance using a BIA 101-F device (Akern/RJL, Florence, Italy), as previously reported [117].

### *3.1.7 Muscle architecture and strength assessment*

To evaluate muscle architecture, a single operator performed right vastus lateralis muscle ultrasound according to a previously published standardized protocol [26]. Particularly, the greater trochanter and the intercondylar notch were identified as landmarks of the upper and lower limits of the vastus lateralis muscle, allowing the measurement of muscle length; the distal 65% length was then identified and marked with a demographic pencil. The ultrasonographic images of right vastus lateralis muscle were obtained using a Philips iU22 xMATRIX Ultrasound system with a 12.5 MHz linear array L12-5 transducer (Philips S.p.A., Milano, Italy) and analyzed with ImageJ Software.

Muscle strength was evaluated by performing the handgrip strength test, using the Hand Grip Dynamometer Kern MAP 80K1S (Kern, Balingen, Germany) [118]. The patients were

instructed to apply as much handgrip pressure as possible; the measurements were repeated three times, and the highest score was recorded in kilograms [119].

### *3.1.8 Laboratory measurements*

Blood samples were obtained from a brachial vein between 08.00 and 09.00 h, after an overnight fast, and immediately processed. Standard laboratory measurements included hemochromocitometric tests, serum glucose, total proteins, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), triacyl glycerides (TGs), creatinine, urea, albumin, and uric acid. Furthermore, analysis of serum AA concentration was performed by tandem mass spectrometry, according to the conventional validated method previously reported [120].

The concentrations of serum cytokines and growth factors, including IL-1a, IL-1b, IL-2, IL-4, IL-6, IL-8, IL-10, tumour necrosis factor (TNF), interferon (IFN)-γ, monocyte chemoattractant protein-1 (MCP-1), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF), were measured using the EV 3513 cytokine biochip array and competitive chemiluminescence immunoassays (Randox Laboratories Ltd., Crumlin, UK), according to the manufacturer's instructions, using the Randox Evidence Investigator [121].

Serum fluorescent adducts formed between peroxidation-derived aldehydes (4-hydroxy-2-nonenal [HNE] and malondialdehyde [MDA]) and proteins were measured by spectrofluorimetry, as previously reported [122].

### *3.1.9 Statistical analysis*

Data were expressed as count and percentages for categorical variables, and as mean ± SDM for quantitative variables. Gaussian distribution of the samples was evaluated by Kolgomorov-Smirnov test. To detect an effect size of 1.11 d in change in hospital LOS [123], with 80% power and a significance level of 5%, a sample size of 40 was calculated. Power calculation was performed with the PS Power and Sample Size Calculations, version 3.

The significance of differences between the two treatment groups (controls versus AA) was assessed by Student's t test (continuous variables) or in contingency tables by Pearson's x2 test and Fisher exact test (categorical variables). The significance of differences between the two treatment groups between the beginning and the end of the supplementation period was assessed by the two-way analysis of variance, to test the main effects of time and treatment as between-subject factor: the
interaction time & treatment was studied, and a Tukey test was applied as post hoc test for multiple comparisons. The correlation analysis between changes in serum IL-4 or IL-10 and serum HNE-protein adducts was performed by using the Pearson's correlation test followed by linear regression. All tests were two-sided, and $P < 0.05$ was considered statistically significant. Statistical analysis was performed with the SPSS version 23 (SPSS, Inc., Chicago, IL, USA) and the package Graph-Pad Prism 6 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

# 3.2 Animal Study

## *3.2.1 Mice and protocol*

18-month-old male C57/Bl6 mice were used for this study. All animals were housed in conditions of Temperature 22 °C ± 2 °C, humidity 55% ± 10%, and 12-h day/dark cycles. Mice were fed *ad libitum* a standard diet (52% carbohydrates, 21% proteins, 4% lipids). Animals were randomized into four different groups based on presence or absence of limb immobilization and AA supplementation:

- Control + Placebo (C+P): n. 5 mice
- Immobilization + Placebo (I+P): n. 5 mice
- Control + AA mixture supplementation (C+A): n. 5 mice
- Immobilization + AA mixture supplementation (I+A): n. 5 mice

After 10 days, muscle function was studied by both endurance running and grip strength tests. At the end of the experiment, mice were euthanized by anesthesia overdose and samples of blood and muscle (tibialis anterior) tissue were taken, weighed and used for isolation of mitochondria.

All procedures were in accordance with the ethical standards and with the formal approval of the Animal Ethics Committee at the University of Foggia.

## *3.2.2 Immobilization of one hindlimb*

Mice included in the I or I + A groups underwent immobilization of one hindlimb by stapling the foot exploiting normal dorsotibial flexion using a skin stapler, as described previously [124]. Hindlimbs were immobilized for 10 day before removing fixing points.

### *3.2.3 AA supplementation*

The AA mixture was administered via oral supplementation, dissolved in a volume of water equal to 52 ± 0.3 mL (according to the estimated amount of daily consumption). This was administered at a dose of 0.1 g / kg / day for 10 days.

### *3.2.4 Running test*

An homemade rodent treadmill was used as previously reported [125]. The running test consisted in a running session at a speed of 9 cm/sec with an inclination of 5°. The speed was increased by 3 cm/sec every 12 minutes. A rigid brush, positioned in the cool-down area at the end of the treadmill track, was used to motivate the animals to perform the exercise.

The test was recorded and the video analyzed to calculate the mice's permanence in the cool-down zone. Mice that needed at least 5 stimuli in the cool-down zone were considered "exhausted" (with conclusion of the exercise), Figure 9 [125].

*Figure 9. Treadmill test with separate lanes for each animal.*


*Figure 10. Grip test using rings with predefined weight.*

### *3.2.5 Muscle strength*

Muscle strength was assessed by using special metal rings with a predefined weight and added from time to time to reach the maximum load tolerated by the animal before its fall, Figure 10 [126].

### *3.2.6 Mitochondria isolation and function analysis*

Mitochondria isolation from the skeletal muscle of the hind leg (100 mg of tissue) was performed by differential centrifugation, as previously reported [127].

The following analyses were performed on isolated mitochondria:

1) Complex I and Complex II-related respiration were assayed in freshly isolated mitochondria by using specific substrates and inhibitors, as previously reported [127].

2) *Mitochondrial HNE-protein adducts*

Protein oxidative damage in mitochondria was assessed by the formed HNE-protein adducts.

A spectrophotometric method described previously was used [128].

3) *Western blot analysis of mitochondrial oxidized proteins*

The qualitative analysis of oxidized proteins was performed by Western blot using the Oxyblot

kit (Chemicon International, El Segundo, CA, USA), as previously reported [129].

### *3.2.7 Statistical analysis*

All data were expressed as means ± SE. To compare all groups, we used two-way analysis of variance (ANOVA) to test the main effect of immobilization and treatment as between-subject factors; the interaction immobilization x treatment was studied, and a Tukey test was applied as a post hoc multiple-comparison test. All tests were two-sided, and $P < 0.05$ was considered statistically significant. Statistical analysis was performed with the package Graph-Pad Prism 6 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

# 4. *Results*

## 4.1. Patients' characteristics

514 patients were evaluated for enrollment. Of these, 94 were finally suggested for supplementation with the AA mixture. Of the 94 patients, 44 accepted and were included into the study group, whereas 50 refused and were considered as the control group, as reported in Figure 11.

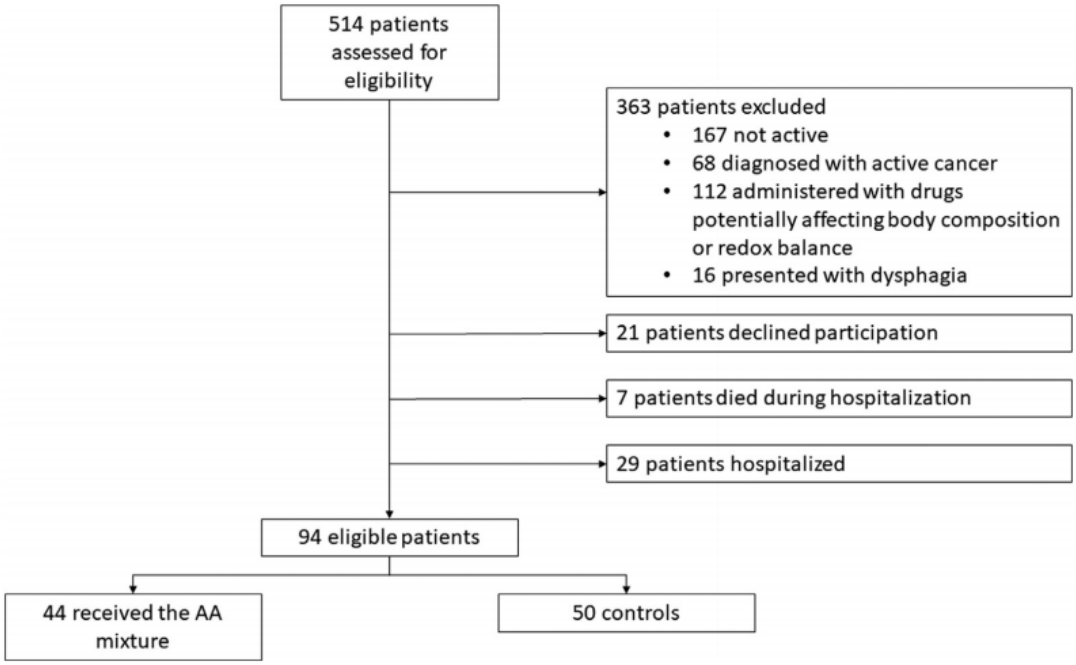


*Figure 11. Participant flowchart. Patients "not active" include those presenting with chronic bedridden conditions, physical handicaps, severe neuromuscular diseases, or cognitive decline.*

Baseline characteristics of the patients included in the control group (76.6 ± 6.8 y of age) or AA group (79 ± 6.8 y of age) are represented in Table 4 and Table 5.

*Table 4. Demographic, anthropometric, biochemical, and nutritional characteristics of patients at baseline, according to study groups. AA, amino acid; BMI, body mass index; HDL, high-density lipoprotein; LDL, low density lipoprotein; WBC, white blood cell. Statistical differences were assessed by Student's t test or Pearson's $X^2$ test. (Modified from Bellanti et al., 2020)*

| | Control (n = 50) | AA (n = 44) | *P*-value |
|---|---|---|---|
| **Demographic** | | | |
| **Age, y** | 76.6 ± 6.8 | 79 ± 7.2 | 0.100 |
| **Sex (M/F)** | 20/30 | 16/28 | 0.832 |
| **Anthropometric** | | | |
| **Body weight, kg** | 61.5 ± 14.3 | 62.1 ± 16.1 | 0.849 |
| **BMI, kg/m$^2$** | 28.7 ± 4.15 | 28.1 ± 3.62 | 0.460 |
| **Biochemistry** | | | |
| **Hb, g/L** | 10.8 ± 2.89 | 10.13 ± 2.21 | 0.225 |
| **WBC, n/mm$^3$** | 9065 ± 3283 | 8578 ± 3275 | 0.474 |
| **Neutrophils, n/mm$^3$** | 6301 ± 3186 | 6005 ± 3256 | 0.657 |
| **Lymphocytes, n/mm$^3$** | 1712 ± 946 | 1621 ± 744 | 0.609 |
| **Glucose, mg/dL** | 114.2 ± 66.6 | 111.7 ± 50.1 | 0.839 |
| **Urea, mg/dL** | 75.6 ± 48.9 | 85.7 ± 28.2 | 0.231 |
| **Creatinin, mg/dL** | 1.21 ± 0.71 | 1.38 ± 0.4 | 0.164 |
| **Uric acid, mg/dL** | 5.11 ± 1.38 | 5.50 ± 2 | 0.269 |
| **Total cholesterol, mg/dL** | 143.3 ± 40.8 | 158.6 ± 57 | 0.135 |
| **LDL cholesterol, mg/dL** | 88.2 ± 34 | 96.4 ± 36.8 | 0.264 |
| **HDL cholesterol, mg/dL** | 39.7 ± 20.7 | 42.1 ± 12.8 | 0.508 |
| **Triacylglycerols, mg/dL** | 133.5 ± 65 | 120.3 ± 42.7 | 0.255 |
| **Total proteins, g/dL** | 6.2 ± 0.6 | 6.1 ± 0.9 | 0.523 |
| **Albumin, g/dL** | 3 ± 0.6 | 2.8 ± 0.7 | 0.139 |
| **Nutritional risk** | | | |
| **Mini-Nutritional Assessment, score** | 21.1 ± 3.6 | 20.8 ± 2.7 | 0.652 |
| **Daily nutritional intake** | | | |
| **Energy, kcal/kg** | 22.9 ± 5.4 | 22.1 ± 4.6 | 0.445 |
| **Protein, g/kg** | 0.92 ± 0.4 | 0.89 ± 0.8 | 0.815 |
| **Lipids, g/kg** | 0.95 ± 0.3 | 0.98 ± 0.2 | 0.575 |
| **Carbohydrates, g/kg** | 2.48 ± 0.7 | 2.65 ± 1 | 0.338 |

*Table 5. Distribution of diseases at baseline, according to study groups. AA, amino acid; COPD, chronic obstructive pulmonary disease. Several patients had more than one diagnosis. Statistical differences were assessed by Pearson's $X^2$ test. (Modified from Bellanti et al., 2020)*

| | Control (n = 50) n (%) | AA (n = 44) n (%) | *P*-value |
|---|---|---|---|
| **Arterial hypertension** | 46 (92) | 40 (90.9) | 1.000 |
| **Diabetes mellitus** | 16 (32) | 22 (50) | 0.094 |
| **Renal failure** | 22 (44) | 15 (34) | 0.399 |
| **Heart failure** | 16 (32) | 12 (27.3) | 0.657 |
| **Atrial fibrillation** | 18 (36) | 22 (50) | 0.211 |
| **Stroke** | 2 (4) | 4 (7.3) | 0.414 |
| **Liver cirrhosis** | 2 (4) | 0 (0) | 0.497 |
| **Ischemic heart disease** | 12 (24) | 7 (13.6) | 0.442 |
| **COPD** | 18 (36) | 16 (36.4) | 1.000 |

The two groups were comparable in terms of clinical, nutritional, and biochemical features. Full compliance (defined as completion of the entire treatment) was recorded for patients who were administered the AA mixture. No AEs were reported in the AA group.

### 4.2. Effects of AA supplementation on primary outcome measure

A shorter hospital LOS was reported in the AA group compared with controls (Table 6).

*Table 6. Hospital length of stay, mortality, falls and readmission, according to study groups. AA, amino acid. Statistical differences were assessed by student's t-test or Pearson's x2 test. *Patients who fell. (Modified from Bellanti et al., 2020)*

| | Control (n = 50) | AA (n = 44) | P-value |
|---|---|---|---|
| *Primary outcome measure* | | | |
| *Length of stay*, d | 11.56 ± 4.29 | 8.62 ± 1.48 | 0.021 |
| *Secondary outcome measures*, n (%) | | | |
| *In-hospital mortality* | 9 (18) | 4 (9) | 0.245 |
| *90-day mortality* | 8 (16) | 3 (6.8) | 0.209 |
| *Overall mortality* | 17 (34) | 7 (15.8) | 0.094 |
| *90-d falls** | 8 (16) | 1 (2.3) | 0.034 |
| *90-d post-discharge hospitalization* | 8 (16) | 0 (0) | 0.006 |

### 4.3. Effects of AA supplementation on secondary outcome measures

Although we did not report any difference between groups related to in-hospital mortality, 90-day post-discharge mortality, and overall mortality, patients supplemented with AA presented with a reduced rate of 90-d falls and post-discharge hospitalization compared with controls (Table 6).

### 4.4. Effect of AA supplementation on circulating AA, anthropometric measures, and body composition

Table 7 summarizes the results related to serum AA levels evaluated at baseline (T0) and after 7 d of low mobility (T1) in older hospitalized patients. A significant effect of treatment was reported for circulating levels of glutamine, valine, and methionine, whereas an interaction effect was described for glutamine, leucine, valine, and methionine. The post hoc analysis showed that the serum level of these AAs was higher in patients undergoing 7 d of supplementation with AA rather than in controls.

No significant differences were found among the two groups when variations of anthropometric parameters between T0 and T1 were considered (Figure 12). Of note, waist circumference was reduced at T1 with respect to T0 in both groups (time factor: $F_{1,184}$ = 7.109, P = 0.0084), indicating the negative effects of low mobility (Figure 12B).

Interestingly, AA supplementation during 7 d of low mobility exerted a significant effect on body composition in hospitalized older patients (Figure 12). The effect of treatment was significant for body cell mass ($F_{1,184}$ = 8.122, P = 0.0049) and skeletal muscle index ($F_{1,184}$ = 13.64, P = 0.0003), whereas a significant interaction effect was recorded for body cell mass ($F_{1,184}$ = 15.61, P = 0.0001), total body water ($F_{1,184}$ = 6.010, P = 0.0152), free fat mass ($F_{1,184}$ = 9.645, P = 0.0022), fat mass ($F_{1,184}$ = 9.243, P = 0.0027), skeletal muscle index ($F_{1,184}$ = 38.02, P < 0.0001), and appendicular skeletal muscle mass ($F_{1,184}$ = 12.13, P = 0.0008). Particularly, a significant increase in body cell mass and free fat mass - and a decrease in fat mass - were observed between T0 and T1 in the AA group rather than in controls; moreover, although a noticeable reduction in skeletal muscle index and appendicular skeletal muscle mass was observed in controls between T0 and T1, these were preserved in AA patients (Figure 13).

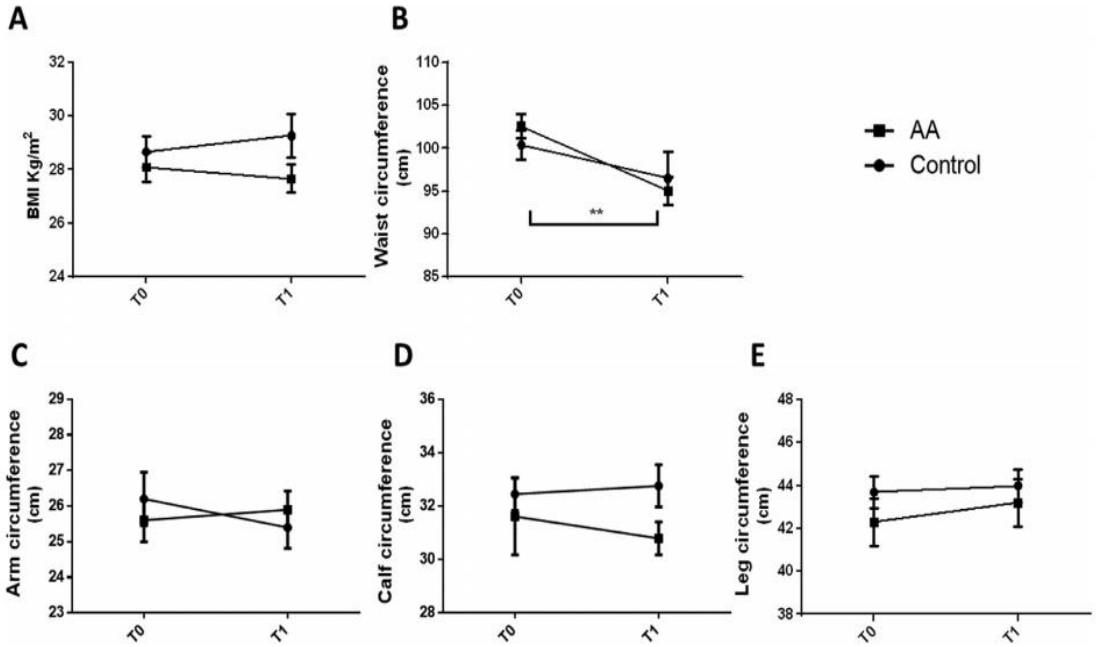


*Figure 12. Variations of anthropometric measures in controls and patients supplemented with AAs between baseline (T0) and end of treatment (T1). Statistical differences were assessed by two-way analysis of variance and Tukey's post hoc test. *P < 0.01. AA, amino acid; BMI, body mass index*

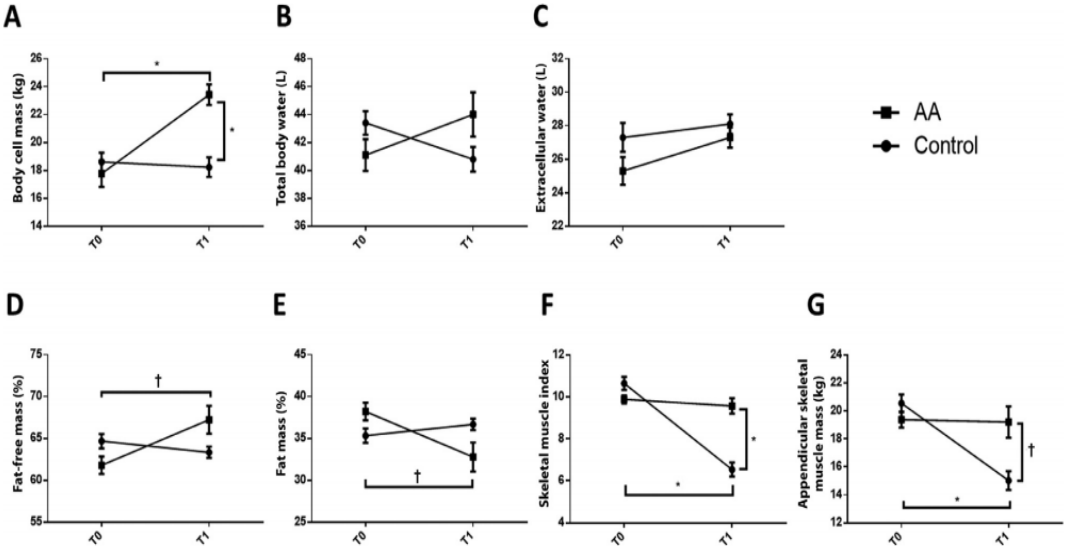


*Figure 13. Variations of body composition parameters in controls and patients supplemented with AAs between baseline (T0) and end of treatment (T1). Statistical differences were assessed by two-way analysis of variance and Tukey's post hoc test. *P < 0.001. yP < 0.01. AA, amino acid*

### 4.5. Effect of AA supplementation on muscle architecture and strength

As represented in Figure 14, measure of right vastus lateralis architecture variations during low mobility resulted in a significant effect of treatment and interaction on both muscle thickness (treatment: F1,184 = 11.95, P = 0.0007; time £ treatment: F1,184 = 9.732, P = 0.0021) and pennation angle (treatment: F1,184 = 13.49, P = 0.0003; time £ treatment: F1,184 = 16.75, P < 0.0001). Similar

results were obtained when handgrip strength was considered (treatment: F1,184 = 7.447, P = 0.0070; time £ treatment: F1,184 = 7.656, P = 0.0062). The post hoc analysis resulted in decreased muscle thickness, pennation angle, as well as handgrip strength in older control hospitalized patients between T0 and T1 as compared with the AA group, which showed no reliable variation for all the considered parameters (Figure 14).

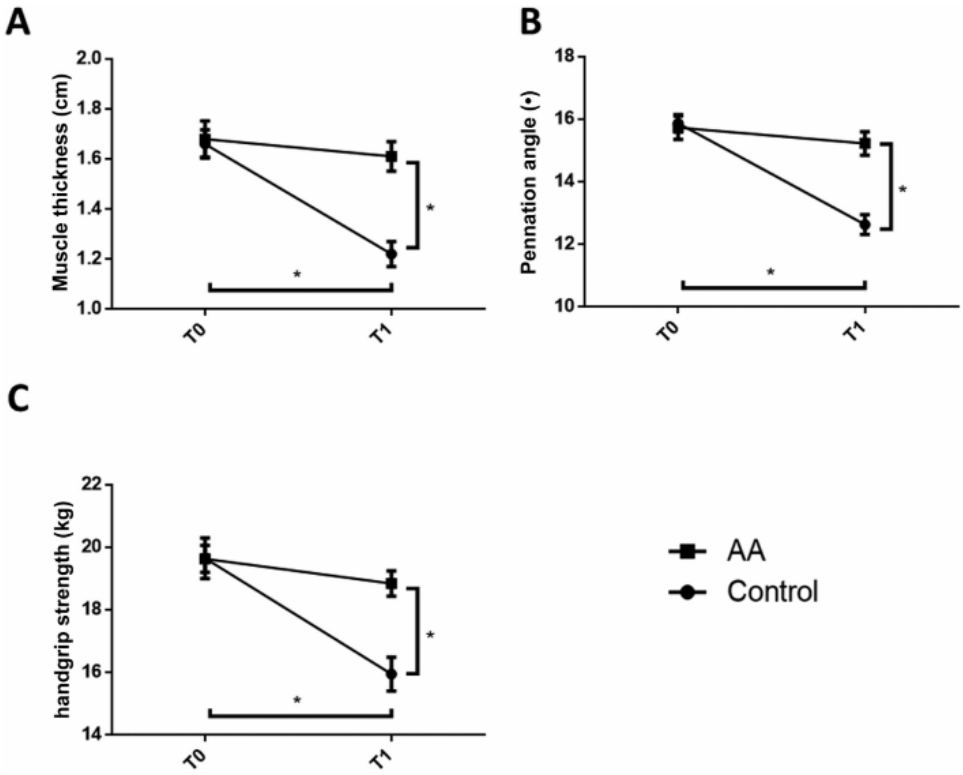


*Figure 14. Variations of muscle thickness (A) and pennation angle (B) evaluated by ultrasonography, and handgrip strength (C) evaluated by dinamometry, in controls and patients supplemented with AAs between baseline (T0) and end of treatment (T1). Statistical differences were assessed by two-way analysis of variance and Tukey's post hoc test. *P < 0.001. AA, amino acid. (Modified from Bellanti et al., 2020)*

### 4.6. Effect of AA supplementation on circulating interleukins and oxidative stress markers

Table 7 summarizes the results related to serum levels of 12 different cytokines and growth factors evaluated at baseline (T0) and after 7 d of low mobility (T1) in the patients. Although no significant differences were reported for most of the outcomes measured, we observed a consistent effect of treatment on circulating levels of IL-4 and IL-10, whereass a time effect was described for EGF. The post hoc analysis showed that both serum IL-4 and IL-10 levels were higher in patients undergoing 7 days of supplementation with AA compared with controls.

*Table 7. Serum concentration of AA levels in the two treatment groups, at the beginning (T0) and at the end (T1) of treatment. Statistical differences were assessed by two-way analysis of variance. *P < 0.05 vs T0 AA. yP < 0.01 vs T1 control. *P < 0.05 vs T1 control (Modified from Bellanti et al., 2020)*

| | | Control (n = 50) | AA (n = 44) | *P*-value Time | *P*-value Treatment | *P*-value Time × treatment |
|---|---|---|---|---|---|---|
| Glutamine (μmol/L) | T0 | 661.3 ± 221.5 | 641.5 ± 202.6 | 0.177 | 0.022 | 0.003 |
| | T1 | 612.8 ± 190.8 | 771.7 ± 208.8*,† | | | |
| Leucine (μmol/L) | T0 | 145.9 ± 31.7 | 140.2 ± 33.9 | 0.749 | 0.173 | 0.014 |
| | T1 | 131.5 ± 41.8 | 151.3 ± 32† | | | |
| Lysine (μmol/L) | T0 | 401.1 ± 133.2 | 391.8 ± 127.8 | 0.931 | 0.442 | 0.192 |
| | T1 | 380 ± 114.3 | 415.9 ± 91.1 | | | |
| Valine (μmol/L) | T0 | 172.9 ± 23.6 | 170.5 ± 33.3 | 0.969 | 0.030 | 0.009 |
| | T1 | 159.4 ± 37.4 | 184.4 ± 44.8† | | | |
| Threonine (μmol/L) | T0 | 18.7 ± 21.4 | 14.8 ± 20.5 | 0.647 | 0.526 | 0.459 |
| | T1 | 15.3 ± 16.9 | 15.6 ± 18.4 | | | |
| Cysteine (μmol/L) | T0 | 9 ± 9.1 | 8.5 ± 9.7 | 0.674 | 0.624 | 0.401 |
| | T1 | 8.4 ± 10.3 | 10.3 ± 9.9 | | | |
| Histidine (μmol/L) | T0 | 191.8 ± 52.7 | 188 ± 63.2 | 0.504 | 0.671 | 0.986 |
| | T1 | 197.4 ± 51 | 193.9 ± 68.2 | | | |
| Phenylalanine (μmol/L) | T0 | 97.4 ± 12.9 | 90.4 ± 15.5 | 0.370 | 0.056 | 0.140 |
| | T1 | 96.2 ± 16.2 | 95.3 ± 10.9 | | | |
| Methionine (μmol/L) | T0 | 45.6 ± 9.8 | 46 ± 9 | 0.675 | 0.005 | 0.011 |
| | T1 | 42 ± 10.7 | 51 ± 15.4† | | | |
| Tyrosine (μmol/L) | T0 | 86.5 ± 17.5 | 83 ± 18.7 | 0.833 | 0.801 | 0.223 |
| | T1 | 84.1 ± 14.4 | 86.4 ± 13.9 | | | |
| Tryptophan (μmol/L) | T0 | 23.8 ± 7.9 | 22 ± 10.2 | 0.569 | 0.776 | 0.319 |
| | T1 | 21.6 ± 8.9 | 22.6 ± 11.3 | | | |

Changes in systemic oxidative stress markers were evaluated by measuring serum HNE- and MDA-protein adducts in patients (Figure 15A, B). Interestingly, we registered a significant effect of treatment and interaction for both serum HNE-protein adducts (treatment: $F_{1,184}$ = 13.27, P = 0.0004; time £ treatment: F1,184 = 18.37, P < 0.0001) and serum MDA-protein adducts (treatment: $F_{1,184}$ = 12.49, P < 0.0001; time £ treatment: $F_{1,184}$

= 16.77, P < 0.0001). As evidenced by post hoc analysis, after 7 d of low mobility, serum HNE-protein adducts were reduced in the AA group compared with controls (Figure 15A). Between T0 and T1, serum MDA-protein adducts were increased in controls, but were reduced in the AA group (Figure 15B).

Notably, focusing on the AA group of patients, bivariate correlation analysis showed that there was a strong correlation between changes registered in the circulating levels of IL-4 and IL-10, and in serum HNE-protein adducts (Figure 15).

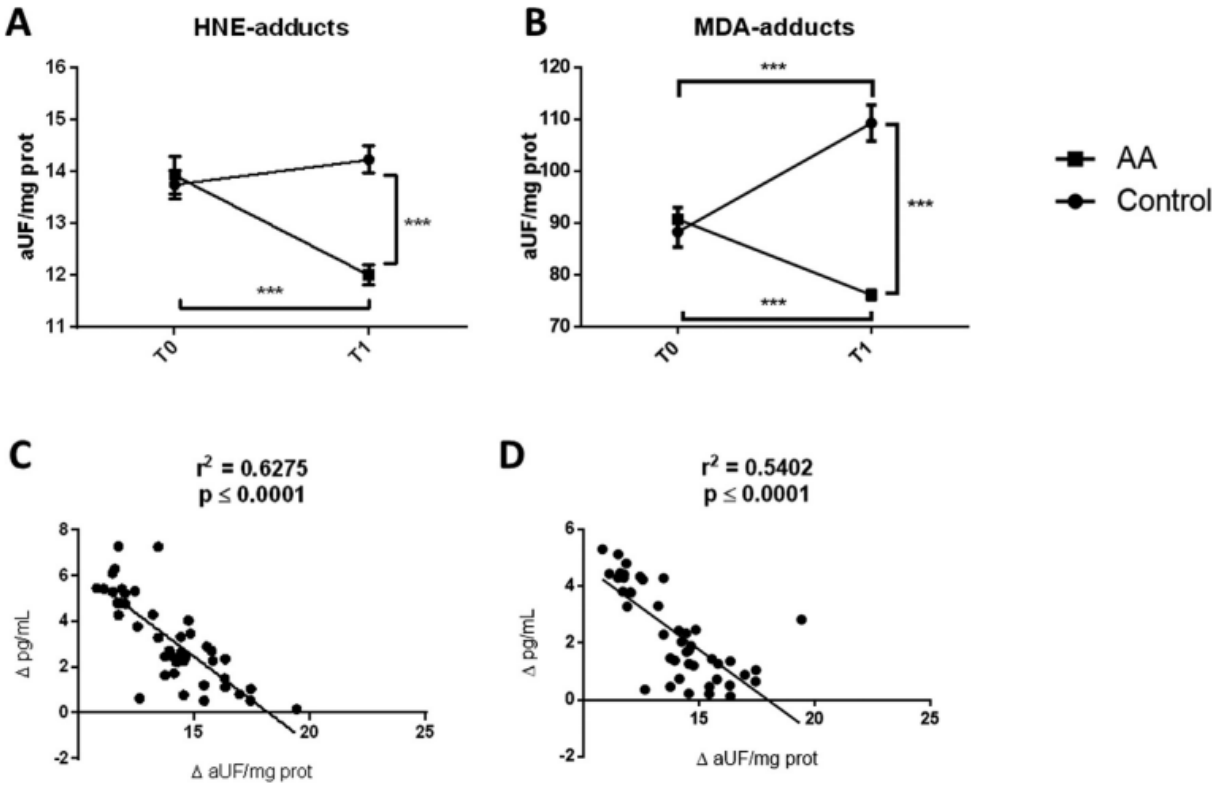


*Figure 15. (A, B) Changes in serum HNE- and MDA-protein adducts measured in controls and patients supplemented with AAs between baseline (T0) and end of treatment (T1). Statistical differences were assessed by two-way analysis of variance and Tukey's post hoc test. *P < 0.001. (C, D) Linear regression analysis between the variation of serum HNE-protein adducts and IL-4 or IL-10 level measured in patients supplemented with AA. AA, amino acid; HNE, 4-hydroxy-2-nonenal; IL, interleukin; MDA, malondialdehyde.*

### 4.7. Effect of AA supplementation on muscle endurance strength, and size after immobilization in mice

With respect to control mice, hindlimb immobilization reduced maximal running time and distance, along with limb grip strength; however, the extent of reduction was lower in I+A than I+P (*Figure 16*). Immobilization resulted in TA atrophy, characterized by a reduction in both wet weight and TA/body weight ratio, and smaller myofibers than controls. Interestingly, these alterations were slightly observed in mice treated with the AA mixture.

### 4.8. Effect of AA supplementation on muscle mitochondria function and oxidation

The mitochondrial yield from TA of I+P mice was lower than controls; of note, the mitochondrial yield from TA of I+A animals was similar to controls. AA mixture administration also preserved mitochondrial bioenergetics in TA muscle, which was disrupted in I+P mice with respect to controls.

In fact, we observed a mitochondrial density reduction of 51.6% in I+P as compared to C+P mice ($p < 0.001$). Oral AA supplementation resulted in a lower reduction of mitochondrial density (44.3%, $p < 0.001$)., Figure 17A and Figure 17B. Respiratory activity from Complex I and II were found significantly lower in I+P with respect to other groups ($p < 0.001$). In particular, Complex I and II activities were 32.8% and 20.5% lower than in C+P, respectively ($p < 0.001$). AA supplementation limited the loss of Complex I and II respiratory activities ($p<0.001$), Figure 17C-Figure 17F. Mitochondrial oxidation was studied by analysing the amount of HNE-protein adducts and carbonylated proteins in freshly isolated samples. As shown in Figure 18A, the amount of HNE-protein adducts was higher in muscle mitochondria extracted from I+P mice, as compared with control

groups; of interest, this was not observed in the I+A group. Similarly, the rise in the amount of carbonylated mitochondrial proteins observed in SM from immobilized mice was limited by the AA mixture (Figure 19 and Figure 20).

**Fig. 16A**

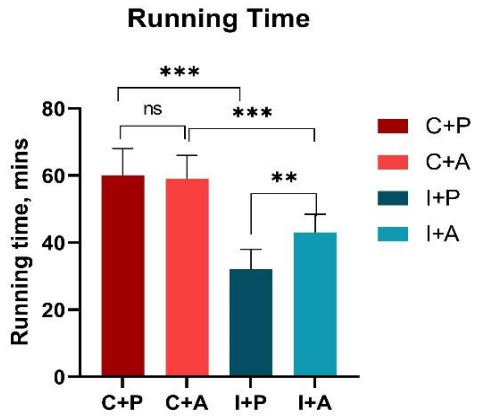


**Fig. 16B**

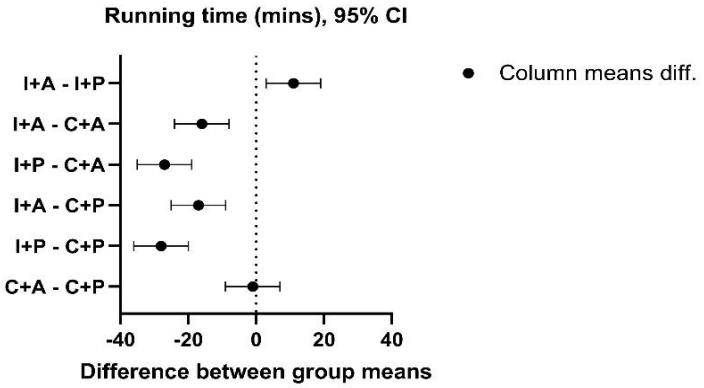


**Fig. 16C**

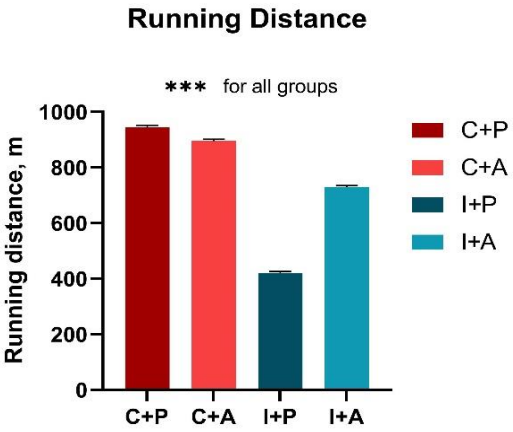


**Fig. 16D**

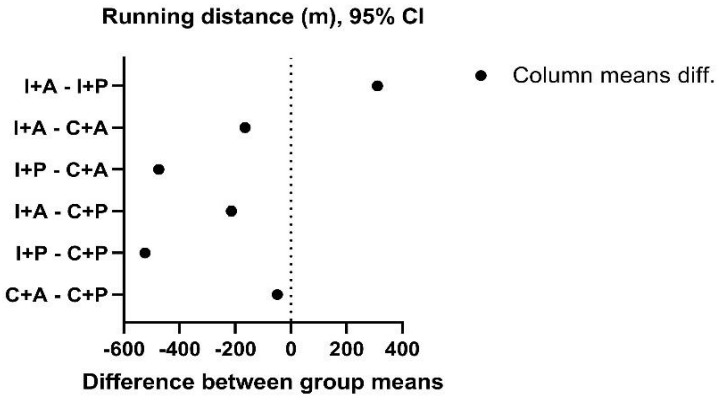


**Fig. 16E**

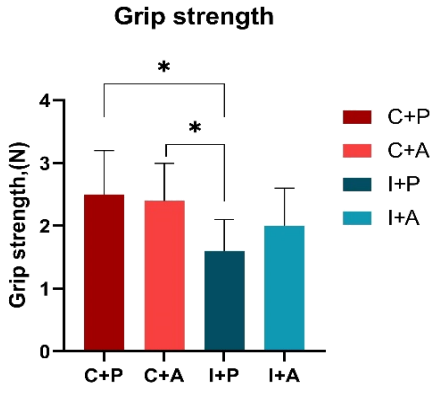


**Fig. 16F**

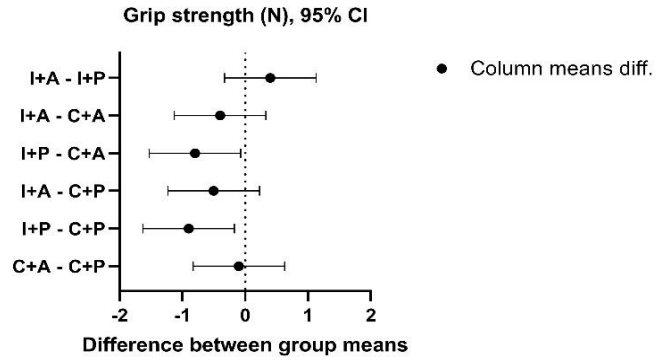


*Figure 16. Comparison of physical performance among animal groups respect to limb immobilization and AA oral supplementation. 9A: differences in running time among the four groups. 9B: forest plot of mean differences between each group. 9C: differences in running distance among the four groups. 9D: forest plot of mean differences between each group. 9E: differences in grip strength among the four groups. 9F: forest plot of mean differences between each group.*

**Fig. 17A**

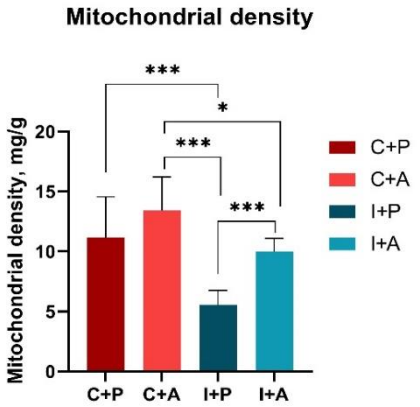


**Fig. 17B**

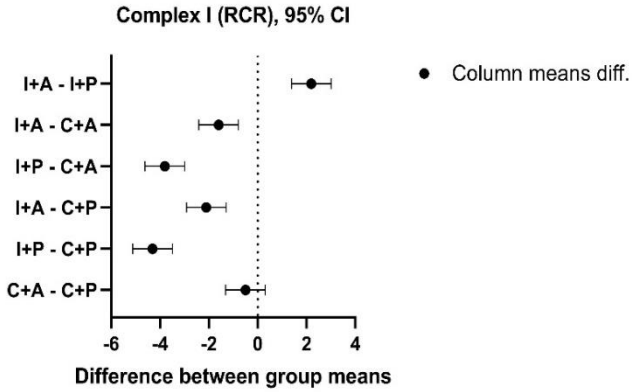


**Fig. 17C**

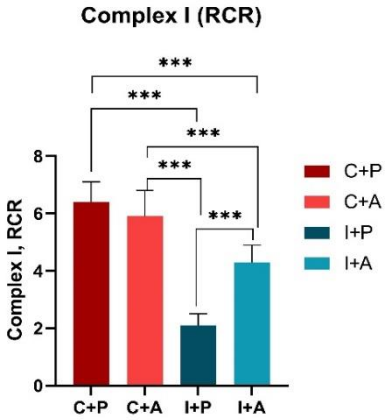


**Fig. 17D**

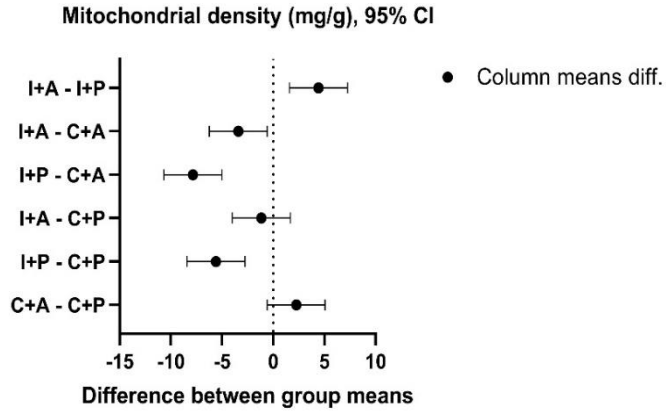


**Fig. 17E**

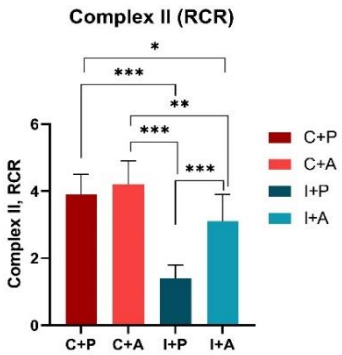


**Fig. 17F**

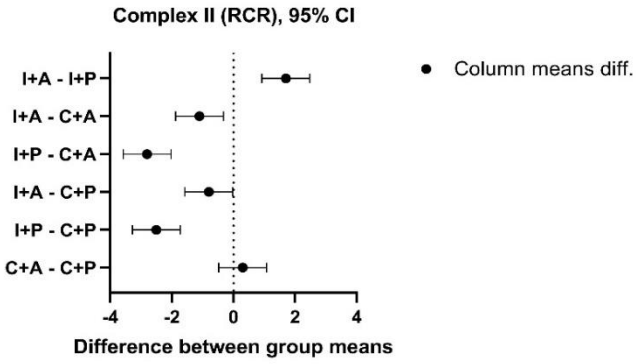


*Figure 17. Differences of mitochondrial density and complex I and II (RCR) according to limb immobilization and AA oral supplementation (Fig A, C and E, respectively); Forest plot of mean differences between each group (Fig. B, D and E, respectively).*

**Fig. 18A**

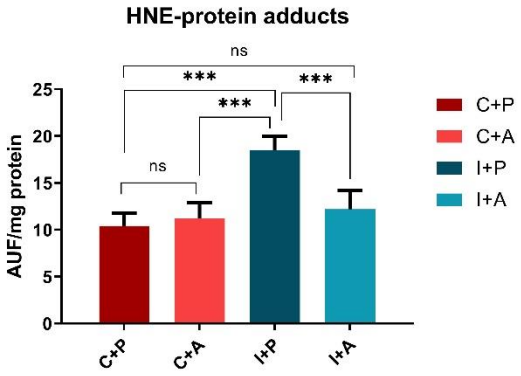


**Fig. 18B**

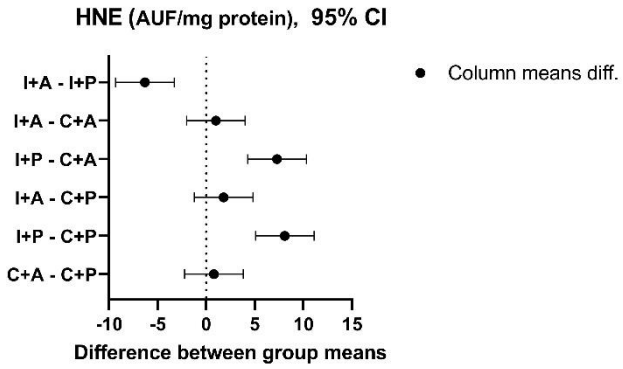


*Figure 18. Adducts formed by hydrxynonenal (HNE) and proteins in mitochondria isolated from tibialis anterior of control (C) or immobilized (I) mice fed a placebo (P) or an amino acid mixture (A). Data are represented as mean ± SD (Fig. 18A); Forest plot of mean differences between each group (Fig. 18B)*

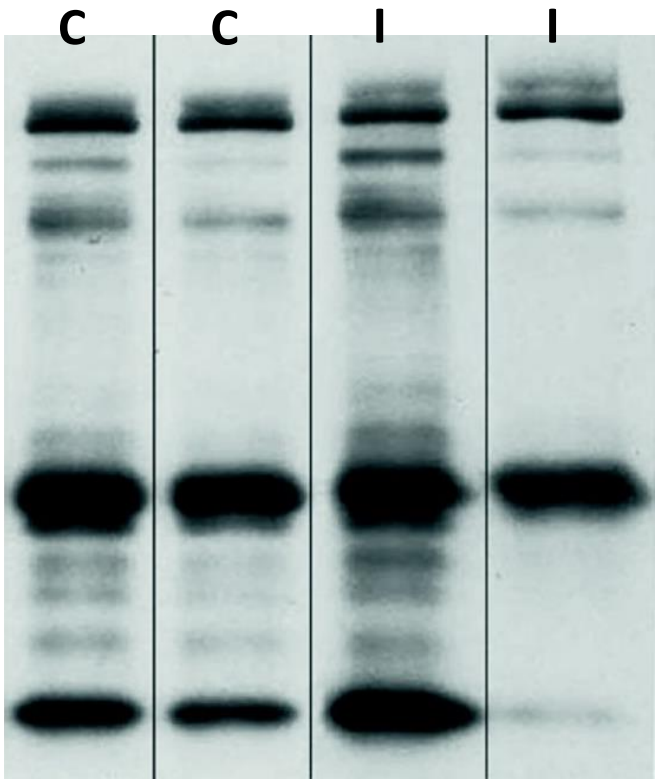


*Figure 19. Representative oxyblot analysis of mitochondrial proteins extracted from tibialis anterior of control (C) or immobilized (I) mice fed a placebo (P) or an amino acid mixture (A).*

**Fig. 20A**

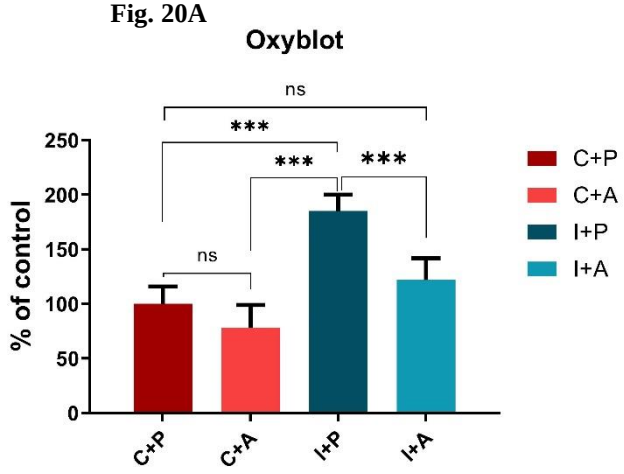


**Fig. 20B**

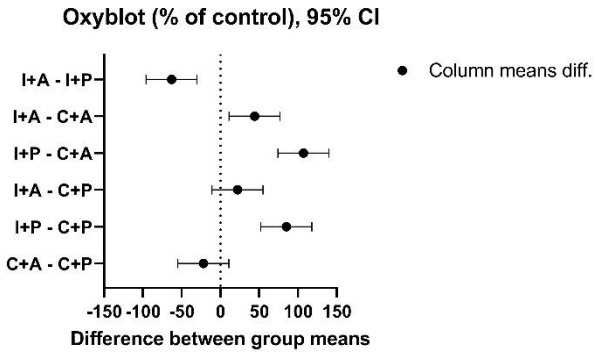


*Figure 20. Densitometric analysis of whole oxyblot lanes from all the mice groups. Data are represented as mean ± SD (Fig. 20A); Forest plot of mean differences between each group (Fig. 20B)*

# *5.Discussion*

The present study demonstrated that supplementation with a mixture of amino acids in older hospitalized patients during 7 days of low mobility was associated with a clinically relevant effect on LOS. Furthermore, the supplementation reduced the rate of post-discharge falls and hospitalizations. The positive effects of AA supplementation were related to the preservation of muscle architecture and strength, and to changes in circulating ILs and oxidative stress markers, probably related to the preservation of mitochondrial function and prevention of mitochondrial oxidation in skeletal muscle.

The average hospital LOS was used as an indicator of efficiency, as a short stay is associated with a reduced hospitalization cost. Although 7 days of a hospital stay could appear excessive, the most recent available data on the average hospital LOS are different in diverse countries, ranging from 5.5 d in the United States to >7 days in several European countries [130].

Hospitalization increases the risk of bed rest or hypomobility. About 23 to 33% older adults undergoes immobilization in bed during hospitalization for acute illness [60]. In turn, this condition is associated with several disadvantageous and clinical significant consequences such as loss of muscle mass with metabolic changes, falls, disability, and higher mortality [131,132].

Older patients admitted to the hospital for an acute medical event frequently undergo a severe decline in health and functional status, and loss of muscle mass and strength is commonly associated with hospitalization [133].

Of note, physical impairment and loss of muscle mass and strength are independently associated with a poor prognosis related to medium-term mortality [134,135]. On the other side, LOS reduction is associated with an improvement in clinical outcomes [123].

To date, several interventions have been tested to prevent or attenuate the consequences related to inactivity and malnutrition caused by hospitalization in older patients [136,137].

Most interventions on older in-hospital patients are physical and rely on different training exercise programs; nevertheless, these protocols show uncertainty and inconsistent results [136,137]. Nutritional interventions, mostly oral AA supplementation, are considered effective to counteract the loss of muscle mass and strength observed during hospitalization [114,138].

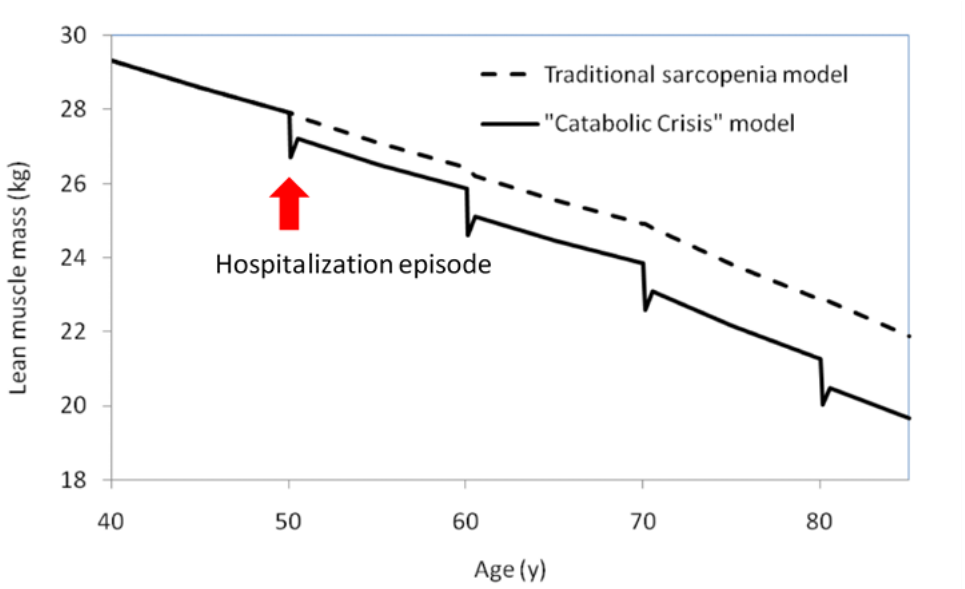


*Figure 21. Muscle loss age related and associated acute acceleration of muscle loss with incomplete recovery occur during acute illness or injury (Modified from English KL et al., 2010 [131])*

Moreover, AA supplementation could be beneficial against systemic inflammation and oxidative stress [139]. EAAs endorse net protein synthesis in skeletal muscle and preserve muscle function during bed rest in older hospitalized patients, promoting functional independence [114,140].

Previous studies have assessed the relationship between AA deficiency and loss of muscle mass, and the effect of AA supplementation in older adults. To our knowledge, this is the first study to investigate the effect of this supplementation on several clinical, functional, and biochemical outcomes in this group of patients. Of note, the AA mixture used in this study was particularly enriched with glutamine, the most abundant free AA in the human

body. Glutamine modulates several functions such as muscle maintenance and immunomodulation [141]. A depletion of glutamine occurs in hospitalized patients, which is dependent on an increased consumption by lymphocytes and immune-stimulated macrophages, and high skeletal muscle turnover during hypercatabolic stress [142]. Glutamine reduction is associated with increased mortality and longer hospital LOS [143]. Furthermore, the present study demonstrated that supplementation with the AA mixture determined a net increase of circulating leucine levels. This AA also can elicit an anabolic response in older adults, protecting muscle integrity during short periods of physical inactivity [144]. Although the AA supplementation in bed-ridden older adults was not associated with a reduced rate of in-hospital or post-discharge mortality, the present study reported a shorter LOS in the supplemented patients. Because mortality was limited in both groups, we could report a significant reduction in the rate of post-discharge falls and hospitalizations in the patients supplemented with AA during bed rest.

This could be related to the positive effect of supplementation on skeletal muscle structure and function. Older individuals facing hospitalization frequently present with changes in anthropometric parameters, predictive of long-term mortality [145]. Moreover, 10 days of bed rest can induce body weight loss and a reduction in muscle mass and strength [146]. In the present hospitalized cohort, we did not report any alterations in BMI and other anthropometric measurements, apart from a reduced waist circumference in both groups, after 7 days of low mobility. This result may be related to recent attention on the nutritional status of older patients in our department. However, interesting differences between the two groups were described in the changes of body composition parameters. Indeed, when compared with controls, the supplemented group exhibited a loss of fat mass but a preservation of skeletal muscle mass, with an increase of body cell mass, the metabolically active cellular compartment. Although the measurement of body

composition in this study was performed through bioelectrical impedance analysis, a previous study demonstrated that this method estimates skeletal muscle mass as accurately as dual-energy x-ray absorptiometry in older hospitalized patients [147].

The positive changes in body composition observed in the supplemented patients were associated with a preserved muscle architecture and strength. In the hospitalized older adults, AA may increase protein intake and maintain muscle protein synthesis, protecting fiber function during muscular inactivity [114]. Furthermore, glutamine may act synergistically to improve the nitrogen balance during hospitalization [148].

Both acute illness and skeletal muscle wasting are associated with alterations in circulating cytokines and oxidative stress markers [122,149]. The present data did not show any modification in the serum level of several cytokines and growth factors during 7 days of low mobility in the participants; however, AA supplementation was associated with increased circulating concentration of both IL-4 and IL-10, which display immunosuppressive activity by inhibiting both activated macrophages and T-helper 1 cells [150]. Of note, IL-4 may play a determinant role in muscle homeostasis [151].

Our preliminary data are suggestive of a further possible mechanism by which AA could preserve muscle structure and function in this population of patients.

Reactive species are produced in inactive skeletal muscle, leading to oxidative stress, which in turn promotes the generation of HNE and MDA. These compounds are lipid peroxidation products able to generate adducts with cellular and circulating proteins; the latter may be used as circulating markers of injury. We have previously described increased circulating serum HNE- and MDA-protein adducts in patients with sarcopenia [122]. AA supplementation in the older hospitalized adults showed a positive effect on systemic oxidative stress. Furthermore, the improvement in circulating HNE-protein adducts was associated with the increase in anti-inflammatory cytokines observed during

the 7-days period. Further basic research is needed to completely elucidate the biomolecular mechanisms underlying such observations. The present study demonstrated that supplementation with a mixture of AAs in older hospitalized patients during 7 days of low mobility was associated with a clinically relevant effect on LOS. Furthermore, the supplementation reduced the rate of post-discharge falls and hospitalizations. Finally, the positive effects of AA supplementation were related to the preservation of muscle architecture and strength, and to changes in circulating ILs and oxidative stress markers. To elucidate a possible mechanism underlying the beneficial effects in humans, we tested the AA mixture in a rodent model of acute immobilization. Results related to muscle performance revealed that the AA supplementation was effective in limiting the loss of endurance and strength caused by disuse. Of interest, while immobilization led to a reduction in mitochondrial yield and function, and an increase in markers of mitochondrial oxidative stress, the AA supplementation was able to limit these damaging effects. Our results confirm that mitochondrial density is lower in skeletal muscle from animals undergoing limb immobilization [152,153]. Both morphological and functional alterations in muscle mitochondria by acute disuse may be influenced by fast changes in cellular energy demand, hormone status, and interference of intracellular redox homoeostasis [152]. Several studies showed that SM atrophy is associated with induction of pro-apoptotic genes and DNA fragmentation, and mitochondria play a crucial role in coordinating these processes [152]. We confirm previous evidence that showed how immobilization resulted in TA atrophy, characterized by a reduction in both wet weight and TA/body weight ratio. The reduction in skeletal muscle fibers observed after acute disuse might be dependent on increased apoptosis in SM, which can be modulated by increased pro-apoptotic (e.g. Bax and Bid) and/or reduced antiapoptotic signals (e.g. Bcl-2 and Bcl-XL), which induce caspase-3 activity, the committed step for cell death [152,154].

Activation of caspase-3 was described in immobilization induced muscle atrophy in rats, and caspase-3 inhibitor prevented soleus muscle from losing CSA [152,155]. Kang et al. found increased caspase-3 activity during 2–3 weeks of muscle immobilization along with steady increases in Bax/ Bcl2 ratio [152]. It is conceivable that mitochondrial dysfunction and oxidation in immobilized SM would trigger the mitochondrial-dependent apoptosis cascade, even though further studies are required to define this pathway, and to verify whether the AA supplementation would be effective on preventing it.

This investigation presented several limitations related to the human study. First, it was not designed as a randomized placebo-controlled trial because the choice of the patients determined the inclusion in the intervention group. Nevertheless, we could not observe a selection bias because the researcher did not influence the treatment decision. However, the confounding effects of eventual imbalance could not be completely removed, so that estimates of treatment effects could be biased. Second, the fact that this study was performed in a single center may have presented some bias, such as slightly larger intervention effects than multicenter studies [156]. Thus, multicenter randomized studies are needed to verify these findings. Third, because the small pilot study was not designed to determine the effects of individual nutrients, we could not refer selective effects to specific components. It is also worth noting that the mixture contained vitamins and a small amount of carbohydrates and lipids, with possible effect on the results. A further point of weakness can be represented by the collection of the 90-d data by the caregivers. Finally, this was an open-label study, which presents limited generalizability.

# *6.Conclusion*

AA supplementation during 7 days of low mobility in hospitalized old patients positively affects several clinical outcomes such as hospital LOS, rate of post-discharge falls and, re-hospitalization. These results are associated with improvements in muscle structure and function, circulating ILs, and oxidative stress markers. Furthermore, we demonstrated that the AA mixture prevents loss of muscle mass and function in skeletal muscle atrophy by protecting mitochondria. Other than providing a further link between mitochondria and proteostatic manteinance to muscle atrophy, these results encourage further research, even designed as randomized placebo-controlled trials, aimed at targeting mitochondria to treat sarcopenia.

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