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EFFICACY OF INNOVATIVE PHARMACOLOGICAL INTERVENTIONS TO COUNTERACT PHYSICAL DECLINE

PhD Student:
Dott.ssa Roberta Lenti

Coordinator:
Chiar.mo Prof. Renzo Luisi

Tutor:
Chiar.ma Prof.ssa Annamaria De Luca

ANNAMARIA
DE LUCA
20.05.2026
11:26:52
GMT+01:00

Co-Tutor:
Chiari.ma Prof.ssa Paola Mantuano

03/06/2026 14:17:39
ROBERTA LENTI

Roberta Lenti

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Abstract

Introduction. Physical decline is one of the major biological and clinical challenges associated with population ageing. Among the conditions that most contribute to the loss of functional independence in older adults, sarcopenia plays a central role. Sarcopenia is a specific form of age-related muscle atrophy characterised by a progressive decline in muscle mass and strength, which can lead to an increased risk of disability, falls and hospitalisation. Furthermore, this condition is frequently associated with comorbidities typical of advanced age, including neurodegenerative diseases, diabetes, and renal impairment, which further worsen the overall clinical picture. Despite its high prevalence in the elderly population, the biological mechanisms underlying muscle decline remain only partially understood, and to date no specific pharmacological therapies are available to effectively counteract its progression. The diagnosis of sarcopenia also presents several limitations: current diagnostic criteria rely mainly on the assessment of muscle strength, physical performance, and muscle mass using instrumental techniques that require specialised personnel and dedicated equipment, which are often not easily available in routine clinical practice. In this context, the identification of reliable circulating biomarkers with potential diagnostic and prognostic value, alongside the development of preclinical studies to improve understanding of the molecular mechanisms underlying sarcopenia, are key to promoting early diagnosis and more effective therapeutic approaches. Within this scientific framework, the present PhD thesis was developed as part of the national research programme Age-It (Ageing Well in an Ageing Society), which is funded by the Italian National Recovery and Resilience Plan (PNRR). The programme's objective is to promote an interdisciplinary approach to the study of ageing processes and the development of innovative strategies to support healthy and sustainable ageing.

Rationale & Aims. The present thesis investigates muscle decline through an integrated approach combining clinical and preclinical studies. In this context, my research activity was directly carried out within Spoke 8 of the Age-It programme, which is dedicated to the development and evaluation of multidomain interventions to prevent functional and cognitive decline in older adults. In line with the programme's interdisciplinary approach, this research also includes preclinical investigations. These are aimed at identifying innovative nutritional and pharmacological therapeutic strategies, as well as elucidating key molecular mechanisms underlying sarcopenia and muscle atrophy.

Aim 1) IN-TeMPO Project: Identification of plasma biomarkers of sarcopenia

In the context of Spoke 8 of the Age-It project, the IN-TeMPO clinical study (ItaliaN with Multidomain Personalized Interventions to Prevent Functional and Cognitive Decline in Community-Dwelling Older Adults; WP1 of Spoke 8) aims to identify sarcopenia biomarkers in community-dwelling older adults. Prior to the initiation of clinical study, I participated in a literature meta-analysis conducted by the WP1 task force. The objective of this analysis was to identify the most relevant circulating biomarkers to be incorporated into the analytical panel for the evaluation of functional and physical decline. According to the available evidence, six biomarkers representative of the main biological processes involved in sarcopenia, including inflammation, metabolic regulation, and endocrine signalling, have been selected: interleukin-6 (IL-6), growth differentiation factor-15 (GDF-15), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), irisin, and ghrelin. The study enrolled 1,662 community-dwelling older adults, recruited through general practitioners, memory clinics, and public awareness campaigns in collaboration with local health authorities. The inclusion criteria encompassed age ≥60 years, a Primary Care Frailty Index between 0.07 and 0.21, a CAIDE score ≥6, a Clinical Dementia Rating (CDR) ≤0.5, and the presence of modifiable risk factors or a family history of dementia. Participants were randomly assigned to receive personalised multidomain

interventions (physical activity, nutritional interventions, cognitive training, and management of metabolic and vascular risk factors) or self-guided interventions supported by digital tools. To obtain a preliminary evaluation of biomarker trends prior to the conclusion of the 12-month follow-up, an intermediate analysis was conducted after 6-months in a subgroup of 53 participants enrolled at the Bari centre. Biomarker analyses were performed using ELISA assays.

Aim 2) Preclinical evaluation of BCAA-based formulations in mouse model of physiological aging

At the clinical level, previous experimental evidence demonstrated the beneficial effects of formulations based on branched-chain amino acids (BCAAs) on muscle function and mass in the context of muscle atrophy. This study therefore focused on evaluating the potential of BCAAs, administered alone or in combination with 2-ALA or Di-ALA, to restore intracellular Ca^{2+} homeostasis in skeletal muscle. A mechanism known to be impaired in sarcopenia. Furthermore, the potential renoprotective and antifibrotic effects were investigated, taking into account the physiological cross-talk between skeletal muscle and kidney. Treatments were administered for a period of 12 weeks in aged wild-type mice. This was achieved using an integrated experimental approach, which included L Q Yrlr Y HR[Yfunctional analyses.

Aim 3) Evaluation of the effects of the growth hormone secretagogue JMV2894 in the D2- P G[mouse model

The D2.B10-'P G[J (D2- P G[) model is widely used for the study of Duchenne muscular dystrophy (DMD), a neuromuscular disorder X-linked recessively inherited that results in the absence of dystrophin. This murine model is characterised by a pronounced pro-atrophic and pro-fibrotic background, which also makes it a valuable experimental model for studying mechanisms underlying muscle atrophy and for evaluating anti-atrophic therapeutic candidates that may have potential translational

relevance for sarcopenia. In light of beneficial effects of growth hormone secretagogues JMV2894 on skeletal muscle reported in previous studies, we investigated the potential therapeutic impact of the compound in this model. Moreover, considering the systemic nature of muscle decline, the evaluation was also extended to renal outcomes.Treatment was administered subcutaneously for six weeks at two different doses (640 µg/kg and 1280 µg/kg), and its effects were evaluated using validated functional, molecular, and histological endpoints, both L Q Yand Y HR[Y L Y R

Results.

Aim 1. Baseline results (n=53) showed no significant differences between sexes, regardless of the biomarker analysed. Therefore, sex was not a relevant confounding factor in the studied population, and the observed variability was mainly attributable to inter-individual heterogeneity. The six-month longitudinal analysis (n=37) demonstrated a trend consistent with the physiological muscle decline associated with ageing, with a tendency toward decreased BDNF, IGF-1, and irisin levels and increased GDF-15 and ghrelin, while IL-6 levels remained substantially stable. Overall, the multidomain intervention did not result in significant changes in circulating biomarkers compared with the control group, although an increase in BDNF levels was observed in male participants undergoing the intervention. These findings underscore the necessity for additional studies employing larger sample sizes and extended follow-up periods to elucidate the diagnostic and prognostic significance of these biomarkers.

Aim 2. In the murine model of physiological ageing, Ca^{2+} imaging in flexor digitorum brevis (FDB) muscle fibres revealed impairment of the store-operated calcium entry (SOCE) mechanism associated with increased resting intracellular Ca^{2+} concentration, confirming altered calcium homeostasis in sarcopenic muscle. In gastrocnemius (GC) muscles, these alterations were accompanied by changes in the gene and protein expression of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1), ryanodine receptor 1 (RyR1), and the proteins mitsugumin-29 (MG29) and mitsugumin-53 (MG53), which are involved in SOCE regulation. BCAAs-based

formulations enhanced muscle contractility and counteracted the age-related shift in the Hz50 parameter, an indirect index of improved Ca^{2+} handling. Following rigorous testing, it was determined that the formulation of BCAAs + 2ALA was the most effective in restoring SOCE activity and modulating the expression of proteins involved in calcium homeostasis. At the renal level, treatment also demonstrated a protective effect against tubulo-interstitial fibrosis, associated with modulation of key mediators of inflammatory and fibrotic processes. Overall, these findings support the potential of BCAAs-based formulations, particularly BCAAs + 2ALA, as a nutritional strategy to counteract muscle decline and some organ alterations associated with ageing.

Aim 3. In the D2-mdx murine model, treatment with the growth hormone secretagogue JMV2894 demonstrated the ability to modulate several pathways involved in muscle degeneration and tissue remodelling processes. Specifically, a decrease in the expression of fibrosis-related genes, including transforming growth factor beta 1 (TGF-β1), matrix metalloproteinase-9 (MMP-9), and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5) along with reduced levels of the pro-atrophic markers muscle atrophy F-box protein (Atrogin-1) and muscle RING finger protein-1 (MuRF-1), was noted. These effects were accompanied by an increase in muscle fibre size and muscle volume, suggesting a potential anti-atrophic effect of the compound. Mechanistically, the results suggest a possible involvement of the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis and the protein kinase B/glycogen synthase kinase-3 beta (Akt/GSK-3β) signaling pathway, which play key roles in muscle growth and regeneration. The analysis was also extended to the renal compartment, where the D2-mdx model showed increased collagen deposition, indicating a systemic pro-fibrotic background. Treatment with JMV2894 demonstrated a tendency to decrease fibrotic processes, also at the renal level, indicating a possible multi-organ protective effect. Overall, these results indicate that JMV2894 can modulate key pathways involved in muscle degeneration and fibrotic mechanisms, opening new perspectives for systemic therapeutic strategies.

Conclusions.

Overall, this PhD work addressed muscle decline from multiple perspectives by integrating clinical and preclinical data. The results indicate that muscle decline should not be regarded exclusively as a phenomenon affecting skeletal muscle, but rather as a systemic condition involving multiple organs and biological pathways. clinical evidence suggests that the study cohort is representative of the target population, as baseline plasma levels of the investigated biomarkers are consistent with those reported in the literature. Similarly, evaluations performed at the T6 time point show an overall trend comparable to that described in previous studies. However, a larger sample size and the integration of clinical outcomes with biological data are required to improve the interpretation of treatment effects and the overall reliable of these biomarkers. Concurrently, preclinical investigations provided important mechanistic insights into the pathophysiology of muscle decline and highlighted the therapeutic potential of innovative pharmacological and nutraceutical strategies. In particular, BCAAs-based formulations have been shown to enhance calcium handling and muscle contractile function, while also demonstrating antifibrotic and protective effects at the renal level. This supports the concept of cross-talk between muscles and organs during the ageing process. In addition, treatment with the growth hormone secretagogue JMV2894 modulated key pathways associated with atrophy and fibrosis, promoted muscle fibre hypertrophy, and demonstrated signs of multi-organ protective activity, reinforcing its potential as a systemic anti-wasting therapeutic approach. In this context, the multidisciplinary approach promoted by the Age-It programme represents a valuable framework for improving our understanding of the mechanisms underlying sarcopenia and for identifying novel diagnostic, preventive, and therapeutic strategies aimed at promoting healthy ageing.

Chapter 1 – Introduction

1.1. Skeletal muscle: structure, function and plasticity

Skeletal muscle is a highly specialised organ that performs essential functions such as generating force for voluntary movement through the transduction of chemical energy into mechanical energy, enabling postural control and protecting bones and internal organs (Frontera & Ochala, 2015a). In addition to its mechanical role, skeletal muscle is an endocrine organ capable of secreting myokines with autocrine, paracrine and endocrine effects (e.g., irisin, IL-6, BDNF) that act as hormones, communicating with other organs and positively influencing metabolism, the immune system, brain health and longevity. From a metabolic standpoint, skeletal muscle contributes significantly to basal energy expenditure, serves as the primary site for glycogen storage and amino acid reserves, and is essential for thermogenesis through heat production associated with muscle contraction and oxidative metabolism (Iglesias, 2025; Schnyder & Handschin, 2015).

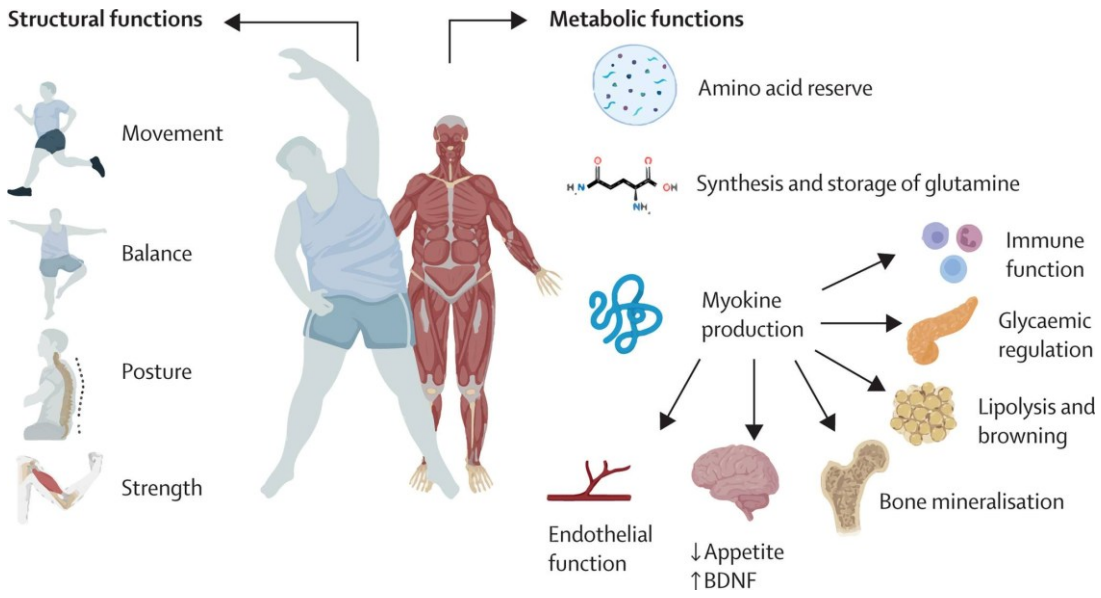

Fig.1: **Structural and metabolic roles of skeletal muscle** 3 U D G R H W D O

1.1.1. Structural organisation of skeletal muscle tissue

From a structural standpoint, skeletal muscle is characterised by a highly specialised hierarchical organisation. The muscle is entirely enveloped by the epimysium, a dense connective tissue that provides mechanical protection and facilitates the transmission of contractile force to the tendons. Internally, the muscle is divided into muscle bundles or fascicles, with an average diameter of approximately 100 μm and a length that can reach 1 cm; each fascicle is surrounded by the perimysium, a connective membrane within which networks of blood vessels and nerve fibres run. Each fascicle consists of numerous muscle fibres (or myocytes), which are elongated, multinucleated cells arranged in parallel. These are covered by the sarcolemma (cell membrane), and a thin layer of connective tissue called the endomysium. A distinctive feature of muscle fibres is their multinucleated, syncytial nature. During the process of embryonic myogenesis, mesenchymal precursor cells undergo a series of cellular changes that result in the formation of mononuclear myoblasts. These cells then proceed to proliferate, a process that is crucial for the activation of myogenic differentiation programmes. At this stage, the expression of specific factors, including myogenin and the fusion proteins myomaker (TMEM8c) and myomerger (miomixer/minion), allows proliferation to arrest and myoblasts to fuse into multinucleated syncytia (Chen et al., 2020). This process leads to the formation of myotubes, which progressively mature into functional muscle fibres containing organised and contractile myofibrils.

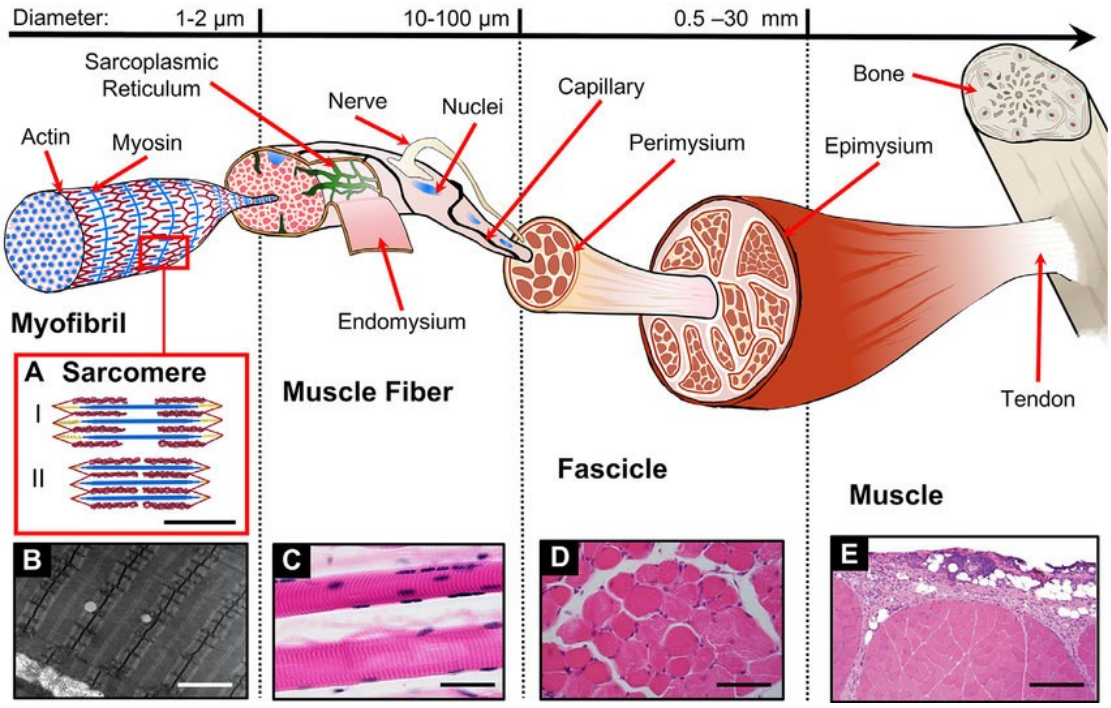

Fig. 2 Hierarchical organization of skeletal muscle tissue. (A) Sarcomere morphology and filament sliding mechanism. (B) Transmission electron microscopy (TEM) image of myofibrils. (C) Phase-contrast microscopy (PCM) image of skeletal muscle fibers; dark purple elliptical structures correspond to myocyte nuclei. (D) Histological image of a transverse section of a fascicle: the wider white bands represent the perimysium; circular structures correspond to muscle fibers, while darker purple dots indicate myocyte nuclei. (E) Histological image of a portion of a transverse section of muscle; the epimysium is visible in the upper region (& R S \ U L J K W (*OR WHHW WL HHUW %D9O

The morphofunctional unit of muscle fibres is the sarcomere, a highly organised structure that is responsible for the contraction of voluntary muscles (Huxley, 1969). It consists of two main systems of myofilaments: thick and thin filaments (Bottinelli & Reggiani, 2000). Their regular arrangement gives muscle tissue its characteristic striated appearance, which is observable under an optical microscope as alternating light and dark bands. This organisation determines the formation of regions with different optical properties:

- Band A: is anisotropic and birefringent and contains both thick and thin filaments. In the central region of band A is the H zone, which consists exclusively of thick filaments. At the centre of the H zone is the M line, which

represents the site of anchoring and alignment of the myosin filaments (Agarkova & Perriard, 2005).

- Band I: is isotropic and monorifractive and is composed exclusively of thin filaments. It is crossed centrally by the Z line, which delimits the sarcomere and defines its structural unit (Cheng & Deatherage, 1989).

Specifically, the thick filaments have a diameter of approximately 12 nm and consist mainly of myosin organised into six bundles, each formed by two heavy chains and four light chains. One end of the myosin contains a globular region known as the head, which exhibits ATPase activity and is responsible for forming cross bridges with actin. The thin filaments have a diameter of 4 nm and consist of two double-helical actin chains that are associated with tropomyosin and the troponin regulatory complex. This complex is formed by the Troponin I (TnI), Troponin C (TnC) and Troponin T (TnT) subunits. These components together regulate the accessibility of myosin binding sites on actin in response to changes in intracellular Ca^{2+} concentration. This allows the activation of the cross-bridge cycle and consequently, muscle contraction. The structural stability and correct positioning of sarcomeric components are ensured by cytoskeletal proteins, like titin, nebulin and the costamer, which includes the glycoprotein complex associated with dystrophin, whose alteration is implicated in neuromuscular diseases such as Duchenne muscular dystrophy (Birnkrant et al., 2018; Capogrosso et al., 2018; Exeter & Connell, 2010; Frontera & Ochala, 2015b).

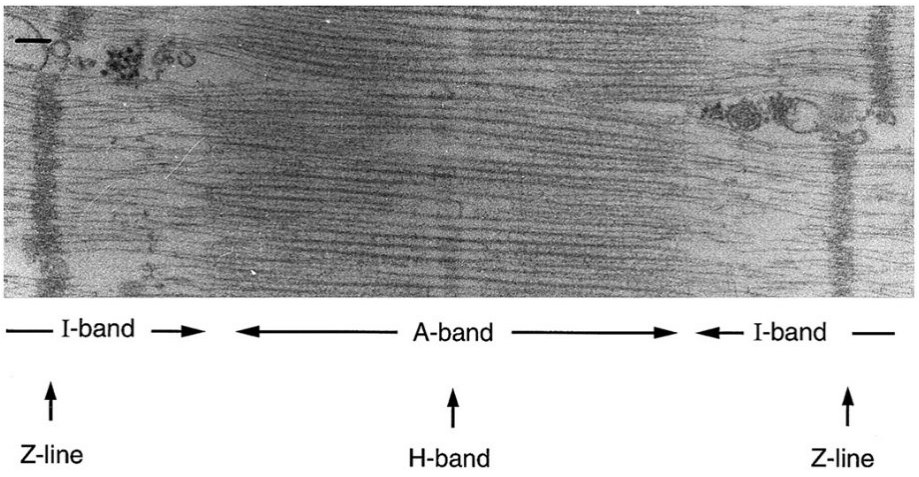

Fig. 3 Electron micrograph of a longitudinal section of a sarcomere from rabbit psoas muscle. The A-band, I-band, H-band and Z-line are indicated. Scale bar (upper left), 100 nm. 0 L O O P D Q

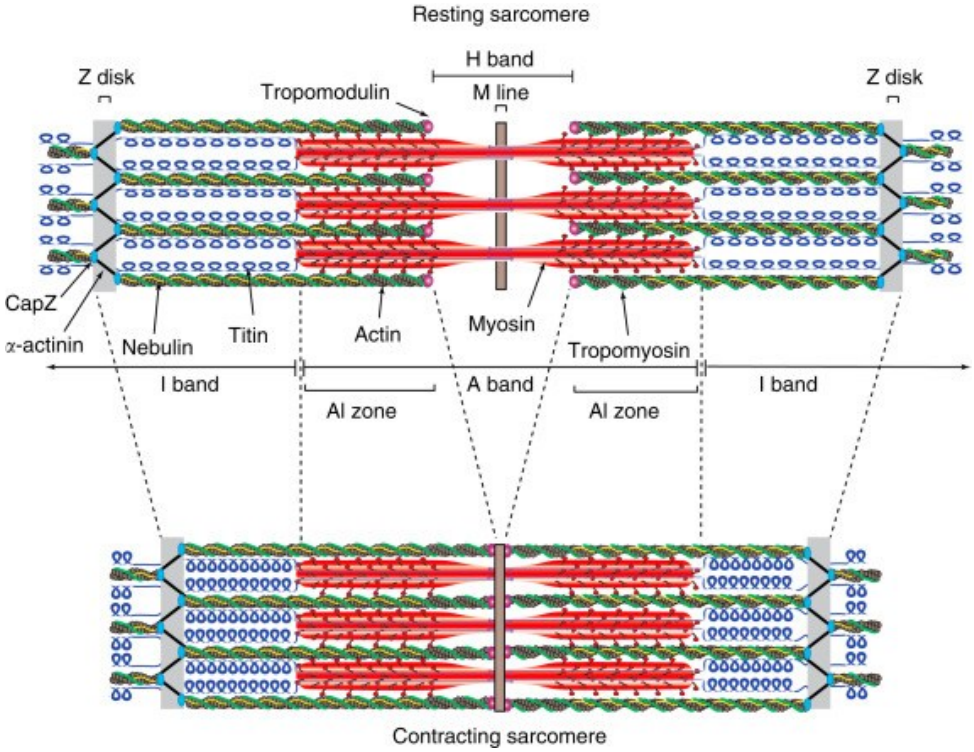

Fig. 4 Sarcomere structure in resting and contracting states & D G R W * R P H V

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In relation to their contractile and metabolic properties, muscle fibres can be classified according to specific functional and molecular parameters (Talbot & Maves, 2016), including:

- contraction velocity (slow or fast fibres);
- metabolic profile (oxidative or glycolytic);
- the myosin heavy chain (MyHC) isoform expressed and its associated ATPase activity.

Based on these criteria, two main categories of muscle fibres can be distinguished: (Figure 6).

- Type I fibres, corresponding to red fibres or slow-twitch fibres (ST), are defined as slow oxidative fibres. They predominantly express the slow myosin isoform (MyHC-I), exhibit a high mitochondrial density, and display a marked capacity for ATP synthesis via oxidative phosphorylation, alongside relatively low glycolytic activity. Their metabolism is therefore predominantly aerobic. These fibres are characterised by a small diameter and rich capillary supply, consistent with their dependence on oxygen to sustain contractile activity. In support of this function, they contain high concentrations of myoglobin, a protein responsible for the reversible binding and storage of oxygen. From a functional perspective, type I fibres generate low-force contractions but are capable of sustaining them over prolonged periods, exhibiting a high degree of fatigue resistance.
- Type II fibres, corresponding to white fibres or fast-twitch fibres (FT), are instead characterised by rapid contraction and a predominantly glycolytic metabolism. They exhibit reduced capillarisation, low myoglobin content, and high activity of glycolytic enzymes, producing ATP primarily through anaerobic pathways. Their larger diameter allows the generation of high force in response to high-intensity stimuli, albeit for short durations, rendering them more susceptible to fatigue.

Type II fibres can be further subdivided into subtypes with distinct functional and metabolic characteristics:

- Type IIa fibres exhibit an intermediate phenotype, characterised by rapid contractile responses and the ability to utilise both oxidative and glycolytic metabolic pathways.
- Type IIb and Type IIx fibres are characterised by rapid contraction and predominantly glycolytic metabolism. This is associated with high shortening velocity and a light colour due to low myoglobin content. While Type IIx fibres share many features with Type IIb fibres, they exhibit a slightly lower contraction velocity and a greater oxidative metabolic component. This gives them a moderately higher resistance to fatigue.

Although different fibre types coexist within a single muscle, they are not activated simultaneously but are organised into distinct motor units and recruited according to a hierarchical order (size principle), depending on mechanical and metabolic demands (Wakeling et al., 2006). This heterogeneity of physiological properties enables skeletal muscle to adapt to a wide range of functional tasks. Furthermore, in response to prolonged or repeated stimuli, muscle fibres can undergo structural, metabolic, and functional modifications due to a fundamental property of muscle tissue known as muscle plasticity (Flück & Hoppeler, 2003).

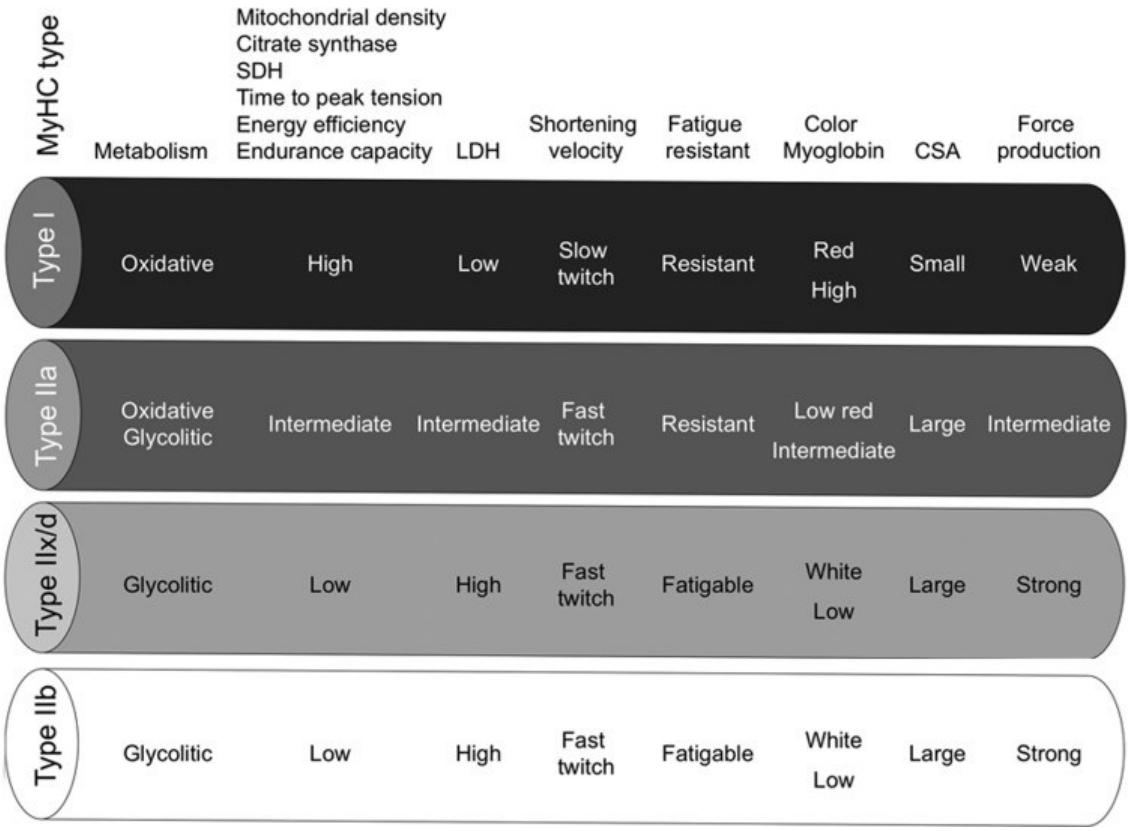

Fig. 5: Characteristics of mammalian skeletal muscle fiber types) H U U D U R H W D O

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1.1.2. Excitation contraction coupling and calcium regulation

At the ultrastructural level, the regulation of intracellular calcium is mediated by triads, which are located at the level of the Z line and represent the fundamental structural units of excitation–contraction coupling. Each triad consists of a central T-tubule, an invagination of the sarcolemma that enables the rapid propagation of the action potential within the muscle fibre, flanked by two terminal cisternae of the sarcoplasmic reticulum, which constitute the primary intracellular Ca^{2+} store.

From a molecular point of view, triads contain a highly specialised complex of proteins that are responsible for regulating calcium release and reabsorption. The dihydropyridine receptor (DHPR), located in the T-tubule membrane, acts as a voltage sensor. In response to membrane depolarisation, it mechanically activates the type 1 ryanodine receptor (RyR1), which is located on the membrane of the terminal cisternae of the sarcoplasmic reticulum. Activation of the RyR1 receptor causes a large release of Ca^{2+} into the sarcoplasm, which is a key event in initiating muscle contraction.

An increase in cytosolic Ca^{2+} concentration promotes the binding of the ion to the TnC subunit of the troponin complex, inducing a conformational change that results in the translocation of tropomyosin along the actin filament and the consequent exposure of myosin binding sites. Under these conditions, the myosin head, previously activated by ATP hydrolysis, interacts with actin to form the cross bridge and generate the power stroke. This event is associated with a conformational transition that involves the rotation of the myosin head from an initial angle of approximately 45° to a configuration of about 90°, accompanied by the release of ADP. The subsequent binding of a new ATP molecule to the myosin head induces dissociation of the actin myosin complex, while subsequent ATP hydrolysis restores the energetic state of myosin, allowing initiation of a new contractile cycle. This sequence of molecular

events is collectively described by the Sliding Filament Theory. (Bolaños & Calderón, 2022; Kuo & Ehrlich, 2015; SANDOW, 1952) (Figure 6).

The cyclic repetition of these events results in sarcomere shortening and the maintenance of muscle contraction, if Ca^{2+} remains bound to TnC. In this context, a fine equilibrium is needed between excitation and dihydropyridine-coupled calcium release by sarcoplasmic reticulum RYR channels, calcium reuptake by the Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) pump, and store-operated calcium entry (SOCE). SOCE is known to involve canonical components such as stromal interaction molecule 1 (STIM1) and calcium release-activated calcium channel protein (ORAI1), plus other proteins such as mitsugumin 53 (MG53) and mitsugumin 29 (MG29), which are emerging as novel key players in the SOCE mechanism (Gruszczynska-Biegala et al., 2021). SOCE is important in limiting fatigue during repetitive high-frequency stimulation (Boncompagni et al., 2017; Cho et al., 2017; Michelucci et al., 2020; Protasi et al., 2023; Wei-LaPierre et al., 2013), whereas its alteration has been associated with the progression of some muscular disorders, including muscular dystrophy (Edwards et al., 2010; Harisseh et al., 2013) and cachexia (E. Conte et al., 2017a), as well as aging. In particular, together with an increase in cytosolic Ca^{2+} concentration (Fodor et al., 2020; Mijares et al., 2021a), a reduction in SOCE activity contributes to age-related muscle weakness (Thornton et al., 2011a; Zhao et al., 2008a) (Figure 7).

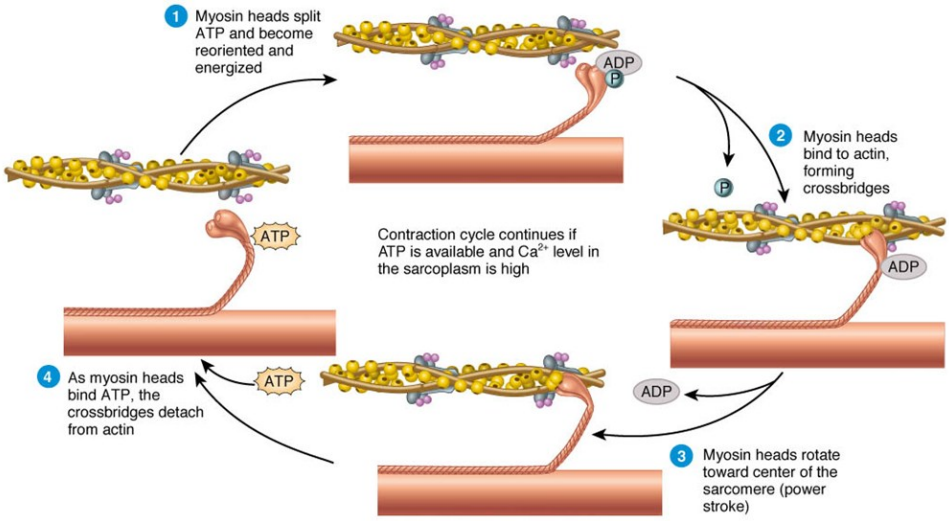

Fig. 6 Schematic representation of the Sliding Filament Theory (W K H 4 W Q H V V W U D L Q H U D F D G H P

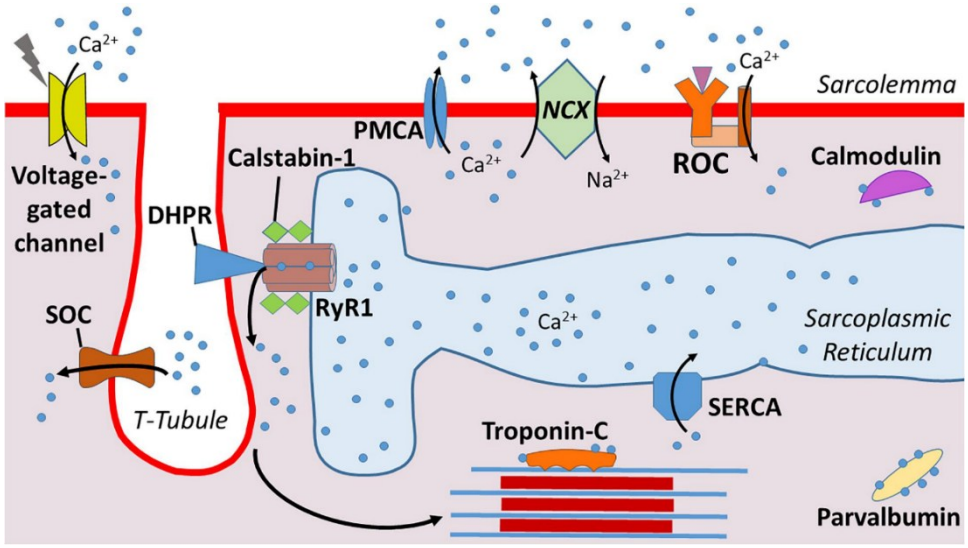

Fig.7 Schematic representation of Ca^{2+} regulation at the sarcolemma. Dihydropyridine receptors (DHPR) contribute to the regulation of Ca^{2+} release from the sarcoplasmic reticulum (SR) into the cytosol through functional coupling with RyR1. Calstabin-1 binds RyR1 and stabilizes the channel, promoting its closed state. Cytosolic Ca^{2+} is re-sequestered into the SR by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). Ca^{2+} influx from the extracellular space occurs through voltage-gated channels, store-operated channels (SOC), and receptor-operated channels (ROC). Ca^{2+} extrusion from the cytosol is mediated by plasma membrane Ca^{2+}-ATPases (PMCA) and the Na^{+}/Ca^{2+} exchanger (NCX). Cytosolic free Ca^{2+} levels are further modulated by Ca^{2+}-binding proteins, including Troponin-C, parvalbumin, and calmodulin + \ D W W 3 R Z H U V

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1.1.3. Muscle plasticity

Muscle plasticity is defined as the ability of skeletal muscle to dynamically adapt to specific functional demands by modifying its structural, functional and metabolic properties. This phenomenon has been observed across all vertebrate species, although with considerable interspecies variability. Skeletal muscle is therefore considered a highly malleable tissue, characterised by a remarkable capacity for remodelling in response to a wide range of physiological and environmental stimuli, including resistance and endurance training (Bodine, 2013; Ferraro et al., 2014), disuse (Bodine, 2013), hypoxia (Lundby et al., 2009) unloading conditions or microgravity (Cannavo et al., 2022), cold exposure (van den Berg et al., 2011; VOGT et al., 2003), nutritional modifications (Vogt et al., 2003) and ageing (Shaikh et al., 2025).

In response to these stimuli, skeletal muscle undergoes coordinated adaptive processes involving changes in myofibre phenotype, contractile function and metabolic profile, together with alterations in neuronal input, endocrine regulation and intracellular signalling pathways. Importantly, muscle plasticity represents a multilevel phenomenon that extends beyond myofibre remodelling and includes adaptations at the neuromuscular junction, activation and differentiation of satellite cells, extracellular matrix turnover, modulation of vascularisation and systemic metabolic adjustments (Flück & Hoppeler, 2003b)

Muscle adaptations can be broadly classified into quantitative and qualitative responses. Quantitative adaptations primarily involve changes in muscle mass, resulting in hypertrophy or atrophy depending on the balance between protein synthesis and degradation (Flück & Hoppeler, 2003b). Qualitative adaptations, on the other hand, consist of phenotypic transitions between different myofibre subtypes mediated by fibre type-specific gene expression programmes and modifications in the expression of myosin light chain (MLC) and MyHC isoforms. These molecular changes lead to

reciprocal transitions between slow- and fast-twitch fibres, ultimately influencing force production and shortening velocity (Pette & Staron, 2000; Puhke et al., 2006).

Physical exercise represents one of the most relevant physiological stimuli regulating muscle plasticity and is largely determined by patterns of neural activation associated with sustained elevations in cytosolic Ca^{2+} levels (Oh et al., 2005; Rose et al., 2007). Among calcium-dependent signalling pathways, calcineurin plays a key role in modulating fibre type-specific transcriptional programmes. Selective activation of calcineurin promotes the expression of genes associated with the oxidative phenotype of type I fibres, whereas its inhibition favours gene expression typical of type II fibres (Oh et al., 2005).

Muscle plasticity also follows a principle of stimulus specificity. Endurance training predominantly enhances oxidative metabolism and ATP production through aerobic pathways by promoting mitochondrial biogenesis and upregulating enzymes involved in the tricarboxylic acid cycle and the electron transport chain (Gnaiger, 2009; Hood et al., 2011), without necessarily inducing substantial increases in muscle fibre size. Conversely, resistance training, characterised by repeated exposure to high mechanical loads, induces significant hypertrophic adaptations, mainly affecting type II fibres and leading to increased myofibrillar content and force-generating capacity (Ferraro et al., 2014).

However, the regulatory mechanisms underlying muscle plasticity do not exclusively lead to beneficial functional adaptations. Under chronic or pathological conditions, such as prolonged inactivity, systemic inflammation, metabolic disorders or neuromuscular diseases, these adaptive processes may become dysregulated, contributing to muscle atrophy, fibrotic remodelling and functional (Grosicki et al., 2022; Stouth et al., 2017) Similar alterations have been described in genetic myopathies, including Duchenne muscular dystrophy (Stouth et al., 2017; Grosicki et al., 2022).

Furthermore, skeletal muscle undergoes continuous remodelling during ageing, even in the absence of significant mechanical stimuli. Age-related muscle decline is often characterised by a preferential reduction in the size and number of fast-twitch fibres and by progressive impairment of functional performance (Grosicki et al., 2022; Shaikh et al., 2025). Ageing is also associated with a reduced sensitivity of skeletal muscle to anabolic stimuli, a phenomenon commonly referred to as anabolic resistance, which increases vulnerability to disuse, chronic disease and systemic inflammation (Pérez-Castillo et al., 2025).

Overall, these observations highlight that the balance between physiological adaptation and maladaptation in skeletal muscle is tightly regulated by complex intracellular signalling networks controlling protein turnover, energy metabolism and cellular stress responses. Therefore, understanding the molecular pathways regulating muscle hypertrophy and atrophy is essential to elucidate the mechanisms of muscle decline and identify potential therapeutic targets. These mechanisms are discussed in the following section.

1.1.4. Molecular pathways involved in muscle hypertrophy and atrophy

Within the framework of muscle plasticity, hypertrophy and atrophy represent two opposing adaptive outcomes of skeletal muscle remodelling in response to physiological, metabolic and pathological stimuli. Muscle atrophy is characterised by a progressive reduction in muscle mass and strength, associated with a decrease in myofibre cross-sectional area (CSA), protein content and contractile capacity. Conversely, hypertrophy manifests as an increase in muscle fibre size and protein synthesis, typically occurring in response to increased mechanical loading or anabolic endocrine and nutritional stimuli (Mukund & Subramaniam, 2020).

The regulation of muscle mass depends on the dynamic integration of anabolic and catabolic signals that converge on the control of protein synthesis, protein degradation and organelle turnover. In this context, the insulin-like growth factor-1 (IGF-1)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signalling pathway represents one of the major regulatory nodes of muscle anabolism. Binding of IGF-1 to its receptor activates a phosphorylation cascade that culminates in AKT activation. AKT promotes muscle growth through stimulation of the mTORC1 complex, which regulates protein translation and ribosomal biogenesis via phosphorylation of downstream targets such as eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and ribosomal protein S6 kinase beta-1 (p70S6K). At the same time, AKT exerts anti-catabolic effects by inhibiting the nuclear translocation of Forkhead box O (FoxO) transcription factors and suppressing the expression of several atrophy-related genes (atrogenes) involved in catabolic processes, including muscle-specific ubiquitin ligases atrogin-1 (MAFbx) and MuRF-1, which are responsible for activation of the ubiquitin–proteasome system (Gingras et al., 2001; Sandri et al., 2004; Schiaffino & Mammucari, 2011).

However, muscle mass regulation does not depend exclusively on the balance between protein synthesis and proteasome-mediated proteolysis. The autophagy-lysosome system also plays a crucial role by contributing to the turnover of damaged proteins and cellular organelles, particularly mitochondria. Autophagy activation is regulated by energy-sensing pathways such as AMP-activated protein kinase (AMPK). Under conditions of metabolic stress, disuse or nutritional restriction, AMPK inhibits mTOR signalling and promotes catabolic processes aimed at restoring cellular energy homeostasis, although its chronic activation may contribute to muscle mass loss.(Sandri, 2013; Xia et al., 2021).

Additional negative regulators of muscle growth include factors belonging to the transforming growth factor-β (TGF-β) superfamily, such as myostatin. Experimental evidence indicates a close interaction between myostatin signalling and the AKT/mTOR axis: pharmacological inhibition of mTOR by rapamycin has been shown to attenuate the hypertrophic effects induced by myostatin blockade, suggesting a functional role of this pathway in the modulation of muscle growth. Moreover, inflammatory pathways mediated by nuclear factor-κB (NF-κB) contribute to muscle wasting by inducing the expression of genes involved in proteolysis, oxidative stress and mitochondrial dysfunction (Sandri, 2013; Vainshtein & Sandri, 2020).

These signalling networks are profoundly influenced by systemic factors such as chronic low-grade inflammation, altered energy metabolism and mitochondrial dysfunction, conditions that are typically associated with ageing and chronic diseases (Baechle et al., 2023). Overall, muscle mass regulation emerges as the result of the interaction of multiple intracellular pathways and systemic signals that control not only protein balance but also energy metabolism, redox homeostasis, mitochondrial function and inflammatory processes. It should be emphasised that the pathways described represent only part of a highly complex and interconnected molecular network involved in the maintenance of muscle homeostasis. An integrated

understanding of these mechanisms is therefore crucial for the identification of novel therapeutic targets aimed at counteracting muscle decline associated with ageing and neuromuscular disorders.

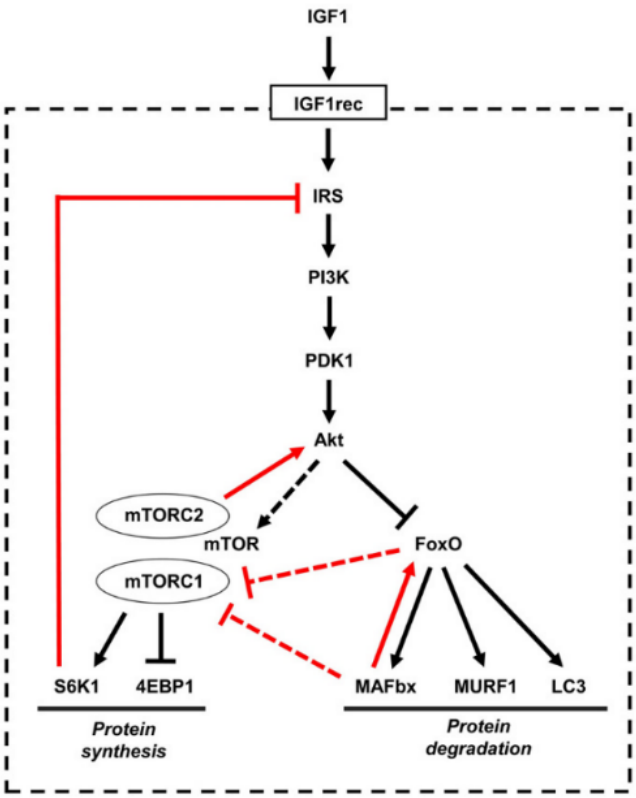

Fig. 8: IGF-1 pathway and regulation of protein synthesis 6 F K L D I I L Q R 0 D P P X F D U L

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1.2. Physical decline and skeletal muscle in ageing

Physical decline is one of the main biological and clinical challenges of adulthood and old age. It is a crucial factor in the loss of independence and reduced quality of life, as well as an increased risk of disability and hospitalisation. This process involves a progressive deterioration of musculoskeletal function, manifesting as a reduction in muscle mass, strength and physical performance. This significantly compromises an individual's ability to respond to physiological and pathological stress. While this clinical condition is partly attributable to biological ageing and associated mechanisms such as low-grade chronic inflammation (inflammaging) and oxidative stress, it is also strongly influenced by modifiable factors including sedentary lifestyle, malnutrition, chronic diseases (cardiometabolic, respiratory and neurodegenerative conditions) and repeated hospitalisation (Ferrucci & Fabbri, 2018; Maldonado et al., 2023; Raffin et al., 2023).

In this context, the progressive ageing of the population represents one of the main challenges for healthcare systems, particularly in high-income countries. This phenomenon is the result of a decline in the birth rate, combined with improved living conditions and survival rates. Italy plays a leading role in this scenario, ranking among the countries with the oldest populations in the world. In 2025, the average age reached 46.8 years, the highest in the European Union. Almost a quarter of Italian citizens are aged 65 or over (24.7%), the number of centenarians has reached a new high (over 23,500) and it is expected that by 2050, the proportion of people over 65 will exceed one third of the population (ISTAT, 2025). This trend is the result of multiple factors, including socio-cultural, environmental, behavioural, economic and demographic elements, such as the structure of family networks, modes of social interaction and the Mediterranean diet. While increased life expectancy is a major public health success, it also poses significant challenges for the national health system, which faces ever-increasing healthcare and welfare costs.

Ageing is a highly heterogeneous process. Individuals of the same chronological age may exhibit profoundly different functional profiles, influenced by lifestyle, daily habits, and the presence of diseases and comorbidities (Tenchov et al., 2024). Given this heterogeneity, further investigation of the molecular mechanisms of ageing and physical decline is needed to develop preventive strategies and innovative interventions aimed at promoting healthy ageing.

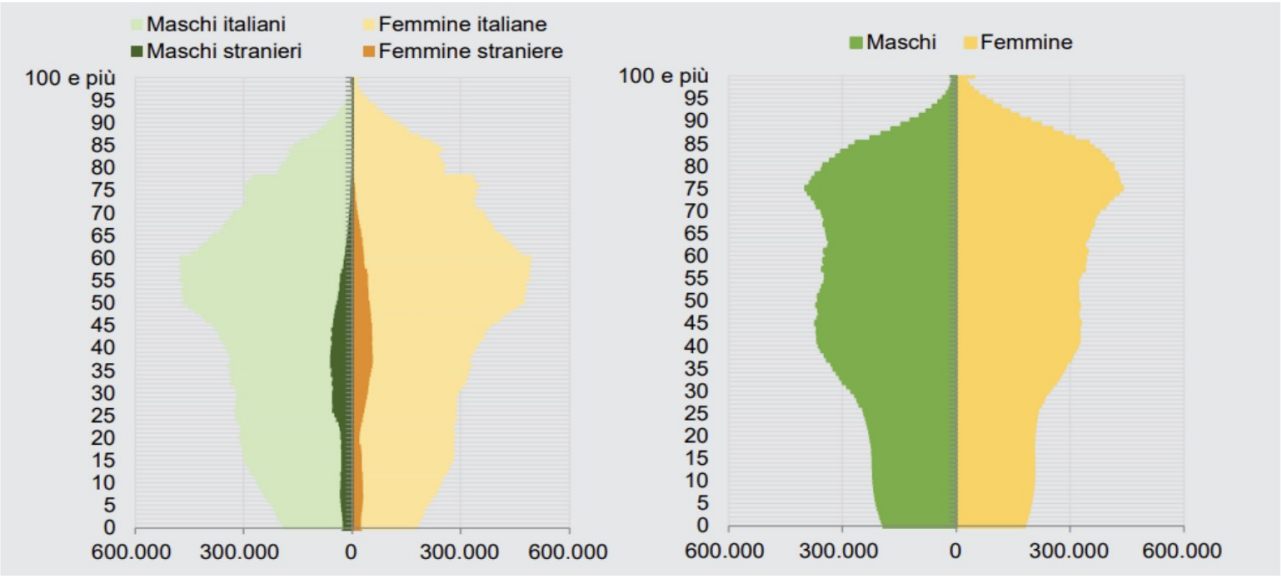

Fig. 9: Age pyramid of the resident Italian and foreign population by sex as of 1 January 2025 (left panel) and projected total resident population by sex as of 1 January 2050 (right panel). In the 2025 pyramid, Italian males and females are represented in light green and light yellow, respectively, while foreign males and females are shown in dark green and orange. , 6 7 $ 7

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1.2.1. Sarcopenia as a paradigm of physical decline

Sarcopenia is a specific form of muscle atrophy that is closely associated with the ageing process. It is characterised by a progressive and generalised loss of muscle mass and strength (Cruz-Jentoft et al., 2010). This condition has been linked to adverse clinical outcomes, including a decline in physical function, a deterioration in quality of life, an elevated risk of falls, and increased mortality (Wiedmer et al., 2021a). Previously, sarcopenia lacked clarity in its clinical definition, and there was an absence of consensus on diagnostic criteria. It was not until 2010 that the European Working Group on Sarcopenia in Older People (EWGSOP) formally recognised sarcopenia as a geriatric syndrome. Subsequently, the condition was included in the International Classification of Diseases (ICD-10-CM, code M62.84) as a muscular disorder, allowing for its recognition (Smith et al., 2022).

From an aetiological perspective, sarcopenia can be classified into primary forms, directly related to the physiological ageing process, and secondary forms, resulting from the interaction of factors such as physical inactivity, chronic diseases and inadequate nutritional intake (Cruz-Jentoft et al., 2010). Age-related muscle mass loss is estimated to occur at a rate of approximately 1–2% per year after the age of 50 (Larsson et al., 2019; Barone et al., 2025). Both forms are frequently associated with multiple comorbidities, including neurodegenerative diseases, renal failure and metabolic disorders, and share a complex biological profile characterised by cellular senescence, mitochondrial dysfunction, oxidative stress, chronic low-grade inflammation, endocrine alterations, reduced regenerative capacity of satellite cells and increased intramuscular adipose infiltration (Amini et al., 2024; Li et al., 2025).

At the molecular level, sarcopenic muscle is characterised by the activation of proteolytic pathways that disrupt protein homeostasis, favouring a shift towards catabolic signalling. Among the main systems involved is the ubiquitin–proteasome pathway, in which transcription factors such as FoxO3 promote the expression of muscle-specific ubiquitin ligases, including MAFbx and MuRF-1, responsible for the

degradation of myofibrillar and sarcomeric proteins (Mankhong et al., 2020a). Additional contributions to proteolysis derive from the autophagy–lysosome system, calcium-dependent calpains and caspases, which are respectively involved in organelle turnover, cytoskeletal remodelling and apoptotic processes (Wiedmer et al., 2021b).

Recent evidence further indicates that dysregulation of autophagy and mitophagy represents a critical determinant of age-related muscle decline. The reduced capacity to eliminate damaged mitochondria and aggregated proteins compromises cellular energetic function and the regenerative potential of satellite cells, thereby promoting muscle atrophy and functional weakness (Baechle et al., 2023; Vainshtein & Sandri, 2020). In parallel, age-related neuromuscular remodelling, characterised by motor neuron loss, instability of the neuromuscular junction and impaired synaptic transmission, contributes to the selective denervation of fast-twitch fibres and the progressive reduction in motor unit connectivity (Hepple & Rice, 2016).

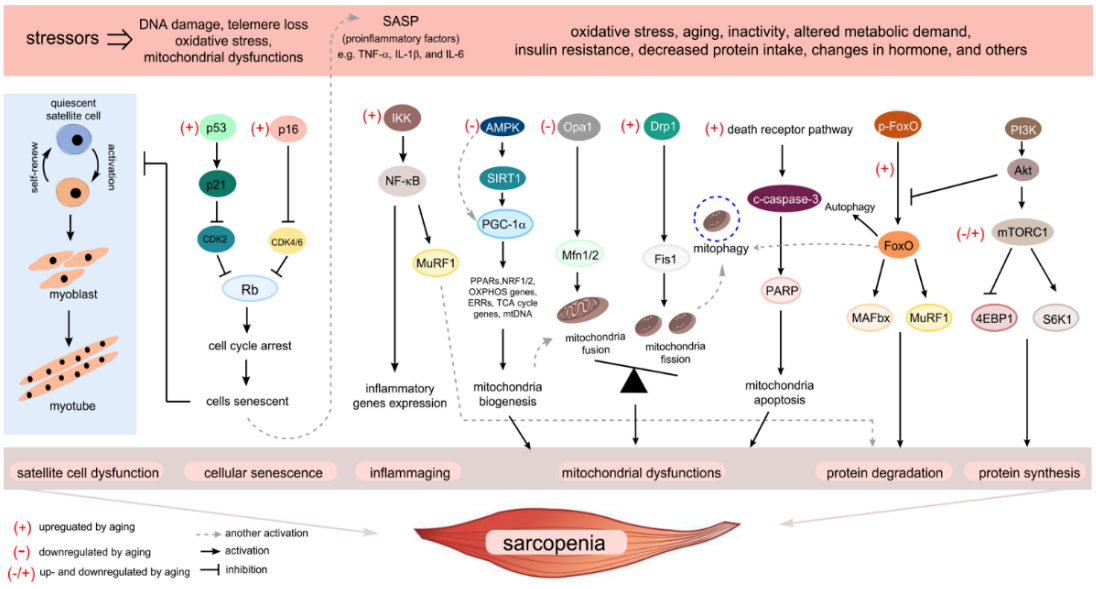

Fig. 10: Pathways associated with sarcopenia 0 D Q N K R Q J H W D O E

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From a morphological perspective, sarcopenic skeletal muscle exhibits a reduction in fibre cross-sectional area, a possible decrease in fibre number and compositional remodelling characterised by increased fibrotic tissue deposition, greater intramuscular fat infiltration and reduced capillary density, ultimately leading to progressive deterioration in muscle quality (Patel et al., 2015). Unlike cachexia, muscle loss in sarcopenia is not necessarily accompanied by a reduction in total body weight but is often associated with a relative increase in fat mass, a condition defined as sarcopenic obesity (Donini et al., 2022; Dunne et al., 2019).

Muscle ageing is also associated with profound metabolic and bioenergetic alterations. Mitochondrial dysfunction, increased production of reactive oxygen species (ROS) and reduced oxidative capacity contribute to the establishment of a chronic pro-inflammatory state that accelerates the decline in muscle mass and function (Fulle et al., 2004). These effects are particularly evident in type II muscle fibres, which are characterised by lower mitochondrial density and greater vulnerability to redox imbalance and metabolic alterations, resulting in a more pronounced loss of strength and power compared with endurance capacity (Amorim et al., 2022).

Finally, emerging evidence suggests a role for circulating myokines in the pathophysiology of sarcopenia, highlighting their potential use as biomarkers and therapeutic targets in age-related muscle decline (Moussaoui, 2025).

Overall, sarcopenia emerges as a multifactorial systemic condition resulting from the interaction between intrinsic molecular alterations within skeletal muscle and systemic determinants, including chronic inflammation, metabolic dysfunction and neuromuscular remodelling. These processes contribute to the progressive deterioration of muscle tissue quality and to the functional decline that characterises ageing.

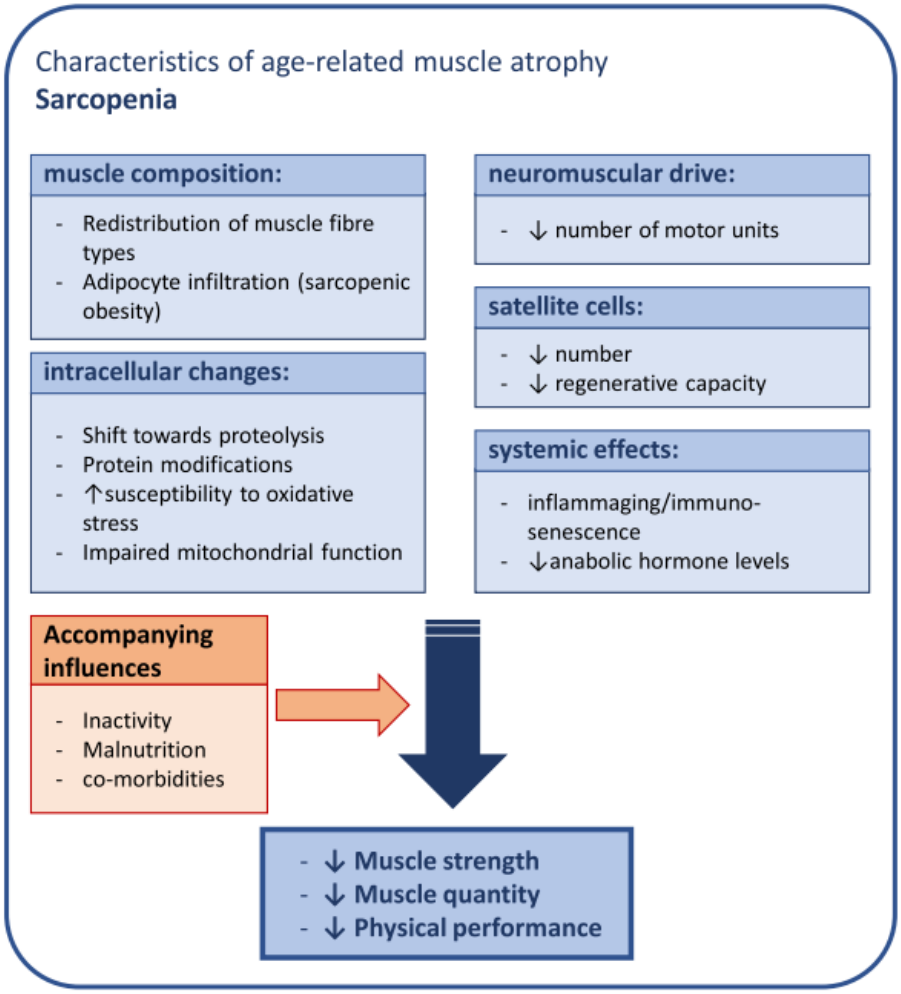

Fig. 11. Characteristics of sarcopenia : L H G P H U H W D O

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1.2.2. Diagnostic criteria of Sarcopenia

Given the complex pathophysiology of sarcopenia, the development of standardised diagnostic criteria is essential for identifying and monitoring the progression of the disease. To enhance the diagnosis of it in both clinical settings and research, the European Working Group on Sarcopenia in Older People (EWGSOP2) proposed an updated definition of the clinical condition in its 2019 revision of the guidelines (Cruz-Jentoft et al., 2019b). This definition identified muscle strength as the primary parameter for predicting adverse clinical outcomes. According to this approach, sarcopenia is considered "probable" in the presence of reduced muscle strength, "confirmed" in the case of concomitant reduction in the quantity or quality of skeletal muscle, and "severe" when the reduction in strength and muscle mass is associated with impaired physical performance. The adoption of multiparametric criteria is attributable to evidence that muscle strength is not exclusively dependent on muscle mass and that the relationship between these parameters is not linear, thus resulting in a definition based exclusively on muscle mass being clinically limited. A plethora of tests and tools are currently available for the characterisation of sarcopenia, the selection of which is dependent on the characteristics of the patient, the availability of technical resources in the healthcare setting (community, clinic, hospital, or research centre), and the purpose of the assessment. The diagnostic process may be preceded by screening tools, including:

- The SARC-F questionnaire: a clinical screening tool that evaluates five key functional domains: strength, walking ability, ability to rise from a seated position, stair-climbing ability, and history of falls. This questionnaire is particularly useful for the early identification of individuals at risk of sarcopenia. The SARC-F is characterised by low to moderate sensitivity and high specificity in predicting reduced muscle strength (Malmstrom et al., 2016).
- The Ishii score: a predictive index that is calculated using an equation that integrates age, handgrip strength, and calf circumference (Ishii et al., 2014).

A muscle strength assessment can predict unfavourable clinical outcomes, including an increased length of hospital stay, greater functional disability, a reduced quality of life and an increased risk of death related to health issues. It is generally performed using:

- Handgrip strength: a test that measures maximum handgrip strength using a dynamometer. This is a reliable indicator of overall muscle strength and the risk of adverse clinical events (Roberts et al., 2011).
- The chair stand test, which is a functional test that assesses the strength and endurance of the lower limbs by measuring the time required to repeatedly move from a sitting to a standing position without using the upper limbs (usually five consecutive repetitions). American Academy of Orthotists & Prosthetists

The diagnosis is then confirmed by assessing muscle mass or quality using instrumental techniques such as:

- Dual-energy X-ray absorptiometry (DXA), an imaging method that allows the quantitative assessment of appendicular muscle mass and body composition (Schweitzer et al., 2015).
- Bioelectrical impedance analysis (BIA) is a non-invasive technique that estimates body composition and muscle mass by measuring the electrical impedance of tissues (Sergi et al., 2017; Yamada et al., 2017).

Finally, a physical performance assessment can identify severe cases of sarcopenia through tests such as:

- Gait speed: a test that measures walking speed over a standardised distance (usually 4 metres) and is an indicator of overall physical function and frailty risk.
- Timed Up and Go (TUG): a test that assesses functional mobility, balance and the risk of falls by measuring the time taken to stand up from a chair, walk three metres, turn around, return to the starting point and sit down again. Ù

- Short Physical Performance Battery (SPPB): a series of tests that assess static balance, walking speed and lower limb strength to provide an overall index of physical performance. The maximum score is 12 points, while a score of ≤8 indicates reduced physical performance.
- The 400-metre walk test, which assesses prolonged walking ability. Participants are asked to complete 20 laps of 20 metres each in the shortest time possible, with up to two short breaks generally allowed during the test.

The integration of screening tools and functional and structural assessments enables earlier and more accurate diagnosis, better clinical risk stratification, and more effective monitoring of disease progression and response to therapeutic interventions. In this context, characterising the phenotype and function of skeletal muscle is a fundamental step towards understanding the biological mechanisms underlying sarcopenia, and towards developing targeted preventive and therapeutic strategies. Currently, the clinical diagnosis of sarcopenia requires the use of dedicated and expensive instrumentation. These operational limitations have contributed to increasing interest in the identification of easily accessible and reproducible molecular biomarkers.

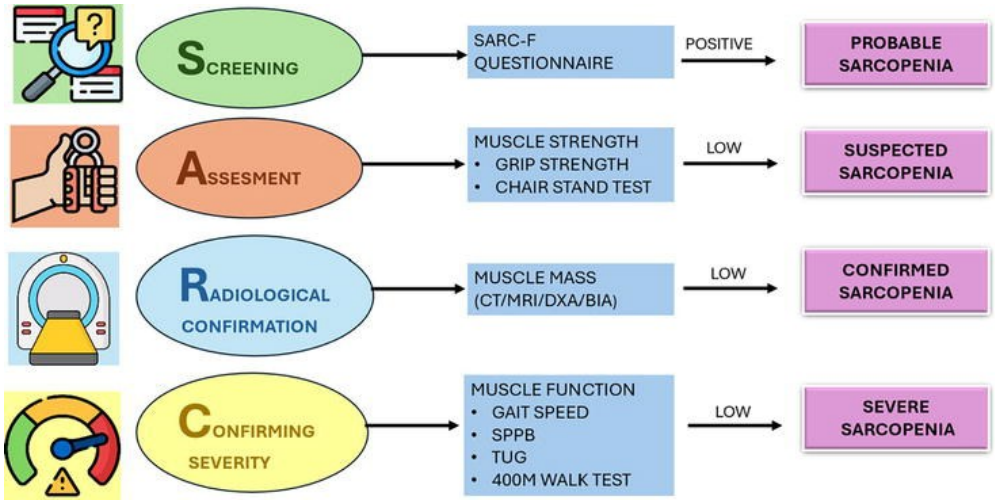

Fig. 12: Schematic representation of the sarcopenia evaluation process %KDQX .DQZDU

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1.2.3. Biomarkers for diagnosis and monitoring of sarcopenia

Sarcopenia is a potentially modifiable condition, so early diagnosis is essential for the implementation of effective preventive and therapeutic strategies. In recent years, the development of consensus definitions and diagnostic protocols has been accompanied by a growing interest in identifying the molecular mechanisms underlying the loss of muscle mass and function. In this context, molecular biomarkers could enhance traditional clinical and functional assessments, improving our understanding of disease progression, facilitating the identification of new therapeutic targets, and supporting diagnostic, prognostic, and risk stratification applications (Picca et al., 2022).

Currently, the analysis of biomarkers in sarcopenia is primarily based on tissue and liquid biopsies, each of which has specific advantages and limitations (Fernández-Lázaro et al., 2020; Wilson et al., 2018). Liquid biopsy, which is based on biofluids such as serum, plasma or urine, is a minimally invasive approach which could be applied on a large scale (Michela, 2021). However, the identification of reliable biomarkers can be affected by variability in the dataset, clinical confounding factors, and differences in the analytical panels used (McDermott et al., 2013). Although muscle biopsy provides highly informative samples for proteomic and molecular analysis, its application in the geriatric population is limited due to clinical issues and acceptability of the procedure (Fernández-Lázaro et al., 2020).

The overall complex pathophysiology of sarcopenia, characterised by the involvement of multiple molecular pathways, makes it improbable that a single biomarker capable of fully describing the condition will be identified. Consequently, current research is focused on identifying multiparametric panels of biomarkers that can be determined through routine blood tests. The objective is to improve diagnostic accuracy, risk stratification, and monitoring of disease progression (Sala et al., 2025).

1.3. Sarcopenia and Age-It project

1.3.1. Age-It project: clinical and preclinical aims

The Age-It research programme (Aging well in an ageing society) is a national research initiative that was promoted within the framework of the Italian National Recovery and Resilience Plan (PNRR), Mission 4 "Education and Research". The programme aims to strengthen Italy's role in the study of ageing processes and to develop innovative strategies to address the challenges associated with demographic transition (Age-It Research Programme, PNRR- Mission 4 "Education and Research", PE0000015). The programme's objective is to establish an integrated research ecosystem capable of generating scientific knowledge and practical solutions in the biomedical, socio-economic, and technological fields. A key priority is to promote the translation of research findings into clinical practice and public policies.

Population ageing is one of the most significant demographic trends of the 21st century. The combination of a rise in life expectancy and a fall in fertility rates has resulted in a significant increase in the proportion of older individuals within the population (Gianfredi et al., 2025a). This has substantial implications for healthcare systems, economies, and social structures. In this context, the Age-It programme aligns with the conceptual framework of active and healthy ageing promoted by the World Health Organization. According to this framework, older age should not be considered merely as a phase of functional decline, but rather as a stage of life in which individuals can continue to contribute to society while maintaining, as far as possible, autonomy and quality of life.

Italy offers a particularly relevant setting for the study of ageing dynamics. The country has one of the highest life expectancies in the world, and the presence of marked regional disparities in socio economic conditions, access to healthcare services and demographic characteristics makes Italy a valuable context for investigating how

biological, clinical, environmental and social factors interact in shaping ageing trajectories.

Despite the scientific advances achieved in recent years, ageing research in Italy has often been characterised by a certain degree of fragmentation. This situation is partly related to the predominance of discipline specific approaches, the limited integration among different research fields, and the difficulty in translating scientific findings into concrete healthcare and policy interventions. The Age-It programme was therefore conceived with the aim of overcoming these limitations by establishing an interdisciplinary research network involving universities, research centres, healthcare institutions, industry partners, and civil society organisations.

From an organisational perspective, the programme is structured according to a hub and spoke model, which allows the coordination of research activities distributed across multiple institutions and research groups while fostering the integration of different scientific competences. Within this framework, the hub provides overall coordination, with the spokes representing the main thematic areas in which the various research activities are developed.

The Age-It programme is organised into ten spokes, with each addressing a specific dimension of the ageing process. Collectively, these spokes facilitate an approach to the topic of ageing that is multifaceted and complementary. In particular:

- Spoke 1: focuses on the study of the demographic determinants of ageing and on the use of data science approaches to support decision making processes.
- Spoke 2: is dedicated to improving the understanding of the biological mechanisms of ageing, with the aim of elucidating the cellular and molecular processes underlying age-related functional decline.
- Spoke 3: investigates the clinical and environmental factors associated with multimorbidity, functional status, and the progression of age-related diseases.

- Spoke 4: examines the trajectories of active and healthy ageing, with particular attention to behavioural and psychological determinants.
- Spoke 5: addresses the organisation and sustainability of care systems in societies experiencing progressive population ageing.
- Spoke 6: focuses on the development of the Silver Economy, including issues related to employment, social participation, retirement, and welfare policies.
- Spoke 7: analyses the cultural and political dimensions of ageing societies, considering how demographic changes influence social and institutional dynamics.
- Spoke 8: aims to develop interventions designed to reduce the burden of age related diseases, disorders, and disabilities.
- Spoke 9: is devoted to the development of innovative technologies and advanced gerontechnologies to support active ageing and improve the quality of life of older individuals.
- Spoke 10: focuses on the integration of scientific knowledge on ageing into decision-making processes and public policies, with the aim of improving the planning and sustainability of welfare systems.

Overall, this organisational structure allows the ageing process to be addressed through a truly interdisciplinary approach, integrating basic research, clinical studies and the development of applied interventions. In this context, the different spokes of the Age-It programme operate in a synergistic and complementary manner, actively collaborating to achieve shared scientific objectives and promoting the exchange of knowledge, expertise and data across the various research domains. Among the various age-related conditions addressed within the Age-It framework, sarcopenia represents a major contributor to functional decline and therefore constitutes a key target for preventive and intervention strategies within the programme. Further information about the Age-It programme is available on the official project website (https://ageit.eu).

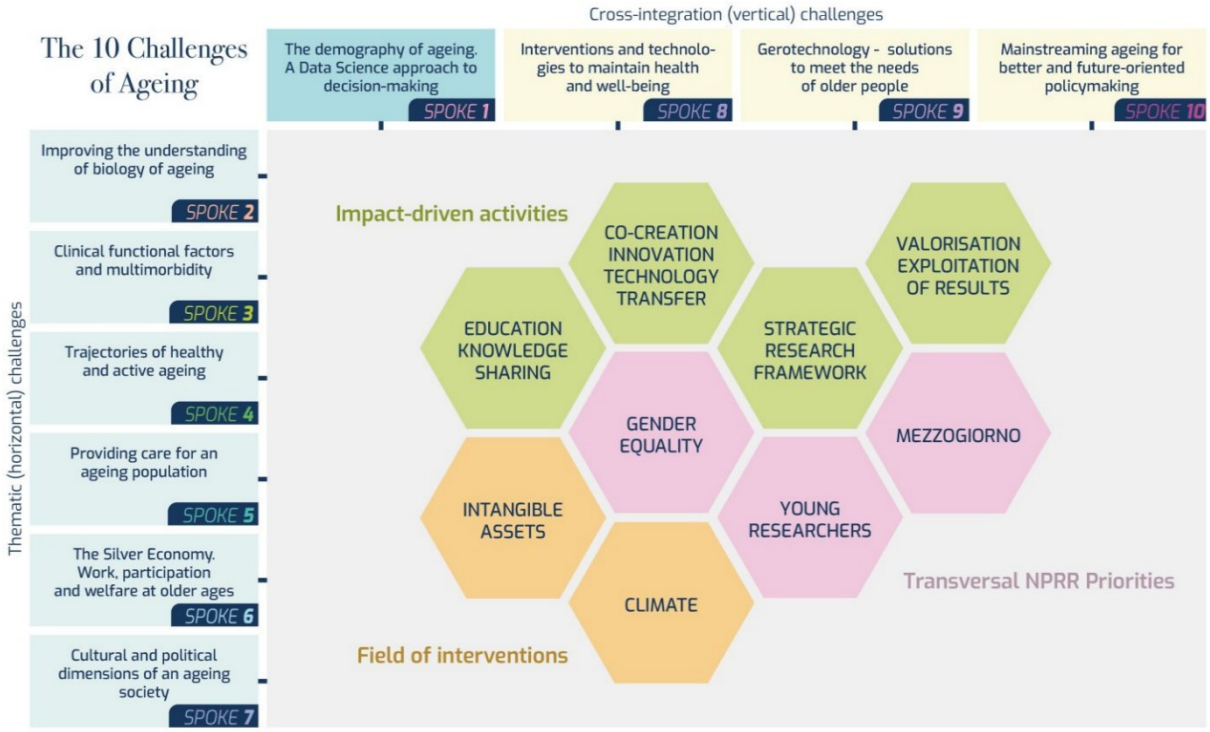

Fig. 12: The ten challenges of ageing addressed by the Age-It programme within its hub-and-spoke research framework K W W S V D J H L W H X

1.3.2. SPOKE 8: multidomain interventions

Within this framework, spoke 8 of the Age-It programme focuses on the development and evaluation of multidomain interventions aimed at preventing cognitive and functional decline and related conditions such as sarcopenia**.** Research activities within this spoke aim to identify the main factors contributing to physical and cognitive decline and to define effective preventive approaches.

In recent years, particular attention has been given to multidomain or multicomponent interventions, which combine several non-pharmacological strategies, including physical exercise, nutritional or dietary interventions, cognitive training, and social engagement. The integration of these approaches allows multiple modifiable risk factors to be targeted simultaneously and represents one of the most promising

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strategies to counteract functional and cognitive decline in older populations. Indeed, several studies have shown that multicomponent intervention programmes may be more effective than single domain approaches in improving health outcomes (Gianfredi et al., 2025a; Ngandu et al., 2015a).

Among the most relevant studies in this field is the FINGER trial (Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability), the first randomised controlled trial to demonstrate the effectiveness of multidomain interventions in preventing cognitive decline (Kivipelto et al., 2020; Ngandu et al., 2015b). The study involved 1,260 Finnish participants, aged between 60 and 77 years, who were at risk of dementia. The two-year intervention combined several non-pharmacological strategies, including dietary counselling, physical activity, cognitive training, and management of vascular risk factors. The results showed a significant improvement in cognitive performance in the intervention group compared with the control group, supporting the potential of multidomain approaches.

Despite these encouraging findings, the overall level of evidence available in the literature remains limited. There are still several challenges regarding the transferability and implementation of such protocols in community-dwelling older populations, where organisational, social and individual factors may influence adherence and effectiveness (Inzitari et al., 2018). Furthermore, the available data remain inconclusive with regard to the optimal duration of multidomain interventions, and it is still unclear whether these strategies can effectively prevent or delay the onset of dementia (Hafdi et al., 2021).

Within this framework, spoke 8 (Age-It) was conceived with the aim of addressing these knowledge gaps through the development and testing of multidimensional interventions targeting the prevention of functional and cognitive decline in older populations recruited across different Italian regions and care settings. The project also includes an in-depth characterisation of participants through the integration of biomarkers (includeing markers of sarcopenia, inflammation, neurodegeneration, and

metabolic dysregulation), omics analyses, genotyping, and environmental factor assessment.

The activities of this spoke are organised into six work packages (WP) and involve collaboration among clinicians, researchers, and technology developers from various Italian universities and institutions. The first three (clinical WPs) focus on implementing intervention studies targeting older individuals across different settings: community, hospital, and LTC facilities. The remaining three WPs (cross-cutting WPs) are dedicated to the development and integration of technological solutions and tools to support intervention delivery, data management, and cost-effective analyses. Overall, specific objectives are:

- To implement and evaluate tailored multicomponent interventions across three care settings, addressing physical, cognitive, nutritional, and psychosocial domains.
- To assess the impact of these interventions on an individual's physical performance, cognitive function, nutritional status, and quality of life over time.
- To evaluate the feasibility and acceptability of technology-supported interventions (e.g., tablets, cognitive platforms, wearable trackers).
- To assess the changes in biomarkers associated with frailty in relation to multicomponent interventions.
- To develop and implement a digital platform for data collection, monitoring, and visualization across trials.
- To assess the cost-effectiveness of multicomponent interventions using micro-costing and health economic modeling from a societal perspective.
- To provide policy-relevant insights for the design of scalable, integrated care pathways for aging populations.

Among these WP, **WP1 represents the central focus of the present PhD thesis** and will be discussed in detail in the following sections. Further information on Spoke 8 and its research activities can be found in Okoye et al. (2025).

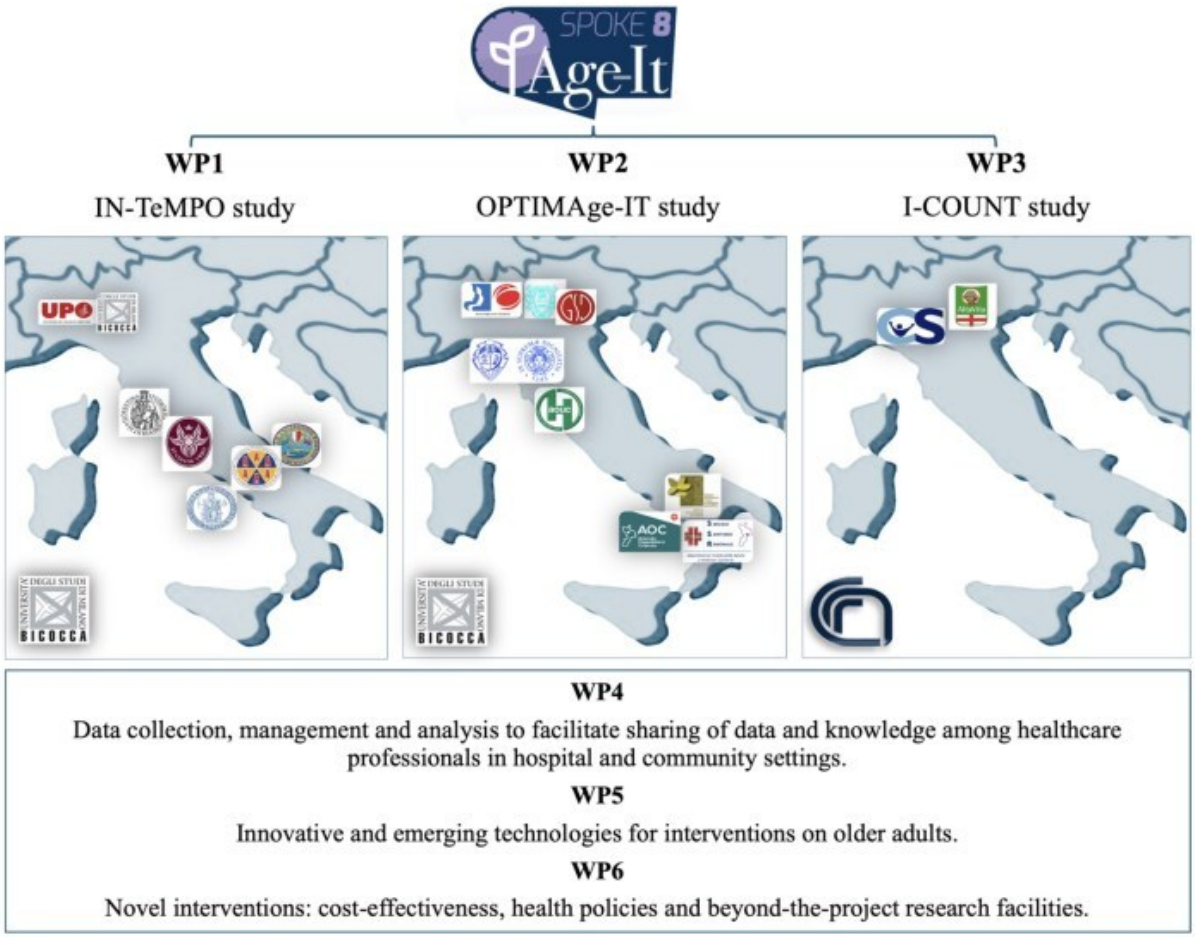

Fig. 13: The Age-It Spoke 8 consortium institutions and partners 2 N R \ H H W D O

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1.4. Preclinical model of atrophy and physical decline

The complexity of the biological mechanisms involved in sarcopenia progression makes it essential to use animal models to study muscle ageing and identify potential therapeutic strategies (Christian & Benian, 2020). Preclinical models enable the controlled analysis of the molecular and cellular mechanisms underlying muscle mass and function loss, as well as the evaluation of the efficacy and safety of new pharmacological and nutritional interventions, even in the presence of comorbidities typically associated with ageing, such as heart disease, obesity, and diabetes (Alonso-Puyo et al., 2024a; Christian & Benian, 2020).

Among model organisms, rodents, particularly mice and rats, are the most widely used experimental subjects due to their significant physiological, metabolic and molecular similarities to humans, and because it is possible to reproduce the ageing process relatively quickly. Indeed, it has previously been demonstrated that murine models and humans exhibit similarities in the ageing process (Dao et al., 2020; Larsson et al., 2019b; Xie et al., 2021a). The laboratory mouse (Mus musculus) has an expected lifespan of around 24 months. Three-month-old mice are comparable to 20-year-old humans, while those aged 18–24 months are comparable to humans aged 56-69 (Yuan et al., 2011). This enables the entire course of muscle ageing to be analysed within a time frame compatible with experimental studies. In murine models of physiological ageing, the first morphological and functional changes in skeletal muscle become detectable at 12-14 months of age and are particularly pronounced in mice aged 22-24 months.

Physiologically, muscle mass increases progressively until around 10 months of age and then gradually decreases after 15 months (Börsch et al., 2021). Comparisons between adult and elderly animals show a significant reduction in muscle mass, contractile strength and functional performance, which is associated with decreased spontaneous motor activity, altered energy metabolism and reduced insulin sensitivity

(Shur et al., 2021). From an experimental point of view, the majority of studies on sarcopenia employ mice aged 18 months or older, a stage at which biomarkers associated with aging are generally detectable (Dutta & Sengupta, 2016). Specifically, 18-month-old C57BL/6J mice exhibit a substantial decline in grip strength, exercise endurance, muscle volume, and muscle mass in comparison to juvenile animals. Research conducted on animal models up to 25 months of age has further corroborated a marked reduction in hind limb muscle mass, maximum muscle strength, and spontaneous activity levels, thereby validating the utilisation of these models as representatives of age-related sarcopenia (van Dijk et al., 2017). In addition, preliminary clinical evidence suggests a possible therapeutic effect of metabolic compounds, such as: 5-aminolevulinic acid (ALA) has been demonstrated to enhance mitochondrial functionality and mitigate metabolic changes associated with sarcopenia (Fujii et al., 2017), while branched-chain amino acids (BCAAs) have been shown to stimulate protein synthesis (Mantuano et al., 2020a; Wu, 2025).Similar to what has been observed in humans, rodents possess muscle satellite cells and show a progressive decline in muscle regenerative capacity with advancing age. (W. Liu et al., 2017). This decline is associated with alterations in the neuromuscular junction and the activation of catabolic and pro-inflammatory pathways. Transcriptomic studies have also highlighted significant changes in muscle gene expression during development and ageing, with alterations in pathways involved in calcium homeostasis, AMPK signalling, and extracellular matrix organisation (Bachman et al., 2018).

However, when interpreting these findings from a translational perspective, it is important to consider differences in muscle fibre composition between mice and humans. Human skeletal muscle typically exhibits a more heterogeneous and mixed distribution of fibre types within the same muscle, whereas individual mouse muscles often show a predominance of either fast- or slow-twitch fibres. This distinction is particularly relevant since sarcopenia predominantly affects type II fibres (Evans & Lexell, 1995; Schiaffino & Reggiani, 2011).

In addition to the established physiological aging model, a range of mouse models have been developed to study specific aspects of sarcopenia. These include accelerated aging models (e.g., SAMP mice), genetically modified models, models of pharmacological or metabolic induction, and experimental models of muscle disuse, such as hind limb suspension, denervation, and immobilization (Alonso-Puyo et al., 2024b; Xie et al., 2021b). While these models facilitate investigation of specific pathogenetic mechanisms, the natural aging model remains the primary reference for studying sarcopenia due to its greater representation of the multifactorial complexity of the condition observed in humans.

Despite the limitations described above, murine models represent an indispensable tool for obtaining information on the alterations of signaling pathways associated with atrophy and maladaptation, as observed during aging. In addition to natural aging, the study of atrophic processes accompanying primary muscle diseases may help identify general mechanisms and provide the basis for pharmacological investigations. In this context, and consistent with the scientific focus of the laboratory where I conducted my thesis work, I became particularly interested in the processes of atrophy and maladaptation occurring in muscles affected by Duchenne muscular dystrophy.

Duchenne muscular dystrophy (DMD) is in fact one of the main models of hereditary muscle atrophy. It is characterised by the progressive degeneration of skeletal muscle, which is caused by mutations in the dystrophin gene located on chromosome X (Xp21) (Duan et al., 2021; Hoffman et al., 1987). The absence or significant reduction in dystrophin results in muscle fibre membrane instability and disruption to the dystrophin-associated glycoprotein complex (DGC), making muscle fibres more susceptible to contraction-induced mechanical damage (Ervasti & Sonnemann, 2008; Wilson et al., 2022). The loss of the structural connection between the cytoskeleton, sarcolemma and extracellular matrix makes muscle fibres more fragile and susceptible to microlesions in the membrane during contractile activity. This ultimately leads to

the leakage of intracellular enzymes, including creatine kinase (Birnkrant et al., 2018; Duan et al., 2021).

From a clinical perspective, diagnosis usually occurs between 4 and 5 years of age, when delayed motor development and progressive muscle weakness become evident. The disease is initially suspected when there is markedly elevated plasma creatine kinase (CK) levels, which are often associated with increased transaminases. This suspicion is subsequently confirmed through genetic testing aimed at identifying causative mutation. In some cases, muscle biopsy may provide additional information through biochemical and immunohistochemical analyses of dystrophin expression (Aartsma-Rus et al., 2019; Birnkrant, Bushby, Bann, Apkon, et al., 2018).

Although DMD is a genetic disorder, several cellular processes involved in its progression show important similarities with those observed in age-related muscle wasting conditions, including sarcopenia. For instance, in DMD, there is persistent activation of the immune response. Specifically, this persistent activation is characterised by increased production of pro-inflammatory cytokines, which contributes to the development of muscle fibrosis (Duan et al., 2021), similar to the state of chronic systemic inflammation that accompanies aging. In this context, repeated episodes of muscle fibre damage and necrosis promote extracellular matrix remodelling and progressive replacement of muscle tissue with fibrotic and adipose tissue.

Another shared feature is mitochondrial dysfunction. In dystrophic muscle, sarcolemmal instability and the presence of membrane micro-ruptures lead to abnormal calcium influx, which may occur through stretch-activated channels and store-operated channels. Research indicates that calcium overload, in conjunction with elevated levels of ROS, can compromise mitochondrial function and disrupt cellular energy metabolism (Allen et al., 2016; Khairallah et al., 2012). Additional alterations involve the sarcoplasmic reticulum, including modifications of the RyR1, which may promote further pathological calcium release. Furthermore, mislocalization of neuronal nitric

oxide synthase (nNOS) contributes to impaired regulation of muscle blood flow, favouring ischemic phenomena and increased oxidative stress (Sander et al., 2000). It should be noted that comparable alterations in mitochondrial function and energy metabolism have also been described in sarcopenia. In this condition, reduced mitochondrial biogenesis, impaired oxidative phosphorylation, and increased oxidative stress contribute to the decline in muscle performance.

Finally, both conditions are characterized by a progressive decline in the regenerative capacity of skeletal muscle. In DMD, repeated cycles of degeneration and regeneration ultimately lead to functional exhaustion of satellite cells (Kodippili & Rudnicki, 2023); in sarcopenia, the reduction in regenerative potential is mainly associated with cellular senescence and chronic oxidative stress. In both cases, alterations in the major systems regulating protein homeostasis are observed, including activation of the ubiquitin–proteasome system, dysregulation of autophagy, and increased apoptotic processes, all of which contribute to the progressive loss of muscle mass and function (Bozzi et al., 2025; Nunes-Pinto et al., 2025).

Overall, these similarities highlight the existence of shared pathogenic mechanisms between DMD and sarcopenia, suggesting that processes such as chronic inflammation, mitochondrial dysfunction, and impaired regenerative capacity may contribute to the development of muscle atrophy in different pathological contexts. In this regard, DMD represents a valuable model for investigating the molecular mechanisms underlying muscle degeneration and for identifying potential therapeutic strategies that may also be relevant to age-related muscle atrophy, including sarcopenia.

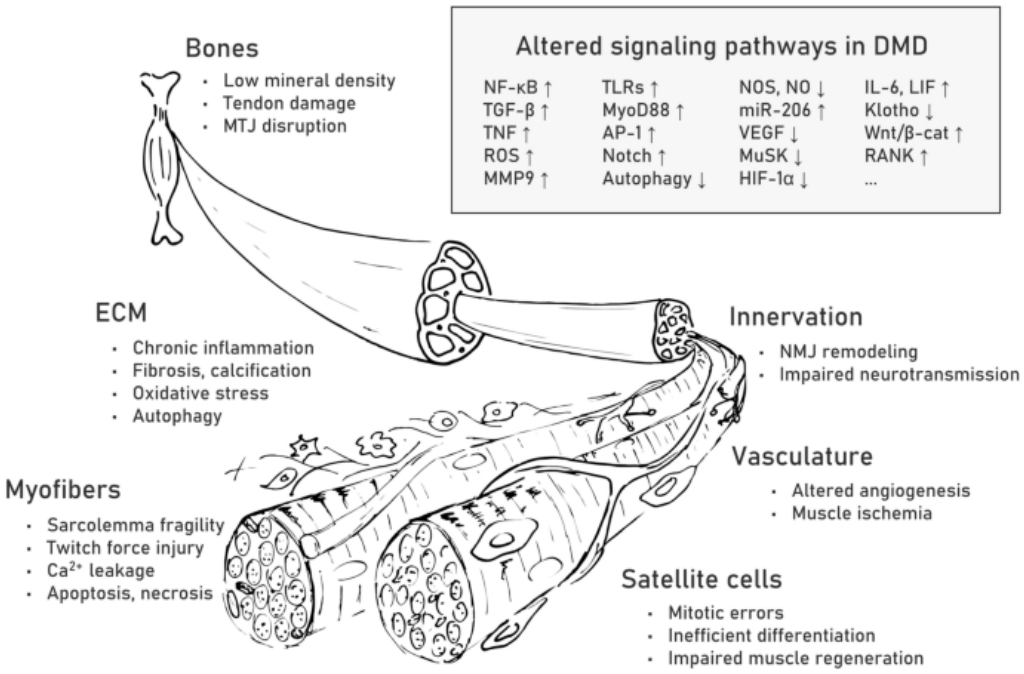

Fig. 14: Pathologenic alterations in DMD 6WDURVWD .RQLHF]Q\

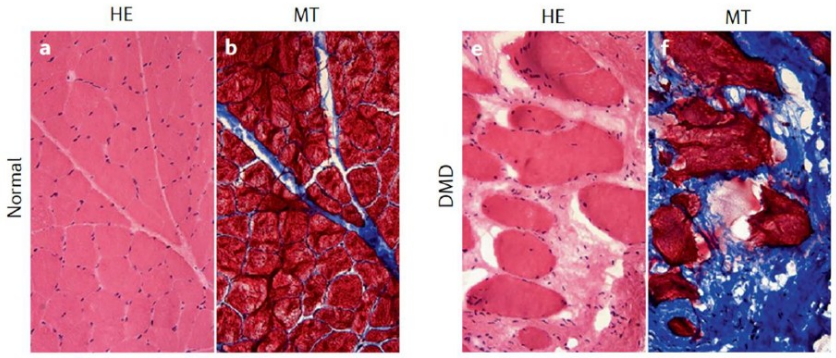

Fig. 15: Histomorphology of healthy and dystrophic muscle. H&E staining (panels a and e) showed centrally nucleated fibers and inflammatory cell infiltration in DMD tissue respect normal muscle. Masson trichrome (MT) staining showed increased fibrosis (blue staining) in DMD muscle compared to healthy muscle (panels b and f). $GDSWHG IURP 'XDQ HW DO 1DW 5

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To investigate the causes of Duchenne muscular dystrophy and evaluate potential therapeutic strategies, several animal models have been developed (Collins & Morgan, 2003). Among these, murine models are the most widely used in preclinical research due to their ease of breeding, relatively low maintenance costs, and significant genetic and molecular homology with the human disease. The classical model is the C57BL/10ScSn-' P G ⁹/J mouse (BL10- P G,[which carries a mutation in the dystrophin gene leading to the absence of the dystrophin protein (Bulfield et al., 1984). However, when compared with the human condition, this model displays a relatively milder phenotype and a greater regenerative capacity of skeletal muscle (Chamberlain & Chamberlain, 2017; De Luca, 2012; Grounds et al., 2008; Stedman et al., 1991).

However, in recent years, a more severe murine model, known as D2.B10-' P G ⁹/J (D2- P G,[has been developed by crossing BL10- P G[mice with the DBA/2J genetic background (Coley et al., 2016). This model exhibits a more pronounced dystrophic phenotype, characterised by increased fibrosis, reduced regenerative capacity, and significant muscle atrophy, thus more closely resembling the pathological condition observed in patients with DMD (Hammers et al., 2020; van Putten et al., 2019). The D2- P G[model is an excellent experimental tool for studying muscle degeneration and the mechanisms that lead to loss of muscle mass and function. In this context, the use of experimental models of muscular dystrophy may also provide important insights into the biological mechanisms underlying other conditions characterised by muscle atrophy, including sarcopenia.

Fig. 16: D2- P G[murine model (D2.B10-Dmdmdx/J) (The Jackson Laboratory).

1.5. State of the art: therapeutic approaches for sarcopenia and atrophy

1.5.1. Possible therapeutic approaches for sarcopenia

Although sarcopenia affects approximately 5–15% of individuals aged 60–70 years and may reach a prevalence of 11–50% in those older than 80 years (Börsch et al., 2021b), no specific pharmacological therapies are currently available that can prevent its onset or significantly modify its clinical course. In clinical practice, the management of sarcopenia therefore relies mainly on non-pharmacological interventions, particularly physical exercise programmes combined with nutritional strategies. The choice of the most appropriate therapeutic approach depends on several factors, including patient age, disease severity, inflammatory status, and the presence of comorbidities (Agostini et al., 2021; Kakehi et al., 2022; Rooks et al., 2020; Wong & Cheong, 2022). These factors often require the development of a personalised treatment plan.

Of all the exercise modalities, resistance training has been proven to be the most effective for improving muscle mass and strength in older adults with sarcopenia. An analysis of 42 randomised controlled clinical trials involving 3,728 patients with a mean age of 72.9 years demonstrated that resistance exercise programmes, either alone or combined with aerobic exercise and balance training, significantly improve functional parameters such as walking speed and performance in the timed up-and-go test. The observed effects are primarily attributed to increased muscle protein synthesis, enhanced neuromuscular activation, and a reduction in intramuscular adipose tissue (Konopka & Harber, 2014).

Despite aerobic exercise's traditionally secondary role to resistance training in the management of sarcopenia, recent evidence indicates that it can offer significant benefits for physical function and metabolic health. Research has indicated that aerobic activity can influence protein metabolism and contribute to the growth of skeletal

muscle tissue. A 12-week comparative study (Konopka & Harber, 2014) found that both aerobic and resistance training produced similar increases in quadriceps muscle volume. Furthermore, the combination of aerobic and resistance exercise has been shown to improve metabolic syndrome and sarcopenic obesity by modulating metabolic and hormonal biomarkers such as insulin, IGF-1, leptin, and adiponectin (Dieli-Conwright et al., 2018). Overall, combined training protocols appear to be the most effective strategy, as they produce greater improvements in muscle function, cardiovascular fitness, and overall physical performance compared with single-modality interventions (Villareal et al., 2017).

In addition to physical exercise, nutritional interventions are integral to supporting muscle health and enhancing exercise-induced adaptations. Research indicates that older adults with sarcopenia require a higher protein intake (1.0–1.5 g/kg/day) than the general adult population. In this context, in preclinical studies, have shown promising results for the supplementation of essential amino acids (BCAAs), particularly leucine-enriched formulations (E. Conte et al., 2024; Mantuano, Boccanegra, Bianchini, et al., 2021a; Mantuano et al., 2020b). Furthermore, a clinical study demonstrated that supplementation with 10.9 g of essential amino acids combined with 4 g of leucine can stimulate muscle protein synthesis and improve muscle function (Hashimoto et al., 2023). Additionally, research indicates that treatment with polyphenol-based mixtures can lead to functional improvements in skeletal muscle, by mitigating the oxidative stress associated with ageing (Pierno et al., 2014).

Vitamin D may also play an important role. Randomised controlled trials have demonstrated that supplementation with active vitamin D (0.75 μg/day) can enhance muscle strength and reduce the risk of falls in sarcopenic individuals (Kawahara et al., 2024). In addition, other nutrients such as omega-3 fatty acids, zinc, selenium, creatine, and β-hydroxy-β-methylbutyrate (HMB) may help reduce inflammation and muscle catabolism, particularly when combined with physical exercise (Hahn et al., 2022; Prado et al., 2022)

Despite the proven efficacy of these non-pharmacological interventions, they are not always easily applicable to frail older patients with multiple comorbidities (Rolland et al., 2023a). Consequently, several pharmacological options have been explored in recent years as potential therapeutic alternatives. However, to date, no drug has been approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA) for the specific treatment of sarcopenia (Rooks et al., 2020; Wong & Cheong, 2022) The main pharmacological classes investigated include myostatin inhibitors, anabolic and androgenic steroids, growth hormone and its analogues, angiotensin-converting enzyme (ACE) inhibitors, troponin activators, appetite stimulants, β-agonists, as well as senolytic and senostatic agents. However, the clinical use of these therapies remains limited due to the occurrence of adverse effects. Large-scale clinical studies have shown, for example, that testosterone therapy may improve muscle strength and functional performance (Midttun et al., 2024). However, its use is limited by the risk of cardiovascular and prostate complications.

Growth hormone (GH) and its analogues have also been extensively investigated. While these supplements may increase muscle mass, this effect does not necessarily translate into improvements in muscle strength or function (Adrian et al., 2019). In this context, the development of growth hormone secretagogues and ghrelin receptor agonists represents a potential therapeutic alternative, showing promising results with a more favourable safety profile compared with direct growth hormone administration (Boccanegra et al., 2023c; Mantuano et al., 2025; Rolland et al., 2023).

In recent years, advances in our understanding of the molecular mechanisms regulating muscle mass maintenance have also promoted the development of novel therapeutic strategies. These include stem cell-based approaches involving satellite cells, muscle stem cells (MuSCs), and induced pluripotent stem cells (iPSCs). In preclinical models, these have demonstrated the ability to promote muscle tissue repair and improve contractile function (Groarke et al., 2024). Concurrently, there are several molecules targeting specific signalling pathways involved in skeletal muscle growth and

metabolism under investigation. Among these, monoclonal antibodies targeting GDF-15 represent a promising therapeutic strategy, as this factor has been implicated in muscle mass loss. These antibodies have been shown to block the catabolic effects mediated by GDF-15 and to reduce systemic inflammation. Phase II clinical studies have also reported significant improvements in muscle strength and physical performance, together with a favourable safety profile (Groarke et al., 2024). Another emerging research area involves gene therapy strategies targeting IGF-1. Studies using modified AAV viral vectors have demonstrated sustained expression of IGF-1 in skeletal muscle, resulting in increased muscle mass and improved function (Sia et al., 2022).

In this context, combinatorial approaches that combine IGF-1 overexpression with myostatin inhibition appear particularly promising due to their synergistic effects (Gellhaus et al., 2023). Also studies investigating the combined effects of chronic exercise, IGF-1 modulation and taurine supplementation, an amino acid known to attenuate calcium-induced muscle degeneration, have also reported beneficial outcomes (De Luca et al., 2003).

Overall, the management of sarcopenia is evolving through the integration of traditional interventions, emerging pharmacological strategies, and personalized medicine approaches. In this context, the identification of more sensitive biomarkers, the optimization of combination therapies, and the development of targeted treatments represent key aspects for improving both prevention and clinical management of the disease.

In the frame of the general interest to identify strategies to counteract or delay sarcopenia, I focused on two recent approaches of our laboratory, deriving from general interest in nutraceutical and anabolic compounds.

1.5.1.1. Nutraceuticals in aging: focus on Branched-Chain Amino Acids

Alongside these pharmacological strategies, which are often associated with potential adverse effects, increasing interest has emerged in recent years toward targeted nutritional interventions, particularly the use of Essential Amino Acids (EAAs), which are able to stimulate protein anabolism and promote the recovery of muscle function impaired in atrophic conditions (Burd et al., 2013). Among these, leucine, isoleucine, and valine, known as BCAAs, stand out for their marked anabolic potential (Figure 17) (Mantuano, Boccanegra, Bianchini, et al., 2021a).

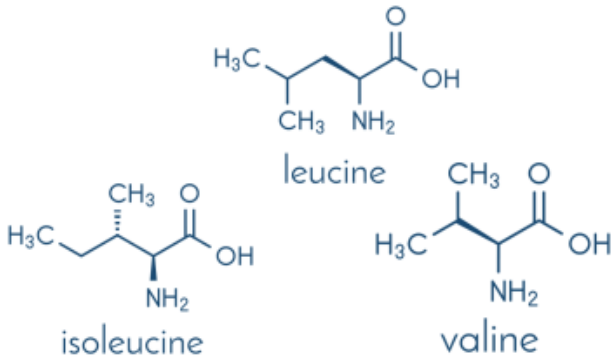

Fig. 17: Structure of branched-chain amino acids (BCAAs)

BCAAs exhibit distinctive metabolic characteristics compared to other EAAs, as they bypass hepatic metabolism and are therefore rapidly available in the systemic circulation, with preferential distribution to extrahepatic tissues, particularly skeletal muscle and the central nervous system (Holeček, 2018). They act both as substrates for protein synthesis and as an energy source (ergogenic effect), while also exerting an inhibitory effect on proteolytic processes. In particular, leucine is able to activate the mTOR signalling pathway, thereby promoting protein synthesis and inhibiting pro-atrophic pathways mediated by Atrogin-1 and MuRF-1 (Bifari & Nisoli, 2017; Maki et al., 2012a). In this context, studies conducted by the research group coordinated by Prof. Annamaria De Luca highlighted the ergogenic potential of BCAAs (2:1:1 ratio), administered alone or in combination with L-alanine (L-ALA), the main amino acid

derived from their catabolism, used to increase their bioavailability. In a murine model subjected to non-damaging chronic exercise protocols lasting four weeks, oral administration of these mixtures resulted in increased muscle mass and reduced fatigue in wild-type mice (Mantuano, Boccanegra, Bianchini, et al., 2021a).

These findings supported the use of the same formulations in a preclinical study using a murine model of disuse atrophy obtained through hind limb unloading (HU) (Mantuano, Boccanegra, Bianchini, et al., 2021b). This model is characterized by marked muscle atrophy, with reduction of myofibre cross-sectional area, mitochondrial dysfunction, increased production of reactive oxygen species (ROS), and reduced protein synthesis. In this setting, a novel formulation containing BCAAs and the dipeptide L-alanine–L-alanine (Di-ALA) was also evaluated (Figure 18). The use of the dipeptide is justified by its faster absorption compared to free amino acids and by its ability to activate the intestinal transporter PEPT1, facilitating oligopeptide absorption at the enterocyte brush border and further increasing the bioavailability of L-ALA and, indirectly, BCAAs (Vig et al., 2006; Young et al., 2000). Administration of these formulations resulted in significant improvements in structural and functional markers in soleus and gastrocnemius muscles, with particularly evident effects in mice treated with the BCAAs + Di-ALA combination (Mantuano, Boccanegra, Bianchini, et al., 2021b). Overall, this evidence supports the potential role of BCAAs as a nutritional approach to counteract sarcopenia, further corroborated by recent studies demonstrating improvements in muscle strength and mass in murine models of physiological ageing (Mantuano et al., 2023).

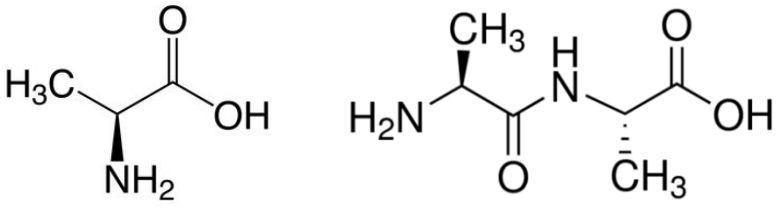

Fig. 18: Structure of L-ALA and the dipeptide Di-ALA

1.5.1.2. Anabolic compounds as a potential therapeutic strategy for muscle atrophy: growth hormone secretagogues

Muscle atrophy is a common feature of several pathological and physiopathological conditions. These include ageing, neuromuscular diseases, cachexia associated with chronic disorders, and prolonged periods of inactivity. Consequently, there has been a growing focus in recent years on therapeutic strategies designed to counteract the processes underlying muscle degeneration. In the field of emerging pharmacological approaches, particular interest has been directed towards growth hormone secretagogues (GHSs).

Ghrelin and des-acyl ghrelin are 28-amino acid peptide hormones primarily produced in the stomach during fasting. These peptides exert their biological effects by binding to the growth hormone secretagogue receptor type 1a (GHS-R1a). Activation of this receptor has been shown to stimulate the release of growth hormone and contribute to the regulation of food intake, adiposity, energy balance, and lean mass accretion (Wren et al., 2001). Beyond their endocrine functions, these peptides exert several extra-endocrine effects, including anti-inflammatory, antioxidant, and anti-apoptotic activities. In addition, they have been shown to modulate fibrotic processes through inhibition of NOX-mediated signalling pathways and the consequent reduction of reactive oxygen species (ROS) production (Angelino et al., 2015). Research has shown that ghrelin and des-acyl ghrelin can play a protective role in preventing muscle atrophy and wasting (E. Conte et al., 2017b). In particular, des-acyl ghrelin has been found to have specific in vitro activities that regulate different stages of muscle regeneration, such as stimulating asymmetric satellite cell division and promoting myoblast differentiation. In vivo studies have further demonstrated that increased des-acyl ghrelin levels in mdx mice are associated with improved muscle function and enhanced satellite cell self-renewal (E. Conte et al., 2017b; Reano et al., 2017a).

However, the direct therapeutic use of these peptides is limited by their poor bioavailability due to their peptide nature. To overcome these limitations, several

synthetic growth hormone secretagogues have been developed. Among them, the pseudopeptide JMV2894 is a particularly promising candidate due to its improved pharmacokinetic properties compared with endogenous peptides. Experimental studies have shown that JMV2894 is able to counteract cachexia associated with several neoplastic conditions and chemotherapeutic treatments, such as cisplatin, through improvement of calcium homeostasis, reduction of oxidative stress, and stimulation of mitochondrial biogenesis in skeletal muscle (Sirago et al., 2017).

A preclinical study was conducted in the Pharmacology laboratories of the research group led by Professor Annamaria De Luca (University of Bari "Aldo Moro") to evaluate the therapeutic potential of two synthetic growth hormone secretagogues, EP80317 and JMV2894, in the BL10- P G [murine model of DMD. The results demonstrated a partial enhancement in muscle function, with more notable effects observed for JMV2894, which exhibited significant anti-fibrotic properties linked with reduced collagen deposition and decreased fibro-adipose tissue accumulation within the muscle (Boccanegra et al., 2023a).

These results provided the foundation for a subsequent evaluation of JMV2894 in the emerging D2- P Gn[urine model, in virtue of its more pronounced pro-fibrotic and pro-atrophic genetic background which translate into a more severe phenotype more closely reproducing the degenerative processes observed in human DMD. The pathological features of this model also make it particularly useful for investigating the mechanisms involved in muscle atrophy. The results of this experimental study will be discussed in this thesis (Mantuano et al., 2025).

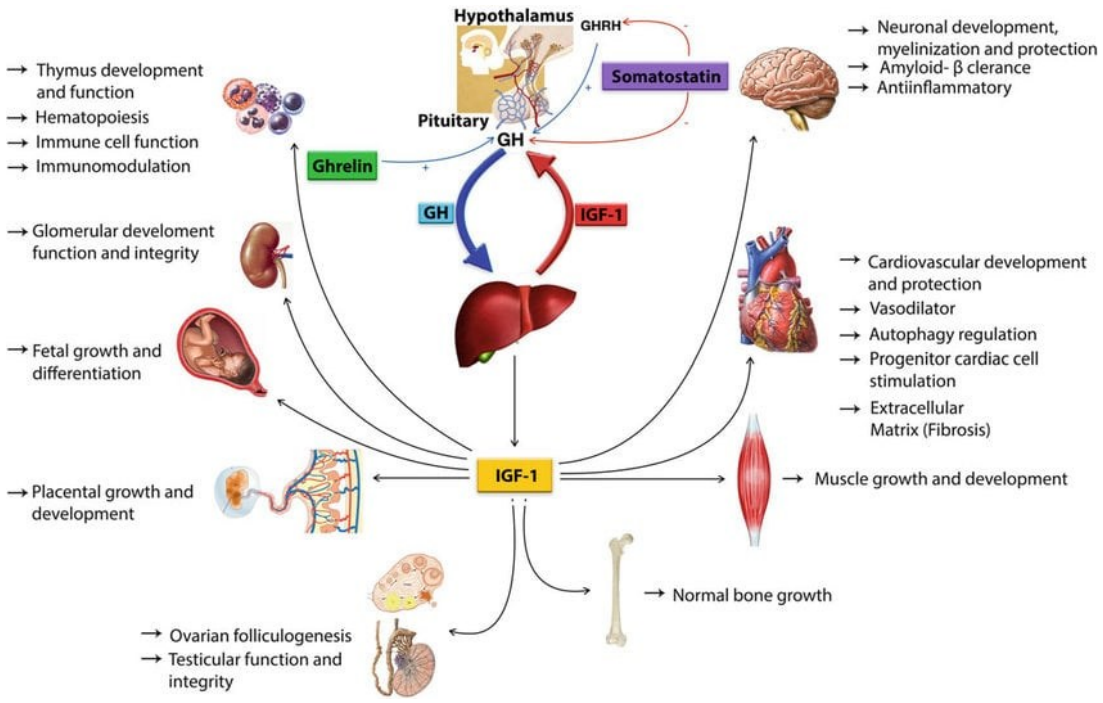

Fig.19: Schematic representation of the GH/IGF-1 axis, $ J X L U U H H W D O

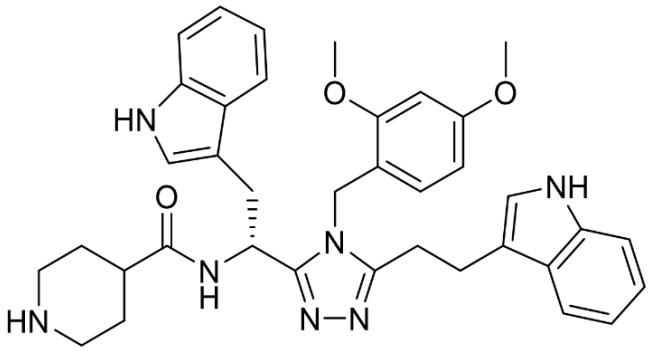

Fig.20: Chemical structure of the growth hormone secretagogue (GHS) JMV2894.

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Chapter 2 – Rationale & Aims

2.1 Rationale & Aims

The progressive ageing of the global population represents one of the major demographic challenges, with important implications for healthcare systems. One of the primary consequences of the ageing process is the onset of age-related physical decline. This clinical manifestation primarily manifests as sarcopenia, a specific form of muscle atrophy characterised by the progressive loss of muscle mass and function. However, it is important to highlight that muscle atrophy is not exclusively associated with the ageing process, but also characterises several pathological conditions, such as muscular dystrophies. Furthermore, sarcopenia is frequently linked to multiple comorbidities, including neurodegenerative diseases, diabetes and renal dysfunction, which can further exacerbate the clinical condition of older individuals. Consequently, the implementation of multi-intervention approaches and therapeutic strategies targeting various levels of the standard conditions of ageing emerges as a highly promising approach.

In light of these considerations, the aim of this thesis, developed within the framework of the Extended Partnership "Age-It", and specifically within Spoke 8, is to identify a panel of circulating biomarkers of sarcopenia, potentially useful as diagnostic and prognostic tools, in order to evaluate the effects of a multidomain intervention on sarcopenia in a population of community-dwelling older adults. In parallel, in line with the multidisciplinary approach of the Age-It project and the collaboration among the different spokes to achieve the common goal of counteracting physical decline and promoting healthy ageing, during my Ph.D. I had evaluated the molecular mechanisms underlying muscle atrophy and the effects of innovative therapeutic strategies in murine models of physiological ageing and in the pro-atrophic and pro-fibrotic D2-mdx model. Given the complexity of the ageing process and the continuous cross-talk among organs and biological systems, these evaluations were also extended to the renal level, as kidney damage represents one of the conditions most frequently associated with functional decline in older individuals.

In detail, the specific aims and approaches of this thesis were the following:

Aim 1) IN-TeMPO Project: Identification of plasma biomarkers of sarcopenia

As part of my PhD programme, which is funded by the Age-It fellowship within the framework of PNRR – Mission 4, Component 2, Investment 1.3 "Extended Partnerships", supported by the European Union – NextGeneration EU, thematic area PE8 "Consequences and Challenges of Ageing" (Project code PE0000015, CUP H33C22000680006), I was involved in the IN-TeMPO project (WP-1; Spoke 8; ClinicalTrials.gov ID: NCT06248723).

The IN-TeMPO project (ItaliaN with Multidomain Personalized Interventions to Prevent Functional and Cognitive Decline in Community-Dwelling Older Adults), is a randomized controlled clinical trial that represents the first Italian study developed according to ICOPE guidelines and the principles of the World-Wide FINGERS (WW-FINGERS) network. The main objective is to evaluate the effectiveness of personalized multidomain interventions in preventing functional and cognitive decline in the community-dwelling elderly population. The project is part of a series of international studies derived from the FINGER trial (Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability), which demonstrated that simultaneous lifestyle interventions in different domains can reduce the risk of cognitive decline (Ngandu et al., 2015b). Based on this evidence, a growing number of international clinical studies have been progressively developed, now coordinated within the global WW-FINGERS network, which involves over 60 countries (Kivipelto et al., 2020).

A distinctive feature of the IN-TeMPO study is the inclusion of blood biomarker analysis. Indeed, a paucity of multidomain studies has been observed to incorporate comprehensive analysis of blood biomarkers with a view to investigating the biological mechanisms underlying response to interventions and identifying potential predictors of efficacy (Boardley et al., 2007; Ha & Son, 2018). To address this knowledge gap,

IN-TeMPO intends to analyse an integrated panel of established and exploratory biomarkers related to Alzheimer's disease, neurodegeneration, inflammation, senescence, sarcopenia and oxidative stress. The objective of this analysis is to improve individual risk stratification and to identify possible biological surrogates of the effect of multidomain interventions. The study is structured in two phases and involves elderly subjects residing in communities: a preliminary feasibility phase, followed by a randomized controlled parallel clinical trial (1:1), comparing guided multidomain interventions with self-guided interventions, conducted in nine Italian clinical centres including our group, coordinated by Prof. Annamaria De Luca, in which I worked as a PhD student.

Participants were randomised to receive multidomain interventions (nutrition, physical exercise, cognitive training, management of metabolic and vascular risk factors, social activities, oral health, and sleep quality) or self-guided interventions supported by digital tools. The use of wearable devices, digital platforms, and artificial intelligence tools facilitate continuous monitoring of clinical parameters and integrated data management. The primary endpoint of the study is the evaluation, at 12 months of the effect of multidomain interventions on cognitive performance through a battery of neuropsychological tests (mNTB). Secondary and exploratory endpoints include the assessment of functional and physical performance, as well as the longitudinal analysis of blood biomarkers associated with cognitive and functional decline.

In the preliminary phase prior to the start of the clinical trial, I participated in a meta-analysis conducted in collaboration with a dedicated task force from WP-1 (Spoke 8). The aim of this analysis was to define an optimal panel of predictive biomarkers associated with frailty, neurodegeneration, and sarcopenia to be included in the experimental protocol. The selection of biomarkers was guided by the scientific consensus of the research team, which analysed the latest evidence available in the literature and identified a number of molecules capable of monitoring the biological pathways that were hypothesised to be modulated by multidomain interventions in the

study. With regard to sarcopenia, biomarkers representative of the multifactorial nature of age-related muscle decline were selected, such as inflammation, metabolic and endocrine alterations. Specifically, the following were selected: IL-6 and GDF-15, which will be measured in the entire cohort (n =1,662); and BDNF, IGF-1, irisin and ghrelin, which will be evaluated in a subgroup (n =100). The subsequent phase of recruitment and randomisation into the two experimental groups was conducted by clinicians.

To obtain preliminary feedback before the conclusion of the 12-month multidimensional treatment, we had analysed the trend of biomarkers in a subgroup of 50 participants from the Bari centre at baseline and after 6 months of follow-up using commercial ELISA kits. This phase of the project was carried out in collaboration with Professor Solfrizzi, affiliated with the Department of Geriatrics at the Policlinico dell'Università degli Studi di Bari "Aldo Moro" and responsible for the randomisation procedure of participants from the Bari centre. To ensure that the blind design was maintained, sensitive clinical data on participants was not disclosed until the end of the project; only information on gender and age, which was necessary for preliminary analyses, was made available. For a detailed description of the experimental design and study protocol, please refer to Sala et al. (2025) and Okoye et al. (2025).

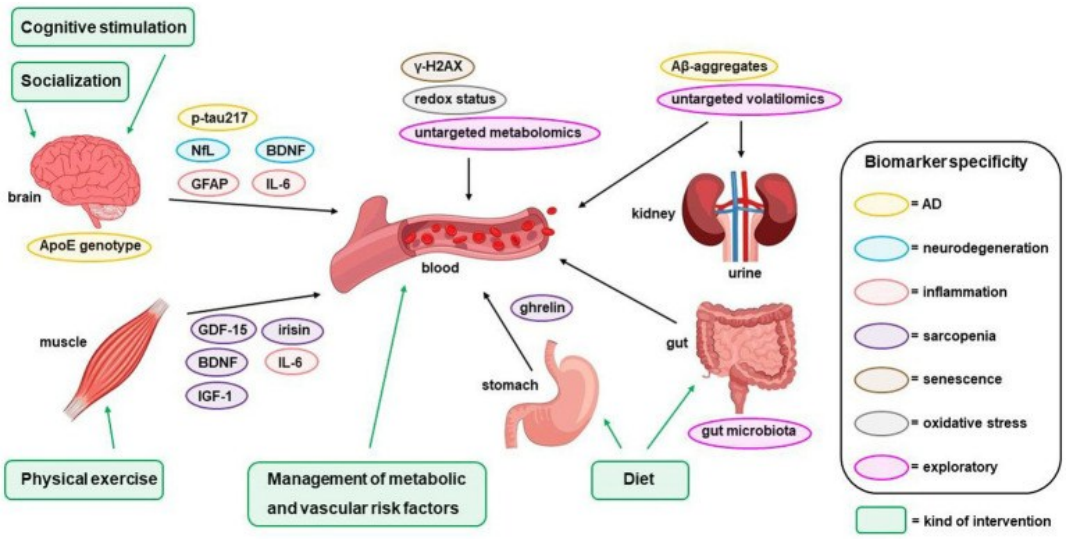

Fig. 21 Blood biomarkers and multidomain interventions of the IN-TeMPO study (Sala et al., 2025)

The following section provides an in-depth analysis of the sarcopenia biomarkers that have been selected for the IN-TeMPO project.

After careful selection, we have identified six explorative biomarkers reflecting the multifactorial pathophysiology of age-related muscle decline, involving inflammatory, metabolic and endocrine pathways. Growth/differentiation factor 15 (GDF-15), a member of the TGF-β superfamily, has emerged as a promising candidate. GDF-15 is highly expressed in muscle tissue, while circulating levels are typically low in healthy young individuals, ranging between 200 and 1,200 pg/mL (Breit et al., 2011). However, its expression markedly increases in the presence of acute and chronic pathological conditions, many of which are age-related (M. Conte et al., 2019, 2020). Elevated GDF-15 levels have also been associated with muscle atrophy, neurodegenerative disorders, mitochondrial diseases and ageing itself, independently of general health status (Lee et al., 2022; H. Liu et al., 2021). Consistent with these observations, several studies have reported a positive correlation between increased circulating GDF-15 levels and reduced skeletal muscle quality, supporting its potential role as a biomarker of sarcopenia (Herpich et al., 2021; Kim et al., 2020; Semba et al., 2020). Nevertheless, the precise mechanistic contribution of GDF-15 to muscle mass and functional decline remains to be fully elucidated.

In parallel, neurotrophic factors such as brain-derived neurotrophic factor (BDNF) have been explored as candidate biomarkers linking neuromuscular integrity and muscle function. BDNF is primarily known for its role in neuronal survival and synaptic plasticity, but it also contributes to muscle repair, regeneration and differentiation processes (Kalinkovich & Livshits, 2015). Circulating BDNF levels vary across the lifespan, with higher concentrations during childhood, a transient increase during early adulthood, and a gradual decline with ageing (Webster et al., 2006). Notably, BDNF levels increase in response to physical exercise, further supporting the role of physical activity as a protective factor against both

neuromuscular and cognitive decline (Coelho et al., 2014; Tsai et al., 2018). These findings suggest that BDNF may represent a potential biomarker reflecting both muscle function and responsiveness to exercise-based interventions.

Hormonal and metabolic regulators also play a central role in sarcopenia pathophysiology. Ghrelin, a gastric peptide with growth hormone secretagogue activity, promotes activation of the GH/IGF-1 axis, stimulates appetite and contributes to energy homeostasis (Pradhan et al., 2013). Circulating ghrelin levels decline with ageing (Rigamonti et al., 2002; Serra-Prat et al., 2007)although the strength and clinical relevance of its association with sarcopenia remain under investigation (Serra-Prat et al., 2015). Similarly, IGF-1, a key anabolic hormone regulating muscle protein synthesis, progressively decreases across the lifespan (Chew et al., 2019). Reduced IGF-1 levels have been associated with impaired muscle strength and function, suggesting a potential role in the development and progression of sarcopenia (Roubenoff et al., 2003).

Among muscle-derived factors, irisin has gained increasing attention as a myokine involved in energy metabolism, through the browning of white adipose tissue, improving glucose tolerance and reducing insulin resistance (S. Liu et al., 2022). Irisin concentrations are positively correlated with muscle mass and strength, and increase in response to physical training, whereas they tend to decline with ageing and under conditions of muscle atrophy and sarcopenia (Chang et al., 2017a; Park et al., 2019). These characteristics support its potential use as a biomarker reflecting muscle functional status and response to exercise interventions.

Finally, chronic low-grade inflammation represents a key hallmark of ageing and sarcopenia. In this context, IL-6 is widely recognised as a biomarker of inflammaging (Franceschi & Campisi, 2014).Elevated circulating IL-6 levels have been associated with several geriatric syndromes, including frailty, sarcopenia, and functional decline (Darvin et al., 2014),supporting the notion that systemic inflammation plays a central role in muscle ageing.

Aim 2) Preclinical evaluation of BCAA-based formulations in mouse model of physiological aging

As previously mentioned in paragraph 1.5.1.1. preclinical studies conducted in the research laboratory of the University of Bari, coordinated by Professor Annamaria De Luca, have shown that supplementation with BCAAs, administered individually or in combination with L-alanine (L-ALA), can improve muscle strength and mass in a mouse model of physiological aging, also resulting in significant increase in gastrocnemius (GC) muscle CSA (Mantuano et al., 2023; Mantuano et al., 2021). I expanded this research during my PhD studies, centering my attention on ascertaining the potential of these formulations in restoring intracellular calcium homeostasis in skeletal muscle (Aim 2a). A mechanism that has been demonstrated to be impaired in sarcopenic muscle (Conte et al., 2024). Moreover, in view of the evidence which documents physiological cross-talk between skeletal muscle and kidney, and the correlation between muscle performance and renal dysfunction (Wang et al., 2023), I also analysed the potential renoprotective and antifibrotic effect of these supplementations (Aim 2b). Renal fibrosis is a prevalent comorbidity associated with sarcopenia, characterised by histological alterations such as interstitial fibrosis, tubular atrophy, reduction in cortical mass, and increased glomerulosclerosis (Ghoshal et al., 2021). Recent evidence indicates that dysregulation of the pathways involved in proteostasis, including the p53/$p21^{Cip1/Waf1}$ and $p16^{INK4a}$/Rb, mTOR, and AMPK pathways, is associated with cellular senescence and pro-inflammatory and pro-fibrotic profiles. From this standpoint, nutritional interventions with the potential to support protein synthesis and maintain cellular homeostasis could assist in counteracting various aspects of tissue senescence, both at the muscular and renal levels.

The present study was conducted on 17-month-old wild-type mice (AGED) for 12 weeks with BCAAs alone, in a 2:1:1 ratio, or in combination with L-ALA or Di-ALA. The outcomes were evaluated by using a multifunctional approach, including L Q Y L Y R

U X

indices of muscle function and [YLYR functional, biochemical and histological readouts. Given that muscle atrophy is a condition frequently observed in neuromuscular disorders (NMDs), experimental procedures were conducted in accordance with the international guidelines for preclinical studies on NMDs, as published on the TREAT-NMD website (https://www.treat-nmd.org/resources-and-support/sop-library/).

Aim 3) Evaluation of the effects of the growth hormone secretagogue JMV2894 in the D2- P Gr[house model

As discussed in Section 1.5.1.2, previous preclinical studies conducted by the research group led by Professor Annamaria De Luca demonstrated the antifibrotic effects of JMV2894 in the BL10-mdx model of DMD (Boccanegra et al., 2023a). Based on these promising findings, during my PhD I extended this line of research to the D2-mdx murine model, characterized by a more pronounced pro-atrophic and pro-fibrotic background, in order to further evaluate the potential benefic effects of this compounds in counteracting muscle atrophy. The first part of the study investigated the effects of JMV2894 on skeletal muscle (Aim 3a). The second part, similarly to Aim 2, evaluated its impact on the renal compartment (Aim 3b). In particular, the six-week treatment involved administering the growth hormone secretagogue JMV2894 subcutaneously to D2- P Gr[mice at two different doses (640 µg/kg and 1280 µg/kg). The experimental procedures were guided by the international guidelines for preclinical studies on NMDs, as published on the TREAT-NMD website (https://treat-nmd.org/research-overview/preclinical-research/) and the treatment were analyzed using validated endpoints of clinical relevance, both L Q Y and Y-R[Y. L Y R

Chapter 3 – Materials and Methods

3.1. Clinical study (Aim 1)

3.1.1. Experimental groups & treatments schedule

A total of 1,662 community-dwelling adults were recruited through general practitioners, memory clinics, and public campaigns conducted in collaboration with local health authorities across the national territory. The inclusion criteria encompassed the following: age ≥ 60 years, Primary Care Frailty Index between 0.07 and 0.21, CAIDE score ≥ 6, Clinical Dementia Rating (CDR) ≤ 0.5, and presence of modifiable risk factors or family history of dementia. Participants were randomized into either the multicomponent intervention group or the self-guided group by the study clinical team. The multidimensional intervention included the following components:

- Physical exercise: three supervised sessions per week for 6 months (72 total sessions, corresponding to 12 sessions per month), including balance, strength, and mobility training according to the Vivifrail protocol (Izquierdo et al., 2021), tailored to the participant's functional capacity and frailty level.
- Digital cognitive training: delivered through the validated BrainHQ platform (Posit Science, San Francisco), aimed at enhancing key cognitive domains such as attention, memory, and executive functions.
- Nutritional counseling: provided based on individual nutritional assessment and functional status.
- Monitoring of vascular and metabolic risk factors: including pharmacological therapy review, blood pressure control, and targeted lifestyle interventions.
- Psychosocial stimulation and group activities: implemented to promote social interaction, improve mood, and enhance overall engagement.

The intervention was coordinated by a multidisciplinary team composed of geriatricians/neurologists, physiotherapists, dietitians, neuropsychologists, nurses, and digital coaches. Digital coaches supported participants in the effective use of the tools and technologies required for the intervention, particularly for cognitive training and

remote monitoring. All team members received specific training to standardize intervention delivery across study sites and to enhance participant adherence. Further methodological details are reported in Okoye et al., 2026.

3.1.2. Sample collection and storage

As shown in Figure 22, 12 mL of blood was collected from subjects participating in the IN-TeMPO study after overnight fasting, in tubes containing K_2EDTA, at baseline and at the end of active or self-guided multidomain interventions (12 months). This was done according to the protocols.

- 3URWRFRO was applied in all clinical centres and the samples collected were used to measure biomarkers in the entire cohort, including IL-6 and GDF-15.
- 3URWRFRO was carried out at the discretion of individual centres, involved collecting additional 2 mL aliquots of whole blood in headspace vials and plasma for analysing additional plasma biomarkers, including BDNF, ghrelin, IGF-1 and irisin. These analyses will be performed on a subgroup of n = 100 subjects recruited at the coordinating clinical centre in Monza.

Regarding the collection of plasma for biomarker measurement by the University of Bari, the blood was centrifuged (2000 × g for 10 minutes at room temperature) to separate the plasma. It was then aliquoted and stored at −80 °C until the blind evaluation.

At the end of each time point (T0 and T12), each centre sent the biological samples collected from the locally recruited subjects to the coordinating centre in Monza, which subsequently redistributed the samples to the other centres participating in the study. Therefore, for each time point, each centre has a total number of samples referable to 1,662 subjects from the entire national territory. Further details regarding sample collection and distribution procedures for centralized and decentralized analyses can refer to the article by Sala et al. (2025).

As the transfer of samples to the recipient centres is still ongoing, the data presented in this work refer to a subgroup of 50 subjects recruited at the Bari centre, for whom samples relating to time points T0 and T6 are currently available.

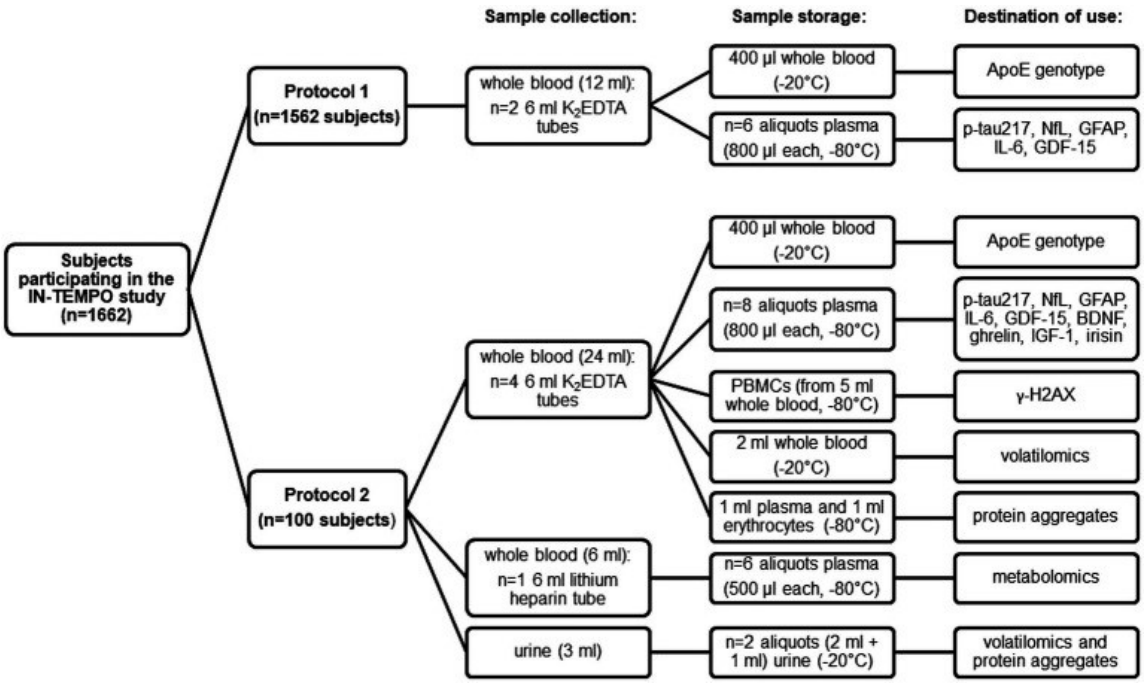

Fig. 22: Schematic representation of the protocols for sample collection, storage, and destination of use.

3.1.3. ELISA test

Plasma levels of selected biomarkers were quantified using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions: Human IL-6 Quantikine ELISA Kit (Cat. No. D6050B), Human GDF-15 Quantikine ELISA Kit (Cat. No. DGD150), Total BDNF Quantikine ELISA Kit (Cat. No. DBNT00), Human Irisin/FNDC5 DuoSet ELISA (Cat. No. DY9420-05), Human IGF-I/IGF-1 Quantikine ELISA Kit (Cat. No. DG100B), and Human Ghrelin DuoSet ELISA (Cat. No. DY8149-05). Absorbance was measured using a VICTOR Nivo™ multimode plate reader (Revvity, Waltham, MA, USA) at 450 nm, and concentrations were determined from a standard curve generated using the supplied standards.

V S

3.2. Preclinical study (Aim 2 and 3)

3.2.1. Experimental groups & treatments schedule (Aims 2-3)

All experiments were conducted in conformity with the Italian law Guidelines for Careand Use of Laboratory Animals (D.L. 116/92), and European Directive (2010/63/UE). Study specific numbers of authorization are detailed below. The experimental procedures used respected the SOPs for pre-clinical tests in P G[hice available on the TREAT – NMD website (http://www.treat-nmd.eu/research/preclinical/dmd-sops/) including the procedures in aim 2, as muscle atrophy is a condition frequently observed in neuromuscular disorders. (Grounds et al., 2008; Willmann et al., 2015, 2020). The procedures were performed in blind by the operators, whenever possible. Animal studies have been reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020). All mice, purchased from The Jackson Laboratory (USA, distributed by Charles River,Calco, Italy), were acclimatized in the animal facility before starting the experimental protocol. Animals were housed in suitable cages (3–5 mice per cage) based on mean body mass (BM), in a single room where appropriate conditions of temperature (22–24 °C), humidity (50–60%), and light/dark cycle (12 h/12 h) were constantly maintained for the entire duration of the study. After acclimatization, mice were randomly assigned to each experimental cohort. Mice were constantly, non-invasively monitored for health and well-being; none of the experimental groups showed signs of pain or distress or macroscopic alterations of vital functions.

Aim 2

The study was approved by the National Ethics Committee for the Welfare of Research Animals of the Ministry of Health (authorization no. 1119/2020-PR). The present experiments were ethically conducted in continuity with the functional and morphological assessments whose results were published in the study by Mantuano et al. (2023) using spared samples from the same set of animals. A total of 32, 17-month-

old male C57BL/6J WT mice (AGED) and 6, 3-month-old male C57BL/6J WT mice (ADULT) were used. After acclimatization, AGED mice cohorts (4 groups of n = 8, each), which were homogeneous for BM and forelimb force, were randomly assigned to each treatment condition, namely the vehicle (filtered tap water), BCAAs or BCAAs combined with 2ALA or Di-ALA. The n = 6 ADULT mice were used as the control group to assess aging-associated sarcopenia outcomes. A sample size was chosen as the best compromise to guarantee a robust statistical relevance, considering both old mice frailty and a long treatment duration (12 weeks, T0–T12). Every week, each formulation was freshly prepared by dissolving the amino acid mixture powder in filtered tap water to obtain the intended final dose, according to mice average BM and water consumption (Mantuano et al., 2018b). Table 1. shows compositions (in weight ratios) and final doses (in mg/kg). These latter translate to equivalent doses in humans, according to an appropriate calculation method for dose conversion (Nair & Jacob, 2016). All mice were fed a daily amount of 5 g/mouse chow (VRF1 standard pelleted diet, Charles River Laboratories) (Mantuano, Boccanegra, Bianchini, et al., 2021c; Mantuano et al., 2018b, 2020b).

Table 1: Composition and daily final dose (mg/kg) for each tested formulation

Formulation	Composition: BCAAs + ALA (Weight Ratio of L-Leu:L-Ile:L-Val:L-ALA/Di-ALA)	Final Dose (mg/kg)
BCAAs	2:1:1	656
BCAAs + 2ALA	2:1:1:2	984
BCAAs + Di-ALA	2:1:1:2	984

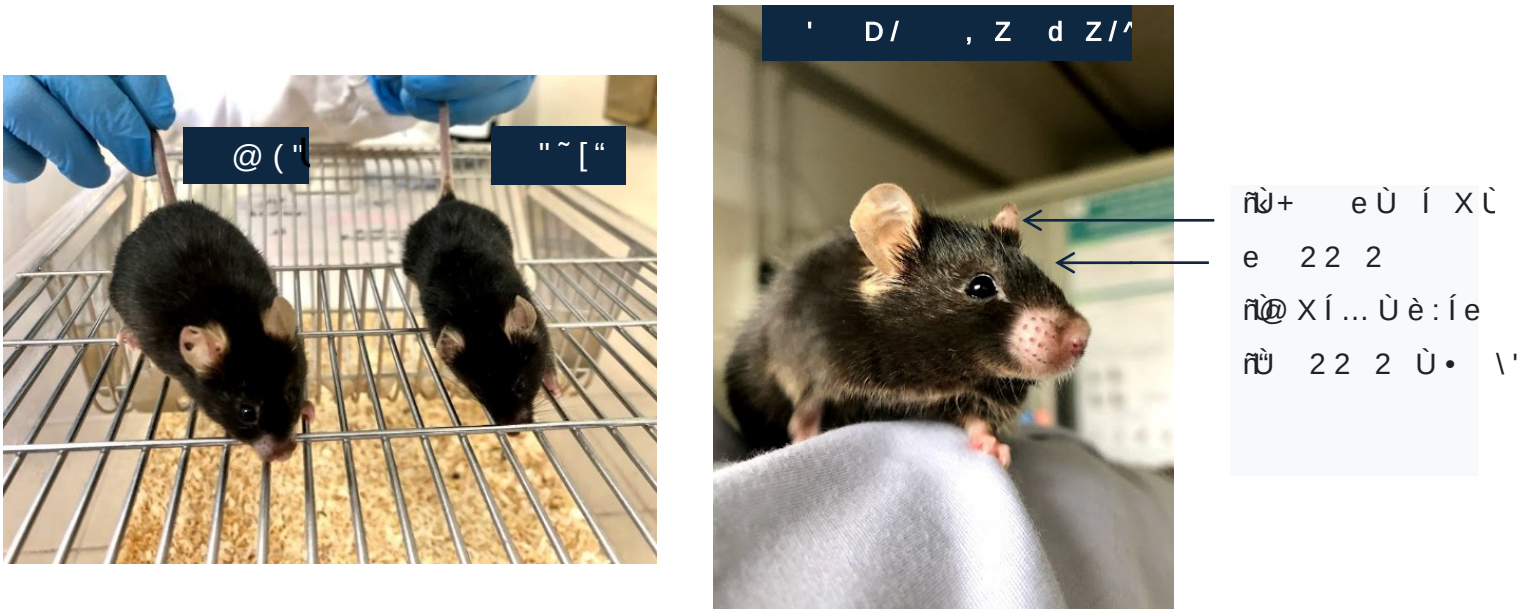

Fig.23: (A) Models used in the study. (B) Characteristics of the AGED model: AGED mice may have grayed coats, thinning whiskers, and slight hair loss.

Aim 3

The study was approved by the National Ethics Committee for the Welfare of Research Animals of the Ministry of Health (authorization no. 1119/2020-PR). Four-week-old, male DBA/2 J wild type (D2-wt; n = 8) and D2- P G([n = 24) mice were used. The D2- P G[were divided into three cohorts (n = 8 mice per group) homogeneous for body mass and randomly assigned to each treatment condition, namely: D2- P G+[vehicle; D2- P G+[JMV2894 640 µg/kg; D2- P G+[JMV2894 1280 µg/kg. D2-WT mice were used as healthy controls. JMV2894 was synthesized in house, as described in previous work (Boccanegra et al., 2023; Moulin et al., 2013). The drug was administered subcutaneously (s.c.) for 6 weeks (T0-T6) at the two selected doses (640 and 1280 µg/kg), 6 days / week; the regimen strategy was chosen on the basis of published studies using JMV2894 or other synthetic GHSs (E. Conte et al., 2020; Demonbreun et al., 2021; Flanigan et al., 2013; Heydemann et al., 2009; Reano et al., 2017b). All mice were non-invasively and longitudinally monitored for health and well-being throughout the total study period. Variations in BM (g) were regularly assessed at the start of each experimental week.

3.2.2 , Q Yp1roVeRdures: hind limb ultrasonography (Aim 3a)

Ultrasonography of the hind limb muscle was performed at T6 via an ultra-high frequency ultrasound biomicroscopy system (Vevo® 2100; VisualSonics, Toronto, ON, Canada). Each mouse, properly anesthetized with isoflurane, was placed on a thermostatically controlled table (37°C) equipped with four copper leads, allowing to monitor both heart and respiratory rate during the imaging session. Body temperature was constantly monitored via a rectal probe. Each hind limb was shaved, secured parallel to the body (foot at 90° with the limb), and covered with ultrasound gel. I assisted to these recordings performed by the main experimenter, Prof. Antonietta Mele. The experimental protocol used is the same detailed in Mele et al., 2021. A three dimensional (3D) volume scan was obtained by acquiring multiple two dimensional (2D) images of the long hind limb axis at regular intervals in Bdimensional mode (B mode) using an MS250 probe (frequency of 21 MHz; lateral and axial resolutions of 165 and 75 mm, respectively). At the end of the procedure, 3D images were reconstructed from previously collected multiple 2D frames and visualized with VisualSonics 3D software, to obtain the total hind limb volume (in mm3). For each mouse 3-4 frames, in which GC muscle was clearly visible, were chosen to evaluate echodensity, using ImageJ® software by creating a grey scale analysis histogram. Echodensity was expressed as percentage variation (%) between D2-WT and D2-P Ggroups (treated or untreated). All data sets were obtained by repeated and independent analyses to avoid intra- and inter-variability, in parallel ensuring the consistency and reproducibility of multiple analyses (Boccanegra et al., 2023b; Mele et al., 2021, 2023).

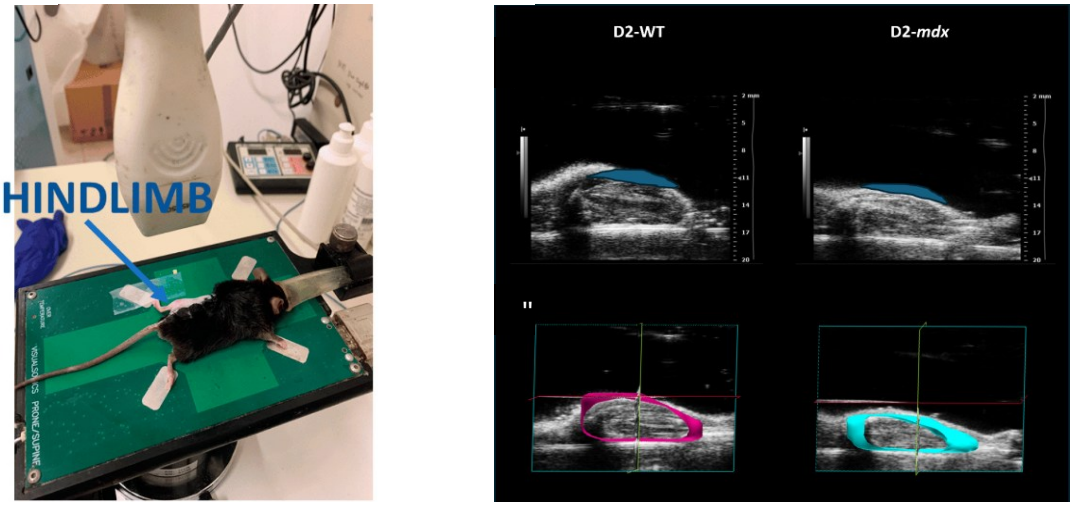

Fig. 24: (A) Acquisition of ultrasound parameters of the hind leg (hind limb) in mouse models with the Vevo® 2100 system (VisualSonics). (B) Sample images from ultrasound acquisitions of the hind paw (hind limb) in D2-WT (left) and D2-mdx (right) mice. (C). Representative frames of B-mode acquisitions of the hind limb, in which the gastrocnemius (GC) muscle was highlighted; B-mode images were used to measure GC echogenicity. (D). Representative 3D images of the hind limb, reconstructed from multiple 2D frames, visualized with VisualSonics 3D software, and used to derive the hind limb volume.

3.2.3. ([Y L Y R procedures

3.2.3.1. Sample collection, processing, and storage (Aims 2 and 3)

The H [Y L Y R experimental phase started at the end of the treatments. Each mouse was anesthetized via intraperitoneal (i.p.) injection with a cocktail of ketamine (100 mg/kg) and xylazine (16 mg/kg). If required, an additional lower dose of ketamine alone (30 mg/kg) was injected to ensure longer and deeper sedation. Several tissues were harvested from each mouse in this phase, following international guidelines, also to fulfil the 3Rs requirements about ethical animal use (Willmann et al., 2015). Based on the experimental needs of each aim, right and left extensor digitorum longus (EDL), gastrocnemius (GC), and soleus (SOL), flexor digitorum brevis (FDB) muscles, as well as vital organs (liver, heart, kidneys, spleen, and brain) were isolated and weighed for a gross examination of drug toxicity and/or effects. Soon after heart removal, DIA

muscle was collected. SOL and EDL muscle of the left limb were carefully prepared for isometric and eccentric contraction recordings (aim 2a). Right GC muscle and kidneys were snap frozen in N2 and stored at − 80 °C until further processing for western blot experiments and RT-PCR analysis (Aims 2 and 3). In parallel, left GC muscle of the left hind limb were embedded in a small amount of Tissue-Tek O.C.T. (Bio Optica, Milan, Italy), immersed in isopentane cooled with liquid nitrogen (N2) for 60 s, and then stored at − 80 °C until being further processed for histology (Aim 3). In addition, left kidney were bisected with a scalpel, rinsed in 1 X PBS, and fixed in 10% buffered formalin overnight. After 24 h, samples were transferred to 50% ethanol and stored at 4 °C until processing. Tissues were then dehydrated through graded alcohols (70–100%), cleared in xylene, and embedded in paraffin blocks, which were stored at −20 °C until sectioning (Aims 2b-3b). In addition, the remaining muscles and organs were snap frozen in N2 and stored at − 80 °C in our mouse tissue repository for possible future analyses (Aims 2 and 3).

3.2.3.2. Isometric and eccentric contraction recordings of isolated muscles (Aim 2a)

EDL and soleus SOL muscles from the left hind limb were isolated and adequately prepared to be used for contractile recordings, as described in full detail by Mantuano et al. (2023). I assisted to these recordings performed by main experimenter, Professor Paola Mantuano. Briefly, each muscle was placed into a horizontal muscle bath (mod. 809B-25, Aurora Scientific Inc.–ASI–Aurora, ON, Canada) (Figure 25) containing 25 mL of isotonic Ringer's solution (mM: NaCl 148, KCl 4.5, $CaCl_2$ 2.0/2.5, $MgCl_2$ 1.0, NaH_2PO_4 0.44, $NaHCO_3$ 12.0, and glucose 5.55; pH 7.2–7.4; 27 ± 1°C), continuously gassed with a mixture of 95% O_2 and 5% CO_2, and thermostatically maintained at 27°C ± 1°C. One tendon was fixed at the bottom of the chamber, and the other tendon was fixed to a force transducer (mod. 300C-LR, Aurora Scientific Inc.–ASI–Aurora, ON, Canada). Electrical field stimulation was obtained by two axial platinum electrodes closely flanking the muscle, connected to a high-power bi-phase

stimulator (mod. 701C, Aurora Scientific Inc.–ASI–Aurora, ON, Canada). Data were acquired [illegible] dedicated signal interface and software (mod. 604A plus DMCv5.415, Aurora Scientific Inc.–ASI–Aurora, ON, Canada). After equilibration (ý30 min), muscle preparations were stretched to their optimal length (L0, measured with an external caliper). Then, the single twitch tension and tetanic contractions at increasing frequencies (10–250 Hz) were elicited according to validated protocols of stimulation (Mantuano, Boccanegra, Conte, et al., 2021; Mantuano et al., 2020b, 2023). This allowed us to calculate using ASI software DMAv5.201 and SigmaPlot 10.0, the Hz50, a calcium-sensitive parameter indicating the frequency at which 50% of maximal sP0 is produced.

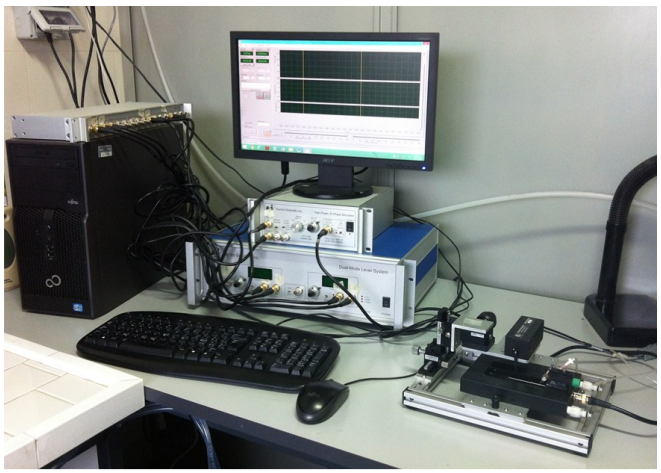

Figure 25. ([Y L [illegible] contraction apparatus used for EDL and SOL muscles.

3.2.3.3. Fiber isolation, Fura2 fluorescence measurements and Store-operated calcium entry measurements (Aim 2a)

I assisted to these recordings performed by main experimenter, Professor Elena Conte. Cytofluorimetric experiments were performed in adult and aged muscle fibers from FDB dissociated using collagenase in normal physiological Ringer's solution, according to previous studies (E. Conte et al., 2021; De Backer et al., 2002). Briefly,

FDB muscles were removed from mice and transferred to a dish containing oxygenated Ringer's solution (mM: NaCl 148; KCl 5.5; $MgCl_2$ 1; $CaCl_2$ 2; $NaHPO_4$ 0.44; $NaHCO_3$ 12; glucose 5.55) to remove residual connective tissue plantaris tendons and then into a dissecting dish containing oxygenated Ringer's solution and 0.2% type IV collagenase (Sigma-Aldrich, United States, C-5138) for 45 min at 37°C. The pH of all solutions was adjusted to 7.3–7.4 by bubbling them with 95% O_2/5% CO_2. After incubation, fibers were washed with Ringer's solution and gently dissociated by several passages through a series of Pasteur pipettes of progressively decreasing diameter. Successively, dissociated fibers were sampled and transferred into a rectangular PRESTIGE 24 × 50 mm glass coverslip (Syntesys disposable labware, cod.372114), coated with PathClear™ extracellular matrix basement membranes (Cultrex, BioTechne, Minneapolis, MN, United States, cod. #3432–005-01). The coverslips were kept in an incubator with 5% CO_2 at 30 °C, and the adhesion of the fibers to the matrix occurred within 2 h. At the end, the coverslips with adhered dissected fibers were transferred into a modified RC-27NE experimental chamber (Warner Instrument Inc., Hamden, United States) for Fura-2 loading and intracellular calcium analysis.
Calcium measurements were performed using the membrane-permeable Ca^{2+} indicator Fura-2 acetoxymethyl ester (Fura-2AM, Thermo Fisher Scientific, Waltham, United States, cod.15455039). Loading of muscle fibers was performed for 1 h in an incubator, with 5% CO_2 at 30°C in normal physiological Ringer's solution containing 5 μm Fura-2AM mixed with 0.05% (v/v) Pluronic F-127 (Thermo Fisher Scientific, Waltham, United States, cod. P3000MP). After loading, muscle fibers were washed, and the experimental chamber was placed on the stage of an inverted Eclipse TE300 microscope (Nikon, Japan) with a 40X Plan Fluor objective (Nikon, Japan) for cytofluorimetric analyses. The QuantiCell 900 integrated imaging system (VisiTech International Ltd., Sunderland, United Kingdom) was used for fluorescence measurements, as previously described (Fraysse et al., 2003; Liantonio et al., 2014).

Pairs of background-subtracted images of Fura-2 fluorescence (510 nm) excited at 340 and 380 nm were acquired at rest, and ratiometric images (340/380 nm) were

calculated for each dissected muscle fiber using QuantiCell 2000 software version 2.0e (VisiTech International Ltd., Sunderland, United Kingdom) and converted to calcium concentration (nM), after the calibration procedure, using the equation: $[Ca^{2+}]i = (R-Rmin)/(Rmax-R)*KD*\beta$, where R is the ratio of the fluorescence excited at 340 nm to that excited at 380 nm; KD is the affinity constant of Fura-2 for calcium, which was taken as 145 nM; and β, Rmin, and Rmax were determined experimentally in situ in ionomycin-permeabilized muscle fibers, as previously described (Fraysse et al., 2003b). Because calibration parameters of Fura-2 can depend on muscle type and experimental condition (Fraysse et al., 2003b) we determined the parameters of Grynkiewicz's equation in each muscle examined for accurate calculation of calcium concentration and used a unique batch of Fura-2 to guarantee no variation in KD between experimental conditions.

A part of dissected FDB fibers adhered on coverslips was used for the SOCE measurement, performed according to previous studies (E. Conte et al., 2017c; Sakai-Takemura et al., 2023). Particularly, fibers were incubated with a calcium free-solution (mM: 148 NaCl, 4.5 KCl, 10 EGTA, 1 $MgCl_2$, 0.44 NaH_2PO_4, 12 $NaHCO_3$, and 5.5 glucose) and 2 μM thapsigargin (Sigma-Aldrich, United States, cod. T9033) to passively deplete Ca^{2+} stores. Successively, calcium-free solution was replaced with normal physiological solution containing calcium (mM: 148 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 0.44 NaH_2PO_4, 12 $NaHCO_3$, and 5.5 glucose) to allow extracellular calcium entry in the cell following the concentration gradient (SOCE protocol). The pH of all solutions was adjusted to 7.3–7.4 by bubbling them with 95% O_2/5% CO_2. All chemicals cited above were purchased from Sigma (St. Louis, MO, United States).

3.2.3.4. Muscle histopathology and immunofluorescence

Aim 3a

Serial cross-sections (10 μm thick) from each frozen left GC muscles were transversally cut into a cryostat microtome set at −20°C (HM 525 NX, Thermo Fisher Scientific, Waltham, MA, USA) and slides sections (SuperfrostTM Plus, Thermo Fisher Scientific) were stained according to the needs (see below). Muscles' morphological features were identified using digital images, acquired with a bright field microscope (CL-I Eclipse Nikon) and the image capture software ImageJ®. For aim 4 was performed Masson's trichrome staining (Bio-Optica), following a validated protocol for the detection of collagen as an index of muscle fibrosis (Boccanegra et al., 2023b). The positive (blue) areas, corresponding to collagen deposition, were quantified using ImageJ® and normalized to the total area of single sections; the results are expressed as percentage (%) of collagen. The analyses were conducted on the entire muscle section, randomly selecting approximately 6 –10 fields per section at 10X magnification for muscle damage and 8 – 12 fields per section at 20X for collagen percentage assessment (Boccanegra et al., 2023b). For immunofluorescence analysis was used to assess to estimate cross-sectional myofibers' area. Tissue sections were fixed in paraformaldehyde for 15 min at room temperature (RT) and permeabilized with 0.1% TWEEN 20 (Sigma-Aldrich) and 0.1% bovine serum albumin (Sigma-Aldrich) in PBS for 45 min at RT. After three washes with PBS, sections were blocked in saturation buffer (0.1% bovine serum albumin in PBS) and incubated with a rabbit polyclonal anti-laminin primary antibody (1:500;L9393S, Sigma-Aldrich) for 2 h at RT in blocking buffer. After three washes in PBS, sections were incubated with 488/594 Alexa Fluor-conjugated secondary antibodies (1:1000; A21206, Thermo Fisher Scientific) for 45 min at RT. Nuclei were stained with PureBlu™ Hoecht 33342 Nuclear Staining Dye (1:50, Bio-Rad Laboratories, Inc., CA, USA). Images were captured using a dark-field microscope (CL-I Eclipse Nikon) at 20×magnification.

Aims 2b and 3b

Kidney samples paraffin-embedded were sectioned at 3 µm by microtome (7 K H U P R 6 F L H, Q 0 V LLFU RMP 355S). Sections were deparaffinized, rehydrated through graded alcohols, rinsed in distilled water. Sirius Red/Fast Green staining was performed to observe renal fibrosis. The slides were incubated for 90 min at room temperature in a Sirius Red/Fast Green solution (1:1 stoichiometric ratio). Slides were then dehydrated and mounted with DPX mounting medium. Collagen fibers stained red, while non-collagen proteins stained green. Whole-slide images of l'AIM2 were acquired with the Aperio ScanScope CS2 (Aperio Technologies, Vista, CA, USA) and analyzed using ImageScope software (v12.1.0.5029, Aperio Technologies). For image quantification, perivascular regions, Bowman's capsule, and section edges were manually excluded using the drawing tools. Results were expressed as the ratio of strongly positive area (NSP) to the total analyzed area (NSP/Area). Mean values were calculated for each animal [Pontrelli et al., 2021]. For AIM 4, however, whole-slide images were acquired using an ECLIPSE Ci-L microscope (Nikon) and analysed using ImageJ software. The perivascular areas, Bowman's capsule and section margins were excluded from the analysis using the drawing tools in the software. Collagen fibres were quantified using ImageJ and the results were expressed as the ratio of the area occupied by collagen fibres to the total area of the analysed section.

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3.2.3.5. Isolation of total RNA, reverse transcription, and real-time PCR

Aim 2a

Gene expression analysis was performed GC muscles of different experimental groups according to a previous study (Boccanegra et al., 2023b). For each muscle sample, total RNA was isolated with TRIzol (Thermo Fisher Scientific, Waltham, United States, cod.10296028). RNA quantification and quality validation (260/280 and 260/230 ratios) were evaluated by using a spectrophotometer (ND-1000 NanoDrop, Thermo Fisher Scientific). Reverse transcription was performed using an iScriptTM gDNA CLR cDNA kit (Bio-Rad, Hercules, CA, United States, cod.1725034). Real-time PCR was performed using the Bio-Rad CFX Connect instrument and custom pre-loaded PCR plates. In addition to the selected genes, all pre-loaded plates included the following controls: DNA contamination control (Bio-Rad, Hercules, CA, United States, Unique assay ID: qMmuCtlD0001004), a positive PCR control (Bio-Rad, Hercules, CA, United States, Unique assay ID: qMmuCtlD0001003), and a reverse transcription control assay (Bio-Rad, Hercules, CA, United States, Unique assay ID: qMmuCtlD0001001). Two technical replicates per biological replicates were performed for each experiment. Amplification of cDNA (3 ng) was performed in a total volume of 20 µL of SsoAdvanced Universal SYBR Green Suprmix (Bio-Rad, Hercules, CA, United States, cod #1725271). The mRNA expression of genes was normalized to the mean of the housekeeping genes: b-actin (Actb). All qPCR analyses performed using SYBR Green were conducted at 50°C for 2 min and 95°C for 2 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min.

Aims 2b, 3a and 3b

Total RNA was isolated from unmounted kidney and GC muscle tissue cryosections collected. Room temperature lysis buffer (200 µL) from miRVana kit (cat. No. A27828, ThermoFisher Scientific) containing β-mercaptoethanol (1.4 µL) was then added

directly to the samples. Solution and tissue were mixed through pipetting to ensure proper lysis. Extraction was performed with KingFisher™ Duo Prime (ThermoFisher Scientific) according to manufacturer's protocols. RNA purity and quantity was assessed via spectrometry (ND-1000 NanoDrop, ThermoFisher Scientific), and only samples that showed 260/280 ratios > 2.0 and 260/230 ratios > 1.8 were considered acceptable. Subsequently, 400 ng of purified RNA were reverse transcribed to cDNA with iScript gDNA Clear cDNA Synthesis Kit (172–5035 Bio-Rad Laboratories, Inc., CA, USA). To each RNA sample, 2 µl of Dnase master mix (volume by reaction: iScript Dnase 0.5 µL, iScript Dnase Buffer 1.5 µL) and nuclease-free water for a total reaction volume of 16 µl were added. The reaction was incubated using a thermal cycler at conditions 25°C for 5 min followed by 75°C for 5 min to inactivate DNAase. 4 µL of iScript Reverse Transcription Supermix 5X were then added, with the reaction incubated at 25°C for 5 min, followed by 46° for 20 min with a final heat inactivation of 95°C for 1 min, according to the protocol provided by the manufacturer. 80 µl of RNAse-free water were then added to the sample in order to obtain a 4 ng/µl cDNA solution. Quantitative Real Time PCR was performed via the CFX384 Connect Real-Time PCR system (Bio-Rad) using Prime PCR assays SYBR® green. Each reaction, carried out in triplicate, consisted of 2 µl of cDNA (8 ng cDNA, assuming 1:1 conversion), with 5 µl of SssoAdvanced Universal SYBR® Green Supermix (172–5271 Bio-Rad), 0.5 µl of PrimePCR assays SYBR® green primers (Tables 2) and 2,5µl of nuclease-free water for a total volume of 10 µl. All qPCR performed using SYBR Green used a hot start (95°C for 2 min) followed by 40 cycles of a two-step reaction (95°C for 5 s, 60°C for 30 s). The mRNA expression of genes was normalized to the mean of two housekeeping genes, for Aim 3a: Actb (qMmuCED0027505 Bio-Rad) and SDHA (qMmuCID0019353 Bio-Rad) and for Aims 2b/3b: Actb (qMmuCED0027505 Bio-Rad) e Ppia (qMmuCED0041303 Bio-Rad). Quantification was performed using the 2ΔΔCt method.

Tab 2. List of PrimePCR™ SYBR® Green Assay IDs

AIM 2a		
Gene	**Gene full name**	**Unique assay ID**
Orai1	ORAI Calcium Release-Activated Calcium Modulator 1	qMmuCID0020628
Cacna1s	Calcium Voltage-Gated Channel Subunit Alpha1 S	qMmuCID0021135
Atp2a1	ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 1	qMmuCID0027023
Atp2a2	ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 2	qMmuCID0005528
Ryr1	Ryanodine Receptor 1	T0PX&,'
Stim1	Stromal Interaction Molecule 1	qMmuCID0021406
AIM 2b		
Cdkn1a	Cyclin-dependent kinase inhibitor 1A	qMmuCED0025027
IL-1β	Interleukin 1 beta	qMmuCID0005641
Tgfb1	Transforming growth factor, beta 1	qMmuCED0044726
Tnf-	Tumor necrosis factor	qMmuCED0004141
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1	qMmuCID0005357
AIM 3a		
TGFb	transforming growth factor beta	qMmuCID0017320
COL1A1	collagen type I alpha 1 chain	qMmuCID0021007
MMP9	matrix metalloproteinase 9	qMmuCID0021296
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif	qMmuCID0017960
FBXO32	E3 ubiquitin-protein ligase atrogin 1	qMmuCID0011869
TRIM63	E3 ubiquitin-protein ligase MuRF1	qMmuCID0014591
IGF1	insulin like growth factor 1	qMmuCED0044388
IGF1R	insulin like growth factor 1 receptor	qMmuCID0005315

W W

3.2.3.6. Protein expression analysis by western blot (Aims 2 and 3)

Protein extraction and immunoblot analysis were performed on left kidneys (Aim 2b) and GC muscles (Aims 2a and 3a) from the different experimental groups. Tissues were homogenized in ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.4 at 4°C), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Roche, Cat# 5056489001). Homogenates were centrifuged at 1,500 g for 15 min at 4°C, and protein concentration was determined using the BCA Protein Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions.

- Aim 2a: For immunoblot analysis, 40 µg of protein were separated on 10% Mini–PROTEAN TGX Stain-Free Protein Gels (Bio-Rad, Hercules, CA, United States, Cat# 4568033) and transferred onto stain-free polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, United States, Cat# 1704156) for 7 min at 1,3A–25 V (Trans-Blot ® Turbo™ Transfer System, Bio-Rad, Hercules, CA, United States). The incubation in primary antibodies was carried out overnight at 4°C, MG29 and MG53 anti-rabbit (1:5000 anti-rabbit IgG, Bio-Rad, Hercules, CA, United States, Cat# 170–6515 (also 1706515), RRID:AB_11125142) horseradish peroxidase-conjugated secondary antibodies were carried out at room temperature. Dilutions of primary antibodies were used as follows: MG29 (dilution 1:1,000, Sigma-Aldrich Cat #SAB3500069, RRID:AB_10604155), and MG53 (dilution 1:700, MyBioSource Cat# MBS8502881, RRID:AB_3086706). The evaluation of the relative amount of each analyzed protein was performed by relating the densitometric value of optical density (OD) units of each protein band to the OD units of the respective vinculin band.
- Aim 2b and 3a. Equal amounts of protein (40 µg) were separated on 10% SDS–PAGE gels and transferred onto PVDF membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad; 7 min, 1.3 A, 25 V). Membranes were

blocked for 1 h in TBS containing 5% non-fat dry milk and incubated overnight at 4°C with the appropriate primary antibodies. For Aim 2b, the following antibodies were used: mouse anti-vinculin (1:800; Sigma-Aldrich, V9131), rabbit anti-AMPK (1:500; CST, #2532), rabbit anti-phospho-AMPKα (Thr172) (1:500; CST, #2531S), rabbit anti-mTOR (1:500; CST, #2972S), and rabbit anti-phospho-mTOR (Ser2448) (1:500; CST, #2971S). HRP-conjugated secondary antibodies were anti-mouse IgG (1:5000; Sigma-Aldrich, A9044) and anti-rabbit IgG (1:5000; Bio-Rad, #170-6515). Phospho-AMPK and phospho-mTOR were normalized to their respective total proteins, detected on the same membrane after stripping (2% SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol, 30 min at 50°C). Total AMPK and mTOR were normalized to vinculin.For Aim 3a, membranes were incubated with mouse monoclonal antibodies (1:100; Santa Cruz Biotechnology): Akt1 (B-1) sc-5298, p-Akt1 (Ser473) sc-293125, GSK-3β (E-11) sc-377213, p-GSK-3β (F-2) sc-373800, and IGF-1R (7G11) sc-81464. HRP-conjugated secondary antibody was anti-mouse IgG (1:5000; Sigma-Aldrich, A9044). Mature IGF-1R (α and β subunits) levels were normalized to pro-IGF-1R. Phospho-Akt and phospho-GSK-3β were normalized to their respective total proteins after stripping and reprobing (buffer containing SDS, Tris-HCl pH 6.8, and β-mercaptoethanol; 30 min at 50°C).

At the end of the experiment, membranes were washed three more times (10 min each) in TBS, developed with chemiluminescent substrate (Clarity Western ECL Substrate, Bio-Rad), and visualized using a Chemidoc XRS+ imaging system (Bio-Rad). Densitometric analysis was performed using Image Lab software (Bio-Rad), which detects chemiluminescence from each protein band to obtain the absolute signal intensity.

3.2.3.7. ELISA test (Aim 3a)

IGF-1 levels in plasma samples were measured using a commercially available ELISA kit (Mouse IGF-1 ELISA Kit, FineTest®, Wuhan Fine Biotech Co., Ltd., Wuhan, China), according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader, and IGF-1 concentrations were calculated from a standard calibration curve generated with the provided standards.

3.2.4. Statistics

Aim 1

Data were expressed as median (IQR) or mean ± SEM, according to graphical representation. Normality was assessed using the Shapiro-Wilk test, indicating a predominantly non-parametric distribution. Comparisons between control and intervention groups were performed using the Mann-Whitney test for independent samples, conducted separately at baseline (T0) and at 6-month follow-up (T6). No within-subject longitudinal comparisons (T0 vs T6) were performed. Statistical significance was set at $p < 0.05$.

Aim 2 and 3

All data were expressed as mean ± SEM. Normality was assessed using the Shapiro–Wilk test; all datasets met the assumptions for parametric analysis within the 95% confidence interval. Statistical analyses were performed using GraphPad Prism 8.

For Aim 2, differences between ADULT and AGED + vehicle mice were evaluated by unpaired Student's t-test, while multiple comparisons among AGED + vehicle, AGED + BCAAs, AGED + BCAAs + 2ALA, and AGED + BCAAs + Di-ALA were performed by one-way ANOVA with Dunnett's post hoc test. For Aim 4, multiple comparisons

among D2-WT, D2- P G-[vehicle, and D2- P Gt[eated with JMV2894 (640 or 1280 µg/kg) were analysed by one-way ANOVA with Bonferroni's correction.

A p-value < 0.05 was considered statistically significant. Samples were excluded only due to overt technical issues. All procedures and analyses were conducted in a blinded manner. When appropriate, treatment efficacy was expressed as Recovery Score (R.S.) according to TREAT-NMD SOP (ID: DMD_M.1.1.001) (Fig. 26-27).

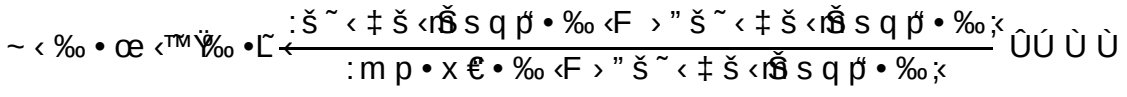

Fig 26. Calculation of recovery score (R.S.) for aim 2

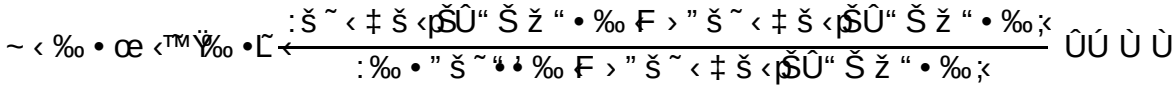

Fig 27. Calculation of recovery score (R.S) for aim 4

RESULTS

4.1. Results Aim 1: Identification of plasma biomarkers of sarcopenia

As part of my PhD programme supported by the PNRR AGE-IT project, I was involved in the IN-TeMPO project (ClinicalTrials.gov ID: NCT06248723). I particularly contributed to the activities related to the secondary objectives of the study, which aimed to identify circulating plasma biomarkers useful for risk stratification and for monitoring the onset and progression of sarcopenia. The study protocol has been published in two scientific journals: Okoye C et al., J Gerontol B Psychol Sci Soc Sc, 2025 Dec (doi: 10.1093/geronb/gbaf186); and Sala et al., Front Aging Neurosci, 2025 May (doi: 10.3389/fnagi.2025.1581892).

4.1.1 Baseline biomarker levels across age groups

At baseline (T0), the correlation between age and circulating plasma levels of selected biomarkers (BDNF, IL-6, GDF-15, IGF-1, ghrelin, and irisin) was evaluated in a subgroup of 53 participants, equally distributed between males and females and aged ≥ 60 years. Correlations were assessed using Spearman's rank correlation coefficient to determine whether the age-related trends of these biomarkers were consistent with those reported in the literature. This would support the representativeness of the selected cohort despite the current limited sample size. The cohort will be progressively expanded in subsequent phases of the study. Overall, the analysed biomarkers exhibited trends comparable to those previously described. Notably, a positive and statistically significant correlation was observed between plasma GDF-15 levels and age, consistent with its established role as a biomarker associated with ageing processes and chronic low-grade inflammation. The remaining biomarkers demonstrated age-related patterns that are consistent with existing evidence, although statistical significance was not reached, likely due in part to the current sample size.

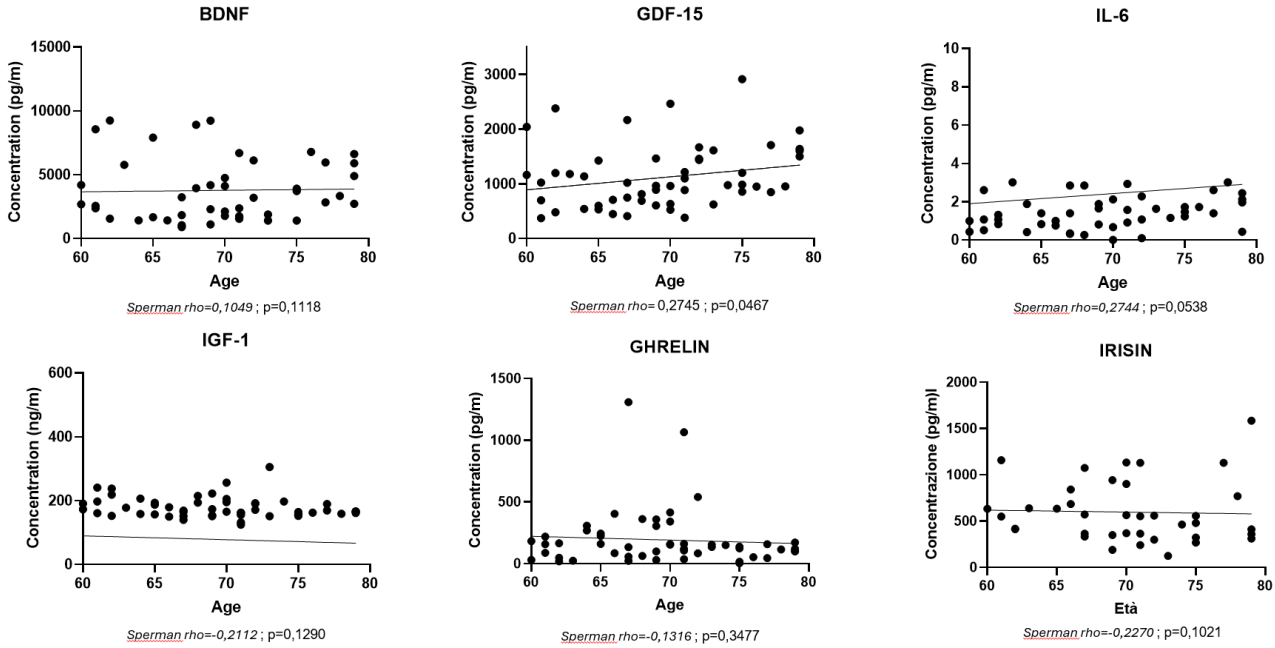

Fig.28: Correlation between age and circulating plasma biomarker levels at baseline (T0). Scatter plots showing the association between age and plasma concentrations of BDNF, GDF-15, IL-6, IGF-1, ghrelin, and irisin in a subgroup of 53 participants (≥ 60 years; equally distributed between males and females). Correlations were assessed using Spearman's rank correlation coefficient. A statistically significant positive correlation was observed between GDF-15 levels and age ($\rho = 0.2745$; $p = 0.0467$). Lines represent linear regression fits.

4.1.2 Distribution of biomarkers stratified by sex at baseline

At T0, plasma biomarker levels were analysed by stratifying the population by sex, in order to assess for any gender differences within the study cohort. Overall, the distributions of BDNF, GDF-15, IGF-1, IL-6, ghrelin, and irisin showed no significant differences between males and females. For certain biomarkers, notably GDF-15, ghrelin and irisin, slight variations in median values and data dispersion were observed; however, no statistically significant differences were identified. The observed dispersion may be attributable to intra-group variability linked to inter-individual heterogeneity in the elderly population. Overall, these results indicate that, at baseline, gender does not represent a significant confounding factor in the distribution of biomarkers analysed in this subgroup. In subsequent steps of the study, expanding the

sample size will allow for more in-depth analyses, including any gender differences in different age ranges.

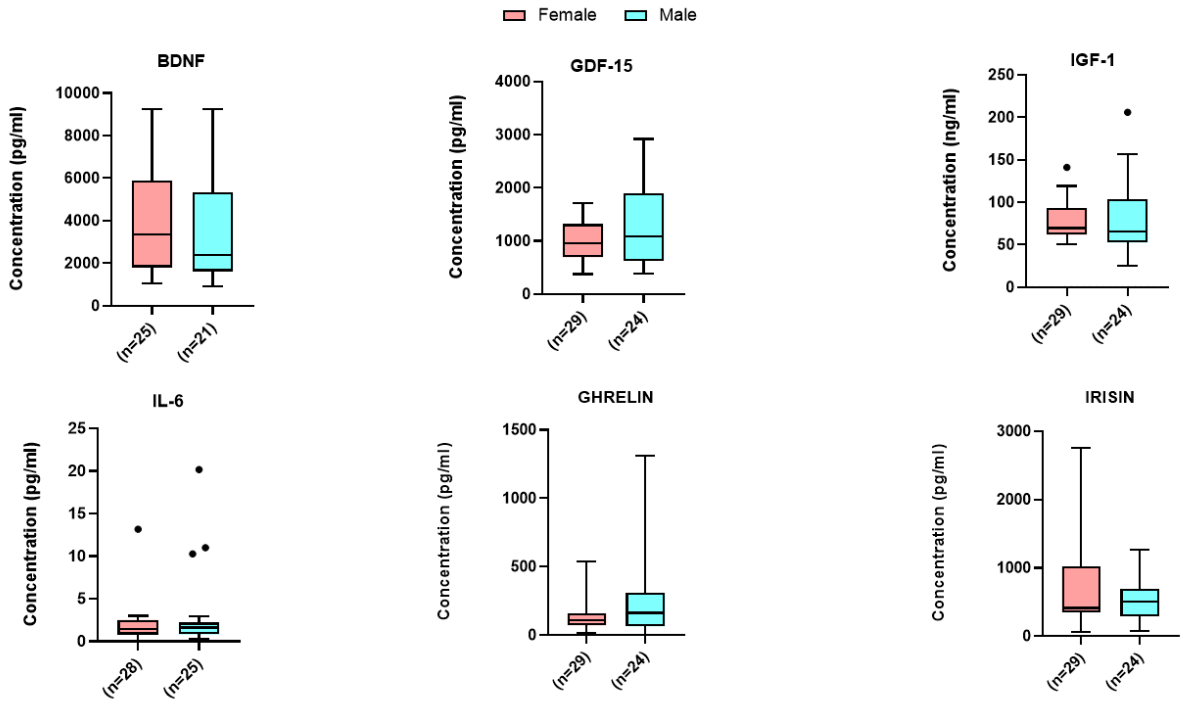

Fig.29: Distribution of circulating plasma biomarkers stratified by sex at baseline (T0). Box-and-whisker plots show the plasma concentrations of BDNF, GDF-15, IGF-1, IL-6, ghrelin and irisin in female and male participants. Data are expressed as median and interquartile range (IQR). The sample sizes for each group are detailed below. Comparisons between sexes were performed using the Mann–Whitney U test. No statistically significant differences were observed for any of the analysed biomarkers.

4.1.3. Six-month longitudinal analysis of circulating biomarkers

Following a 6-month follow-up (T6), changes in circulating plasma biomarker concentrations were evaluated relative to T0 within the two study groups: the control group, which followed self-guided interventions supported by apps, web platforms, or video tutorials, and the intervention group, which participated in a structured multidomain intervention guided by a multidisciplinary team. At T6, the sample size was reduced (37 participants vs. 53 at baseline) due to drop-outs occurring during follow-up, a factor that may have affected the statistical power of the analysis. At T6,

the analysed plasma biomarkers demonstrated temporal patterns consistent with the expected trends. BDNF, IGF-1, and irisin decreased, GDF-15 and ghrelin increased, while IL-6 levels remained stable. The results at T6 showed no significant differences between the control and intervention groups when compared to the results at T0. This indicates that the intervention did not cause any substantial changes in the circulating biomarker profile of the overall cohort.

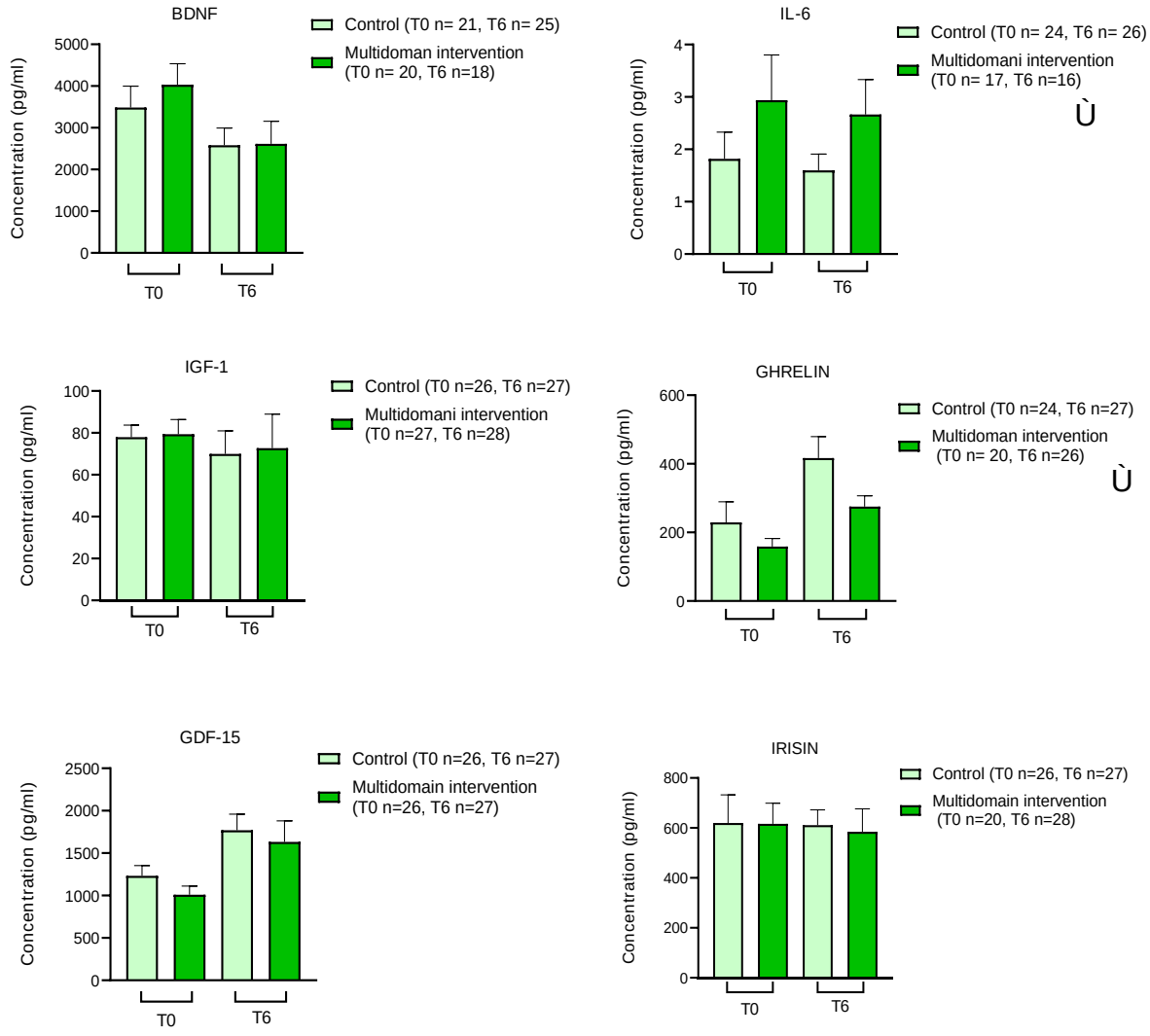

Fig. 30. Temporal changes in circulating plasma biomarker levels at baseline (T0) and after 6 months of follow-up (T6) in the control and multidomain intervention groups. The graphs show circulating concentrations of BDNF, IL-6, IGF-1, ghrelin, GDF-15, and irisin in the two study groups at the two time points. Data are expressed as mean ± SEM. Statistical comparisons between groups were performed using appropriate tests, and no statistically significant differences were observed in the overall cohort. Sample size for each group is indicated in the legend.

The subsequent analysis explored the potential influence of treatment in relation to sex. At T6, male participants in the control group showed a reduction in plasma BDNF levels compared to the baseline, while those in the multidomain intervention group showed an increase relative to T0. Given that baseline medians were comparable between the two groups, the reduction observed in the control group appears consistent with an age-related trend, while the increase in the treated group may reasonably be attributed to the effect of the intervention rather than to pre-existing differences between groups. The observed increase in BDNF may be associated with adaptive mechanisms induced by physical activity and/or cognitive training included in the multidomain intervention. However, at this stage of the study, data regarding changes in muscle mass, muscle strength, or other functional parameters have not yet been made available; therefore, the underlying biological mechanism cannot be definitively established. No significant longitudinal changes were observed for the other analysed biomarkers in relation to gender.

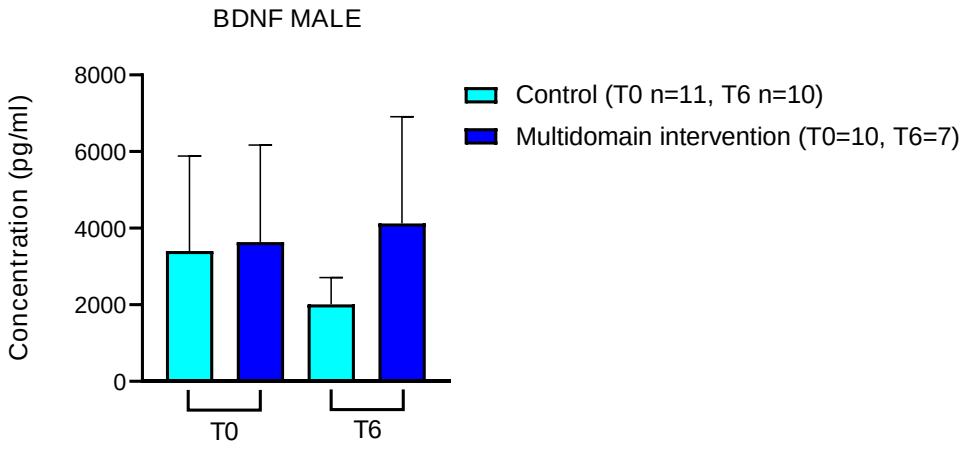

Fig.31: Plasma BDNF levels in male participants at baseline (T0) and after 6 months of follow-up (T6) in the control and multidomain intervention groups. Data are expressed as mean ± SEM. Sample sizes are indicated in the legend for each group and time point. The difference at T6 was not statistically significant (Mann–Whitney U test).

4.2. Results Aim 2: Preclinical evaluation of BCAA-based formulations in mouse model of physiological aging

Previous studies published in Mantuano et al., 2023 showed that supplementation with BCAAs alone or in combination with potentiating molecules reduces histological markers of atrophy and improves muscle weakness L Q Yand YHR[Y in Yan Relderly mouse model. To investigate the molecular mechanisms through which BCAAs counteract age-related functional muscle alterations, we examined the impact of these formulations on intracellular Ca^{2+} levels and the SOCE mechanism in sarcopenic skeletal muscle (Aim 2a). This study was conducted in collaboration with the pharmaceutical company Dompé and I was involved in this evaluation during my PhD. The results were published in Conte et al., Front Pharmacol June 2024 (DOI: 10.3389/fphar.2024.1393746). The second part of this study was conducted in collaboration with Professor Pontrelli at the Department of Precision and Regenerative Medicine and Ionian Area (DIMEPRE-J), University of Bari "Aldo Moro", and aimed to evaluate the potential protective effect against kidney damage associated with the ageing process (Aim 2b).

4.2.1. Results Aim 2a:

4.2.1.1. Resting intracellular calcium and SOCE

As expected (Fraysse et al., 2006a; Mijares et al., 2021b), the resting intracellular calcium concentration, $[Ca^{2+}]_i$, was significantly higher in FDB aged muscle fibers than in fibers from adult mice (AGED + vehicle: 94 ± 5 nM vs. ADULT: 53 ± 3 nM). Treatments with all mixtures were able to significantly prevent this increase by bringing the intracellular calcium levels to values almost overlapping those of the adult mice (recovery scores toward ADULT value: AGED + BCAAs 53%; AGED + BCAAs + 2ALA 71%; and AGED + BCAAs + Di-ALA 61% (Fig. 32 A). To evaluate SOCE activity, SR Ca^{2+} stores of FDB muscle fibers were depleted with thapsigargin in the

absence of extracellular Ca^{2+} and then extracellular Ca^{2+} was applied to muscle fibers to measure SOCE. As shown in Fig 32. B according to previous studies (Thornton et al., 2011b; Zhao et al., 2008b), aged muscle fibers showed an SOCE value significantly reduced with respect to adult muscle fibers (AGED + vehicle: 138 ± 19 nM vs. ADULT: 450 ± 50 nM). A trend of increased SOCE was observed with the treatment with BCAAs and BCAAs + 2ALA, with the latter formulation being the most effective (recovery scores toward ADULT value: AGED + BCAAs 29% and AGED + 2ALA 48%). In contrast, no significant effect was exerted by BCAAs + Di-ALA, with the SOCE value completely overlapping that of aged untreated mice.

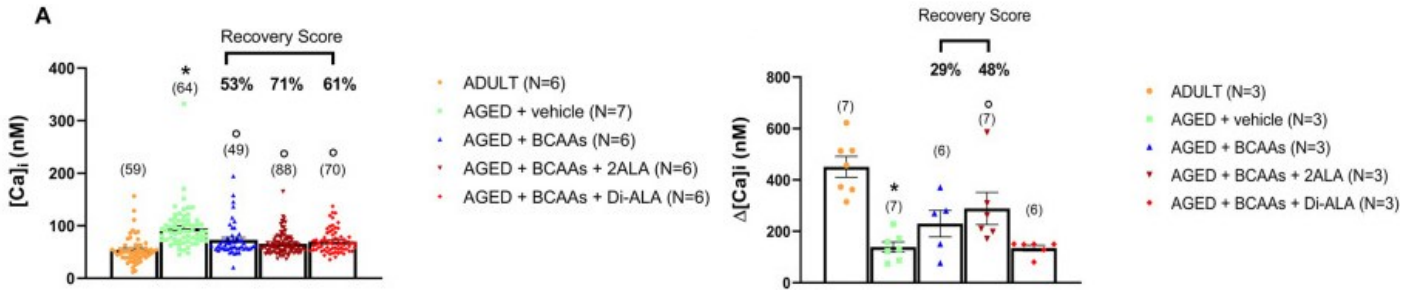

Fig. 32: Calcium homeostasis characterization of adult, aged, and treated FDB fibers. (A) Resting calcium concentration. A statistically significant difference was found by unpaired Student's t-test for AGED + vehicle vs. ADULT (* S< 0.0001). A statistically significant difference was found among AGED mice groups by one-way ANOVA (F = 14.87; S< 0.0001). Dunnett's S R V WestKuRe F to compare each mixture-treated group to the vehicle group, is as follows: °vs. AGED + vehicle (S< 0.0001). Values are expressed as the mean ± SEM of the number of fibers analyzed (indicated in brackets over the bars) derived from a number of 6/7 mice in each group. **(B)** Amplitude values of $[Ca^{2+}]i$ observed with the SOCE protocol. A statistically significant difference was found by unpaired Student's t-test for AGED + vehicle vs. ADULT (* S< 0.0001). A statistically significant difference was found among AGED mice groups by one-way ANOVA (F = 3.589; S= 0,0318). Dunnett's S R V WestKuRe F to compare each mixture-treated group to the vehicle group, is as follows: °vs. AGED + vehicle (S< 0.035). Values are expressed as the mean ± SEM of the number of fibers analyzed (indicated in brackets over the bars) derived from a number of 3 mice in each group. The recovery score toward the ADULT value, calculated for each treated group, is indicated above the bars.

To investigate the outcome of the improved Ca^{2+} homeostasis at the functional level, we focused on calcium-dependent functional indices calculated during isometric contraction recordings in isolated fast-twitch muscle (i.e., EDL) and particularly on the frequency of stimulation to obtain a half maximal tetanic contraction (Hz50). In fact, in parallel with a significant decline in the maximal specific tetanic force (Mantuano et al., 2023), EDL muscles from aged mice also exhibited a significant shift of the Hz50 toward lower frequencies (i.e., a train of pulses of lower frequency is needed to get a half maximal contraction) with respect to adult mice. Importantly, a significant amelioration of Hz50 was observed in aged mice treated with all three formulations (Figure 33), with BCAAs + 2ALA being especially effective (recovery scores toward ADULT value: AGED + BCAAs 100%; AGED + BCAAs + 2ALA 162%; and AGED + BCAAs + Di-ALA 150%).

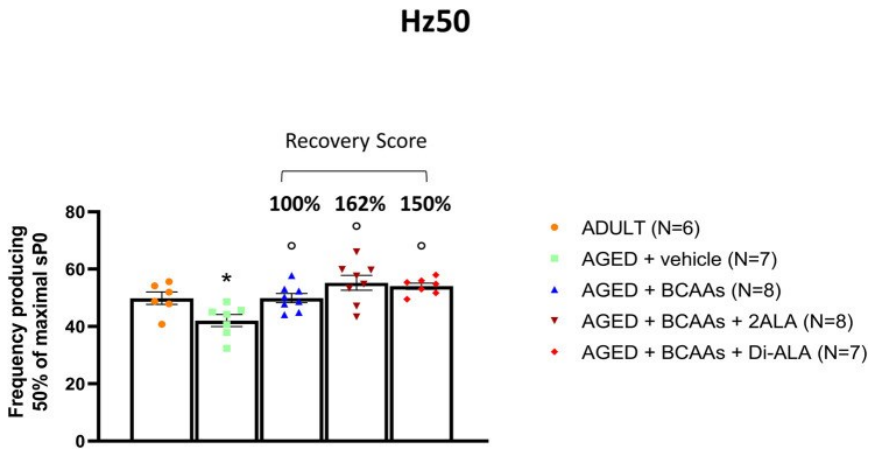

Fig.33: Hz50 of isolated EDL muscle. Values for Hz50, a contractile index related to calcium homeostasis indicating the frequency at which 50% of maximal isometric specific tetanic force (sP0) is produced, measured in extensor digitorum longus (EDL) muscles isolated from ADULT, AGED + vehicle, AGED + branched-chain amino acids (AGED + BCAAs), AGED + branched-chain amino acids +2 L-alanine (AGED + BCAAs + 2ALA), and AGED + branched-chain amino acids + L-alanyl-L-alanine (AGED + BCAAs + Di-ALA). All values are expressed as mean ± SEM from a number of 6/8 mice in each group. A statistically significant difference was found by unpaired Student's t-test for AGED + vehicle vs. ADULT mice ($p < 0.03$). A statistically significant difference among AGED mice groups was found by one-way ANOVA ($F = 8.9$, $p = 0.0003$). Dunnett's post hoc test, used to compare each mixture-treated group to the vehicle group, is as follows: °vs. AGED + vehicle ($0.0002 < p < 0.03$). The recovery score toward the ADULT value, calculated for each treated group, is indicated above the bars.

4.2.1.2. Expression profile of genes involved in SOCE mechanism and calcium buffering

To gain insight into the molecular mechanism underlying the capability of BCAAs of interfering with SOCE, a gene expression analysis about the key components of SOCE has been performed on GC muscles. As shown in Figure 34 the expression values of U \ U (encoding for the ryanodine receptor 1, RyR1) and D W S (encoding for the sarcoplasmic/endoplasmic reticulum Ca^{2+}-transporting 1, SERCA1) genes were significantly decreased in untreated aged muscle compared with adult ones. Similarly, although not in a significant manner, D W S (encoding for the sarcoplasmic /endoplasmic reticulum Ca^{2+}-Transporting 2, SERCA2) mRNA levels had reduced expression. In contrast, no alteration of V W L P R U and L F D V F U Q (encoding for dihydropyridine receptor) mRNA levels were observed, suggesting that the age-related decrease in SOCE activity is not likely due to changes in the relative abundance of these SOCE components.

Interestingly, BCAA and BCAAs + 2ALA treatments efficaciously counteract the decrease of U \, UD W S, and D W S gene expression, resulting in the expression values of treated animals overlapping or being even greater than the related values of adult animals. Furthermore, the same mixes induced an increase of F D F Q gene V expression. By contrast, no effect has been observed for BCAAs + Di-ALA treatment.

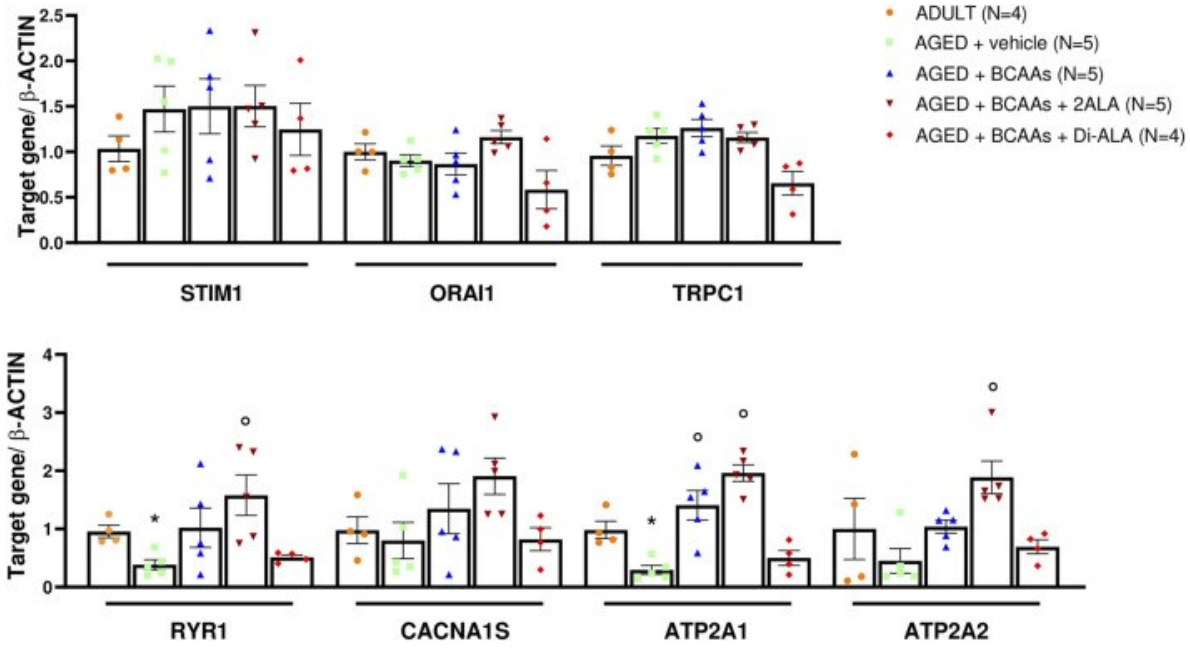

Fig. 34: mRNA expression levels of selected genes involved in the SOCE mechanism. Relative content of mRNA levels for ORAI calcium release-activated calcium modulator 1 (R U), stromal interaction molecule 1 (V W), transient receptor potential cation channel subfamily C member 1 (W U) genes (upper panel), ryanodine receptor 1 (U \ ), calcium voltage-gated channel subunit alpha1 S (F D F Q) (encoding for dihydropyridine receptor, DHPR), ATPase sarcoplasmic /endoplasmic reticulum Ca^{2+} transporting 1 (D W S) (encoding for sarcoplasmic/endoplasmic reticulum calcium ATPase1, SERCA1), and ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2 (D W S) (encoding for sarcoplasmic/endoplasmic reticulum calcium ATPase2, SERCA2) genes (bottom panel) involved in store-operated calcium entry (SOCE) mechanism and calcium homeostasis, respectively, normalized to D F W gene in gastrocnemius (GC) muscles isolated from ADULT, AGED + vehicle, AGED + branched-chain amino acids (AGED + BCAAs), AGED + branched-chain amino acids +2 L-alanine (AGED + BCAAs + 2ALA), AGED + branched-chain amino acids + L-alanyl-L-alanine (AGED + BCAAs + Di-ALA) groups. A statistically significant difference was found by unpaired Student's t-test for AGED + vehicle vs. ADULT (* S = 0.042 for ryr1; * S = 0.032 for atp2a1). A statistically significant difference was found among AGED mice groups by one-way ANOVA for D W S (F = 22.24, S < 0.0001), D W S (F = 9.979, S = 0.0007). Dunnett's S R V W test was as follows: °vs. AGED + vehicle (0.0002 < S < 0.004). Data are expressed as fold-difference compared with the ADULT group, and values are expressed as mean ± SEM from a number of 4/5 mice in each group.

4.1.2.3. Expression analysis of MG29, MG53

In addition, we evaluated the protein and gene expression levels of MG29 and MG53. These are key regulators involved in the control of the SOCE mechanism in skeletal muscle (Gruszczynska-Biegala et al., 2021). MG29 is a protein located in the T-tubules that functions as a modulator of SOCE and RyR1 activity (Weisleder et al., 2006; Woo et al., 2015; Zhao et al., 2008a) MG53 is a protein involved in Ca^{2+} handling and muscle membrane repair processes (Baumann et al., 2016; Benissan-Messan et al., 2020; Cai et al., 2009; Ryan et al., 2008). As demonstrated in the figures, we observed a significant reduction in MG29 expression, concomitant with an increase in MG53 levels, in the GC muscles of AGED + vehicle mice compared to ADULT mice. All tested formulations, particularly those enriched with ALA, were able to counteract the alterations in MG29 and MG53 at both the gene and protein expression levels, although to different extents.

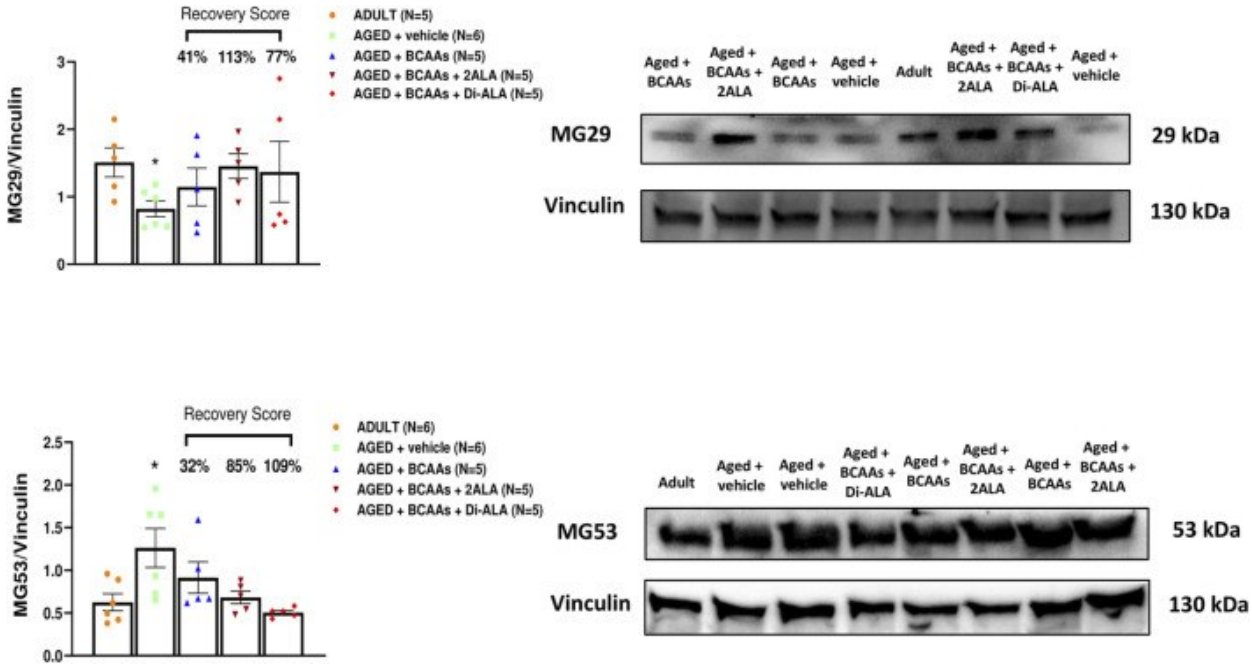

Fig. 35: MG29 and MG53 protein expression analyses in gastrocnemius muscles. Densitometric analysis and representative Western blot of mitsugumin 29 (MG29) and mitsugumin 53 (MG53) bands normalized to vinculin of ADULT, AGED + vehicle, AGED + branched-chain amino acids (AGED + BCAAs), AGED + branched-chain amino acids +2 L-alanine (AGED + BCAAs + 2ALA), and AGED + branched-chain amino acids + L-alanyl-L-alanine (AGED + BCAAs + Di-ALA) groups. Blots were loaded by an operator unaware of the experimental groupings of samples. A statistically significant difference was found by unpaired Student's t-test for AGED + vehicle vs. ADULT (* S= 0.0163 for MG29; * S= 0.0284 for MG53). Values are expressed as mean ± SEM from a number of 4 mice in each group. The recovery score toward the ADULT value, calculated for each treated group, is indicated above the bars.

4.2.2. Results Aim 2b

4.2.2.1. Effects of BCAAs and L-ALA supplementation in aged kidney fibrosis

For a macroscopic assessment of renal status, kidney weights were compared between adult and aged mice, both treated and untreated. Despite the finding that aged kidneys exhibited a marginal increase in weight compared to adult kidneys, this discrepancy did not attain statistical significance. Moreover, the therapeutic intervention administered did not result in a quantifiable effect on kidney weight (data not shown). In order to evaluate the effects of treatment on tubulointerstitial fibrosis, Sirius Red/Fast Green staining was performed on the cortex, medulla and glomeruli (see Figure 36). Aged kidneys exhibited significant fibrotic remodelling, characterised by prominent collagen deposition, tubular dilation, and interstitial inflammatory infiltrates affecting both cortical and corticomedullary regions. The administration of treatments containing either 2ALA or Di-ALA resulted in a substantial enhancement of these structural abnormalities. The findings indicate that both combinations significantly reduced cortical fibrosis in comparison to untreated aged controls (see Figures 36 and 37). The BCAAs+2ALA mixture exhibited the most substantial reduction (2.4-fold; $p=0.01$). BCAAs alone also mitigated fibrosis, though to a lesser extent (1.5-fold reduction), suggesting a dose- or synergistic benefit of alanine enrichment. No significant antifibrotic effects were observed in the medulla or glomeruli of treated mice in comparison to the AGED-VEH group (data not shown).

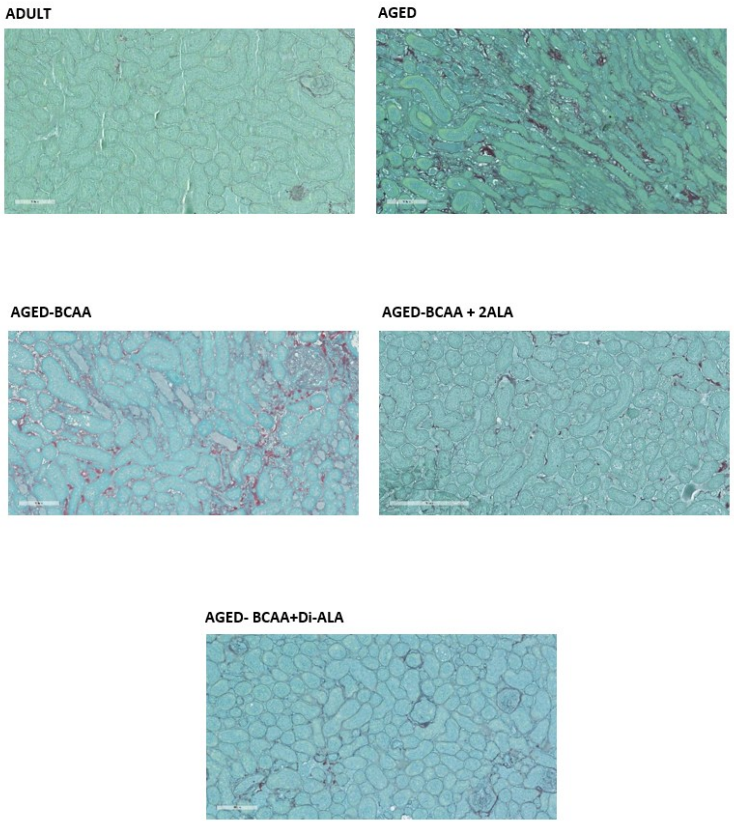

Fig. 36: Sirius Red/Fast Green staining of renal sections from adult, aged, and aged mice treated with BCAA mixtures, BCAA + 2-alanine (2ALA), and BCAA + alanine dipeptide (Di-ALA). Collagen fibers, abundantly present in fibrotic regions of the kidney, appear stained in red. Whole-slide scanning was performed using the Aperio ScanScope CS2 system (Aperio Technologies, Vista, CA, USA), highlighting in particular the tubulointerstitial region of the renal section.

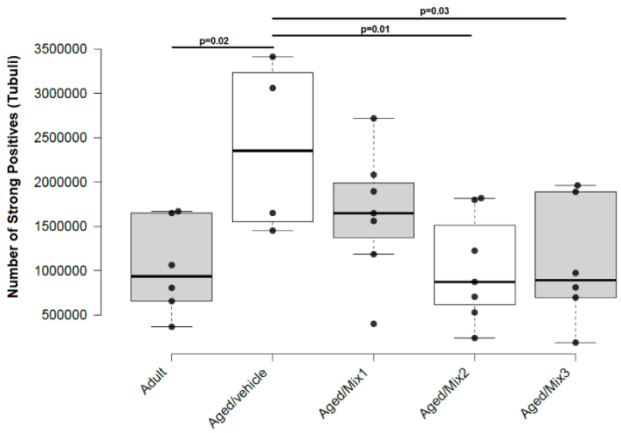

Fig. 37: Quantitative analysis of renal fibrosis. Staining was quantified using the 3 R V L W L Y H 3 L [H O algorithm combined with computer-assisted image analysis. Data are expressed as median (IQR). Values represent normalized strong positive pixel counts in the tubulointerstitial region (4-7 samples per group).

P O V

4.2.2.2. Expression profile of genes involved in inflammation and fibrosis

To gain insight into the molecular mechanism by which BCAAs counteract the fibrotic process, gene expression was analysed in aged kidneys. As shown in Figure 38, the expression value of the TGF-β1 gene (a key pro-fibrotic biomarker) was significantly higher in AGED-VEH mice than in ADULT mice ($p < 0.0229$). A clear trend towards reduced gene expression was observed in the treated aged kidneys, particularly with BCAAs + 2ALA (recovery score ≥ 90% vs. adult), although this was not significant. The kidneys of AGED-VEH mice showed significant overexpression of both TNF-α and NF-κB compared with adult mice ($p=0.0082$; $p=0.0322$). TNF-α levels were reduced by all three treatments. Notably, the expression of the TNF-α gene was significantly reduced in aged kidneys treated with BCAAs + 2ALA ($F=3,597$; $p=0.0433$). Similarly, BCAAs formulations effectively counteracted the increase in NF-κB gene expression, particularly the 2ALA-enriched formulation (R.S. vs. adult: 98%), which showed values that overlapped with those observed in adult animals. In accordance with previous studies (Anders, 2016) and the pro-inflammatory environment of the aged kidney, IL-1β expression levels were higher than in the adult kidney ($p=0.0003$). Interestingly, IL-1β was significantly downregulated by treatment in the following order: 2ALA > BCAAs + Di-ALA ≥ BCAAs ($p = 0.0051 > p > 0.0115$) Chronic inflammation and the resulting fibrosis can induce cellular senescence. Accordingly, we observed a significant increase in the expression of the CDKN1A gene, which encodes the p21 protein. This protein is a cyclin-dependent kinase inhibitor that causes cell cycle arrest and is a canonical marker of cellular senescence. It contributes to the persistence of the inflammatory and fibrotic state (Yang et al., 2024). Figure 38 shows significantly higher CDKN1A gene expression in AGED-VEH mice compared with ADULT controls ($p < 0.0012$). Interestingly, the formulations 2ALA and Di-ALA reduced gene levels efficaciously ($0.0035 < p < 0.0060$), whereas BCAAs alone had no effect.

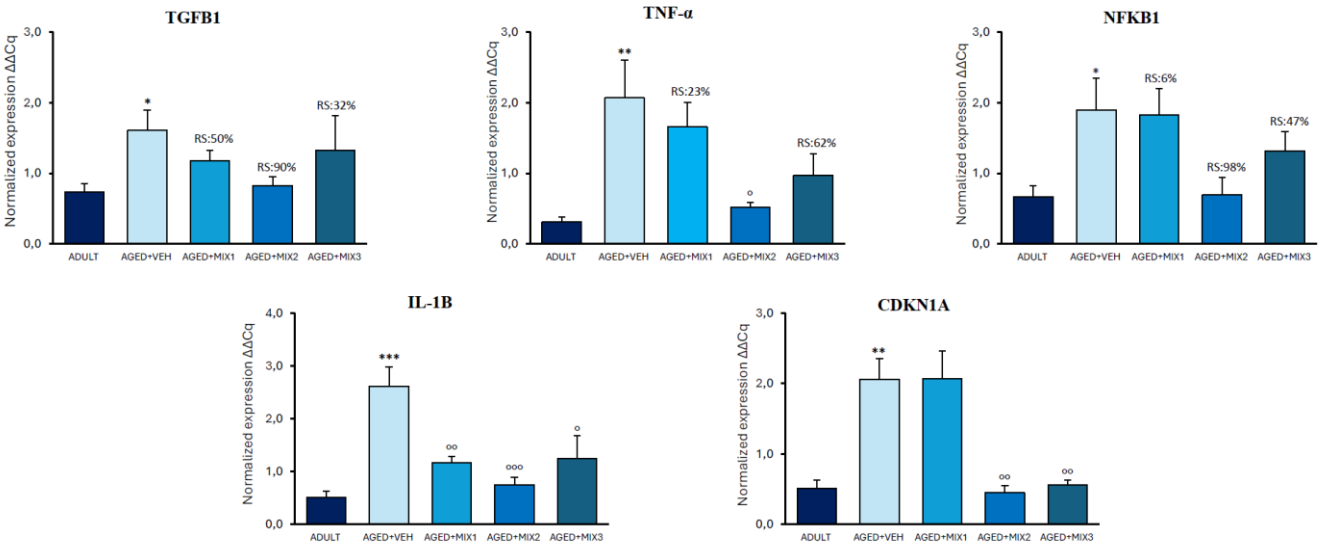

Fig. 38: Transcriptional levels, measured by qRT-PCR in kidneys of genes related to inflammation, fibrosis and metabolism. Values are expressed as mean ± SEM from 3 to 5 samples per group, normalized to the mean of housekeeping genes PPIA, ACTB. A statistically significant difference was found by unpaired Student's t-test for AGED + vehicle vs. ADULT (*p<0,0322). A statistically significant difference was found among AGED mice groups by one-way ANOVA for TNF-α (F=3,597; p=0,0433), IL-1β (F= 8,843; p= 0,0013) and CDKN1A (F=10,12; p=0,0008). Dunnett's post hoc test used to compare all AGED groups °vs. AGED + vehicle is as follows: TNF-α (p=0,0276), IL-1β (0,0006<p<0,0115) and CDKN1A (0,0035<p<0,0060). Recovery scores (RS) toward WT values are indicated above the bars.

4.2.2.3. Modulation of AMPK and mTOR protein expression by BCAAs and L-ALA supplementation

Furthermore, given the pivotal role of the AMP-activated protein kinase (AMPK) and mechanistic Target of Rapamycin (mTOR) signalling pathways in regulating energy metabolism and cellular stress responses (Zhou et al., 2020) we quantified by western blot the levels of both total and phosphorylated forms of AMPK and mTOR.

In aged kidneys, total AMPK protein levels (normalised to vinculin) in AGED-VEH mice were significantly lower than in ADULT mice. However, the p-AMPK/AMPK ratio was, paradoxically, elevated, in line with the increase in LKB1 expression. These unexpected increases may be indicative of stress-induced kinase hyperactivation and an attempt by the body to compensate, despite protein loss. All tested formulations partially counteracted this deficit, restoring AMPK expression and at the same time reducing p-AMPK levels towards normal. In contrast, total mTOR was found to be upregulated in aged mice, accompanied by a reduced p-mTOR/mTOR ratio, suggesting altered complex assembly or activity. BCAAs and BCAAs+2ALA significantly lowered total mTOR and modestly restored its phosphorylation, whereas BCAAs+Di-ALA induced only a slight decrease in total mTOR with negligible effect on phosphorylation.

Overall, these findings suggest that BCAA/L-alanine formulations may have the potential to counteract the alterations of the AMPK–mTOR axis associated with aging. The treatment restores energy-sensing homeostasis in aged kidneys by replenishing AMPK protein levels, attenuating its hyperactivation, and restraining mTOR overexpression.

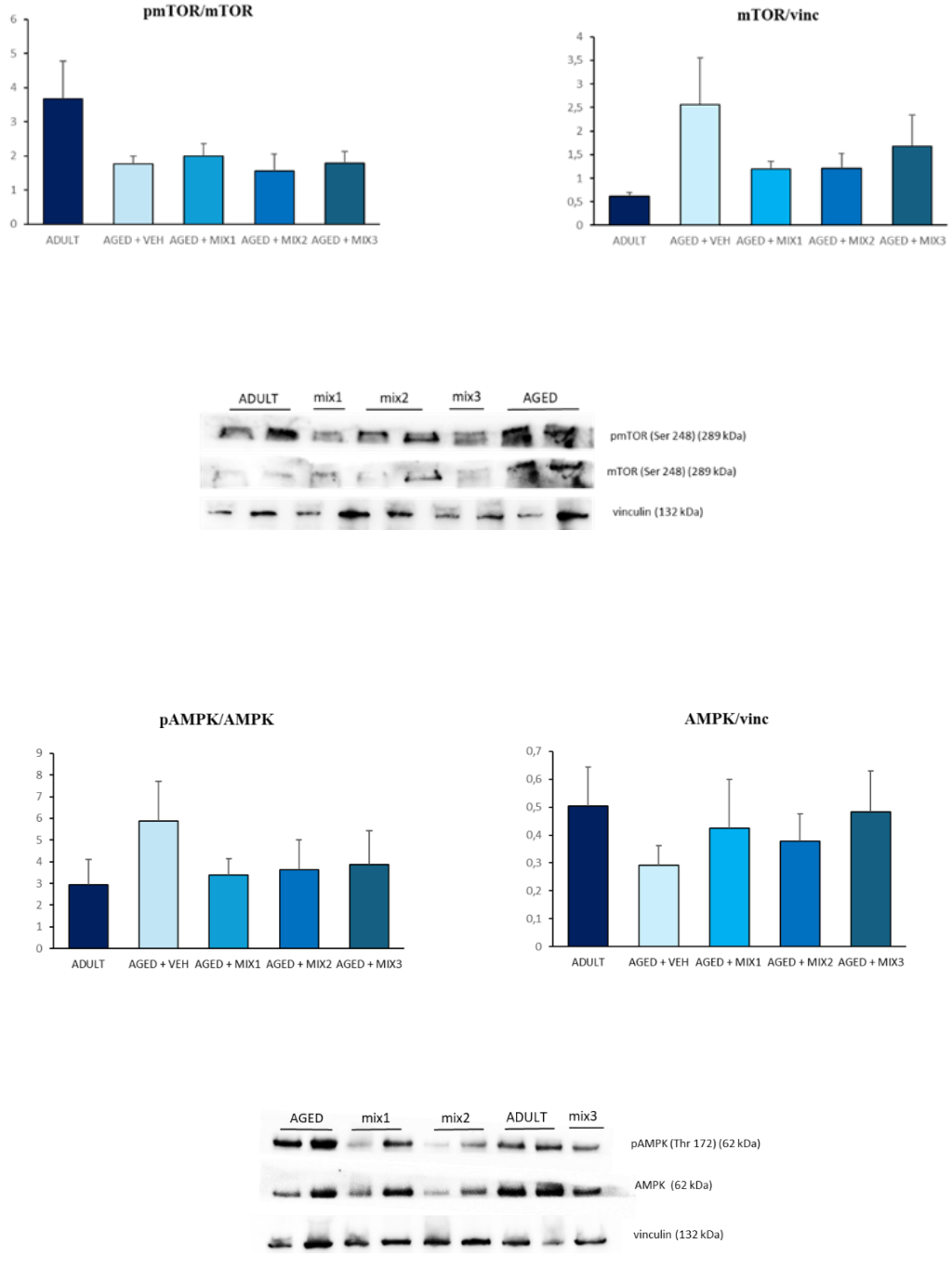

Fig.39: Western Blot analysis of pAMPK/AMPK, pmTOR/mTOR ratio and total AMPK and mTOR in GC muscles of different experimental groups. Blots were loaded by an operator unaware of the experimental groupings of samples. Values are expressed as mean ± SEM from the number of mice (n) indicated in brackets. No significant difference was found by student t-test between AGED mice + vehicle vs. ADULT mice, and one-way ANOVA followed by Dunnet post-hoc test between all AGED groups.

4.3. Results Aim 3: Evaluation of the effects of the growth hormone secretagogue JMV2894 in the D2- P Gmouse model

The present project, to which I contributed during my PhD, aimed to evaluate the potential effects of JMV2894 in the skeletal muscle (Aim 3a) of the D2- P Gmouse model. The results of this study were recently published in 0 D Q W X D Q R H W % L R P H G L F L Q H 3 K D U P D F R O R J \ (DOI: 10.1016/j.biopha.2025.118767). Subsequently, considering that JMV2894 modulates intracellular pathways shared by muscle and kidney, we expanded the study to evaluate the potential effects of this molecule also at the renal level (Aim 3b).

4.3.1 Results Aim 3a

4.3.1.1. Effects of JMV2894 on in vivo readouts in D2- P Gmice

In D2- P Gmice, ultrasound assessment of the hind limbs confirmed that D2- P Ga exhibit an atrophic phenotype, characterised by a significant reduction in limb volume compared to D2-WT controls ($p = 0.007$). Notably, administration of JMV2894 at the lower dose exhibited a protective effect against hind limb volume loss in D2- P Gmice, achieving an R.S. of 118% relative to WT values. However, this difference did not reach statistical significance ($p=0.11$) (Fig. 51A). In parallel, the echodensity of GC muscle was significantly higher in D2- P Gmice versus control mice ($p = 0.004$); on this index, a trend towards reduction was observed with JMV2894 at either dose, with R.S. up to 34 % ($p = 0.09$) (Fig. 51B).

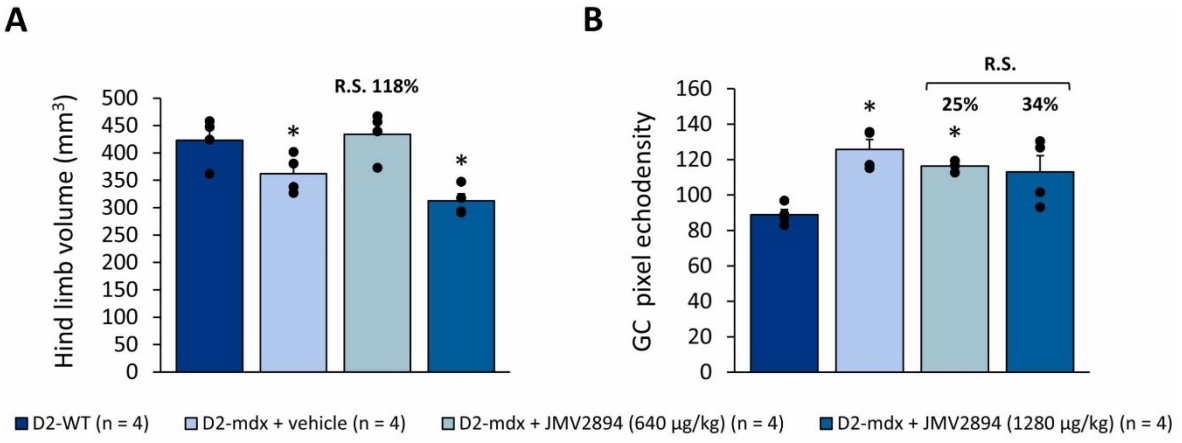

Fig. 51: Hind limb volume (mm³; A), gastrocnemius (GC) muscle echodensity (B). All measured by ultrasound in all mice cohorts at T6. Values are expressed as mean ± SEM from the number (n) of mice indicated in brackets. A statistically significant difference among groups was found by RQH ZDANOVA for A (F = 9.15, S= 0.002), B (F = 7.7, S= 0.004), and C (F = 17.05, S< 0.0001). Bonferroni post hoc test for individual differences between groups is as follows: * vs. D2-WT (0.0003 < S< 0.03), # vs. D2- PGC[S= 0.01). Recovery scores (R.S.) towards WT values are indicated above the bars.

4.3.1.2. Histological analyses

Histological analyses were conducted at the conclusion of the treatment to evaluate the fibrosis and myofibre CSA of GC muscles from mice in the experimental group. Visible increases in fibrotic areas were observed in the D2- PGmuscle groups (Fig. 52A). This was confirmed by quantitative analysis, which showed significantly higher levels of collagen deposition in all dystrophic cohorts ($p < 0.001$). Treatment with JMV2894 did not improve the fibrosis of D2- PGmuscles, although there was a mild reduction in collagen deposits in GC muscles from mice treated with the lower dose (R.S. 19%, $p > 0.99$) (Fig. 52B). However, JMV2894 partly counteracted myofiber atrophy ($p = 0.02$) (Fig. 52C), as shown by the increased myofiber CSA in mice treated with the lower (R.S. 33%, $p > 0.99$) or higher (R.S. 37%, $p > 0.99$) dose (Fig. 52D).

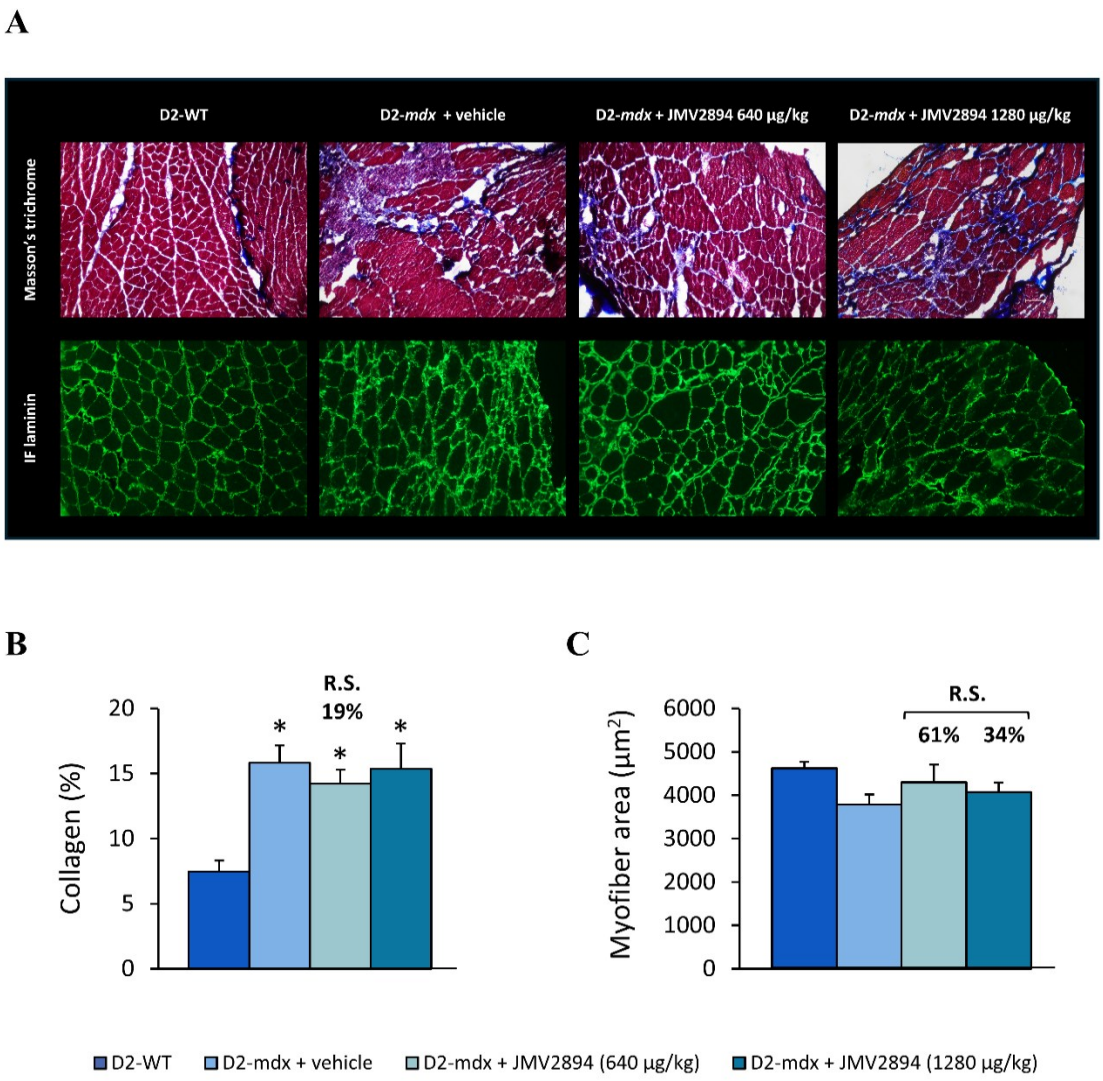

Fig. 52: Histological stains. Representative sections of the gastrocnemius muscle (GC) are shown in (A), stained with Masson's trichrome stain (magnification 10×; upper panel), and in immunofluorescence (IF) for laminin (magnification 20×; lower panel), for each experimental group (D2-WT, D2-mdx + vehicle, D2-mdx + JMV2894 at doses of 640 and 1280 µg/kg). Masson's trichrome staining allows for the highlighting of collagen deposits (in blue), calculated as a percentage (%) of total muscle area, as shown in (B). Values are expressed as mean ± SEM of n = 6–8 mice per group. A statistically significant difference between groups was identified using one-way ANOVA ($F = 9.78$, $p = 0.0002$); Bonferroni's post hoc test showed individual differences between groups as follows: * vs. D2-WT ($0.0003 < p < 0.001$). Immunofluorescence for laminin allows estimating the cross-sectional area (CSA) of myofibers, expressed in µm², as shown in (C). Values are expressed as mean ± SEM of n = 4–6 mice per group. No statistically significant differences were found using one-way ANOVA and Bonferroni post hoc tests. The recovery score (RS) values towards the D2-WT are indicated above the bars.

4.3.1.3. Gene expression

Subsequently, we evaluated the expression levels of genes involved in muscle remodeling and fibrosis (TGF-β1, COL1A1, MMP9, and ADAMTS5). As expected, all these markers were significantly upregulated in vehicle-treated D2-mdx mice compared to D2-WT controls. In contrast with the findings obtained from Masson's trichrome–stained histological sections, JMV2894 treatment induced a marked reduction in TGF-β1 expression in treated animals at both doses (with recovery scores of 57% and 63%, respectively). Similarly, a partial decrease in COL1A1 levels was observed (with an R.S. of 65% at the higher dose), along with a reduction in the expression of matrix metalloproteinases MMP9 and ADAMTS5. Regarding the effects on muscle atrophy, treatment, notably at the lower dose, resulted in a reduction in the expression of the genes ATROGIN and MuRF1, which were markedly upregulated in dystrophic mice. In JMV2894-treated D2-mdx mice, ATROGIN and MuRF1 levels were reduced compared to untreated counterparts (R.S. 143% and 54%, respectively). These findings are consistent with the increase in myofiber CSA observed by immunofluorescence analysis. Furthermore, the impact of treatment on IGF-1 and its receptor expression was assessed to explore the potential mechanism of action of the compound. JMV2894 at the lower dose significantly increased IGF-1 expression, while no changes were detected in the mRNA levels of its receptor, IGF-1R.

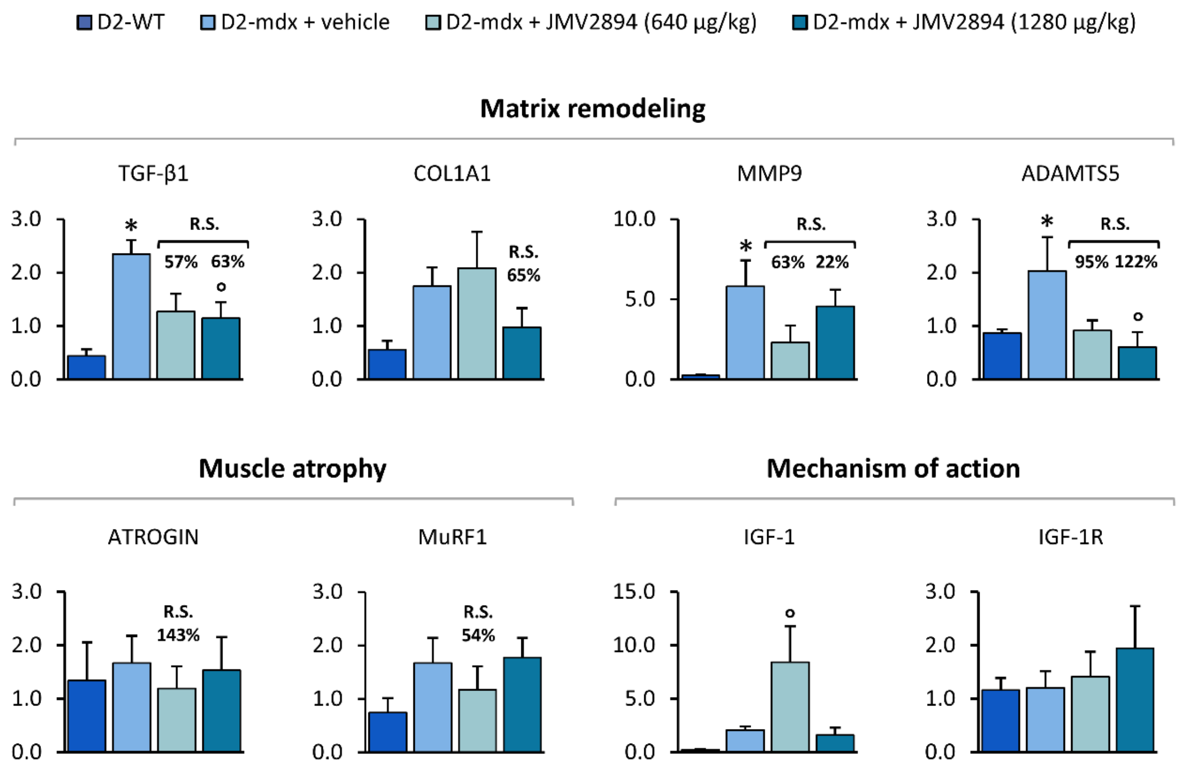

Fig. 53: Transcriptional levels of genes involved in muscle remodeling and fibrosis (7*) &2/$ 003 $'$076), muscle atrophy ($752*,1 0X5)) and drug mechanism of action (,*) ,*)5) measured by real-time PCR in gastrocnemius (GC) muscle for all mice cohorts (D2-WT, D2-PG[vehicle, and D2-PG[treated with dose 1 – 640 µg/kg, or dose 2 – 1280 µg/kg of JMV2894). Data are expressed as mean ± SEM from n = 4 – 8 values mice SHU group, normalized to the mean of housekeeping genes (DFWLQ6,-$). Statistically significant differences among groups were found by RQHZD ANOVA for the following genes: 7*) (F = 7.7, S = 0.003), 003 (F = 5.8, S = 0.005), $'$076 (F = 4.9, S = 0.01), ,*) (F = 6.3, S = 0.003). Bonferroni post hoc test for individual differences between groups is as follows: * vs. D2-WT (0.0004 < S < 0.05); # vs. D2-PG[(S < 0.01). Recovery scores (R.S.) towards WT values are indicated above the bars.

4.3.1.4. Evaluation of protein expression related to the mechanism of action

To further characterize the potential mechanisms underlying the pharmacological actions of JMV2894 in our experimental model, the expression of proteins involved in the Akt/GSK-3β signaling axis (upstream regulated by IGF-1) was assessed via western blot analysis in treated D2- P Gmice vs. untreated ones (Fig. 54). In detail, the levels of GSK-3β were expressed as ratio of its phosphorylated (inactive) form to the total protein (p-GSK-3β/GSK-3β); similarly, the levels of the phosphorylated (active) form of Akt were quantified relative to total Akt (p-Akt/Akt), while the levels of mature IGF-1R were expressed relative to the immature form (pro-IGF-1R). The treatment with the lower dose of JMV2894 induced a trend toward increased levels of both p-GSK-3β (+25 %, S= 0.31; Fig. 54A) and p-Akt (+43 %, S= 0.46;Fig.54B). Additionally, both doses promoted a 65 % increase in the expression of the mature form of IGF-1R (Fig. 54.C).

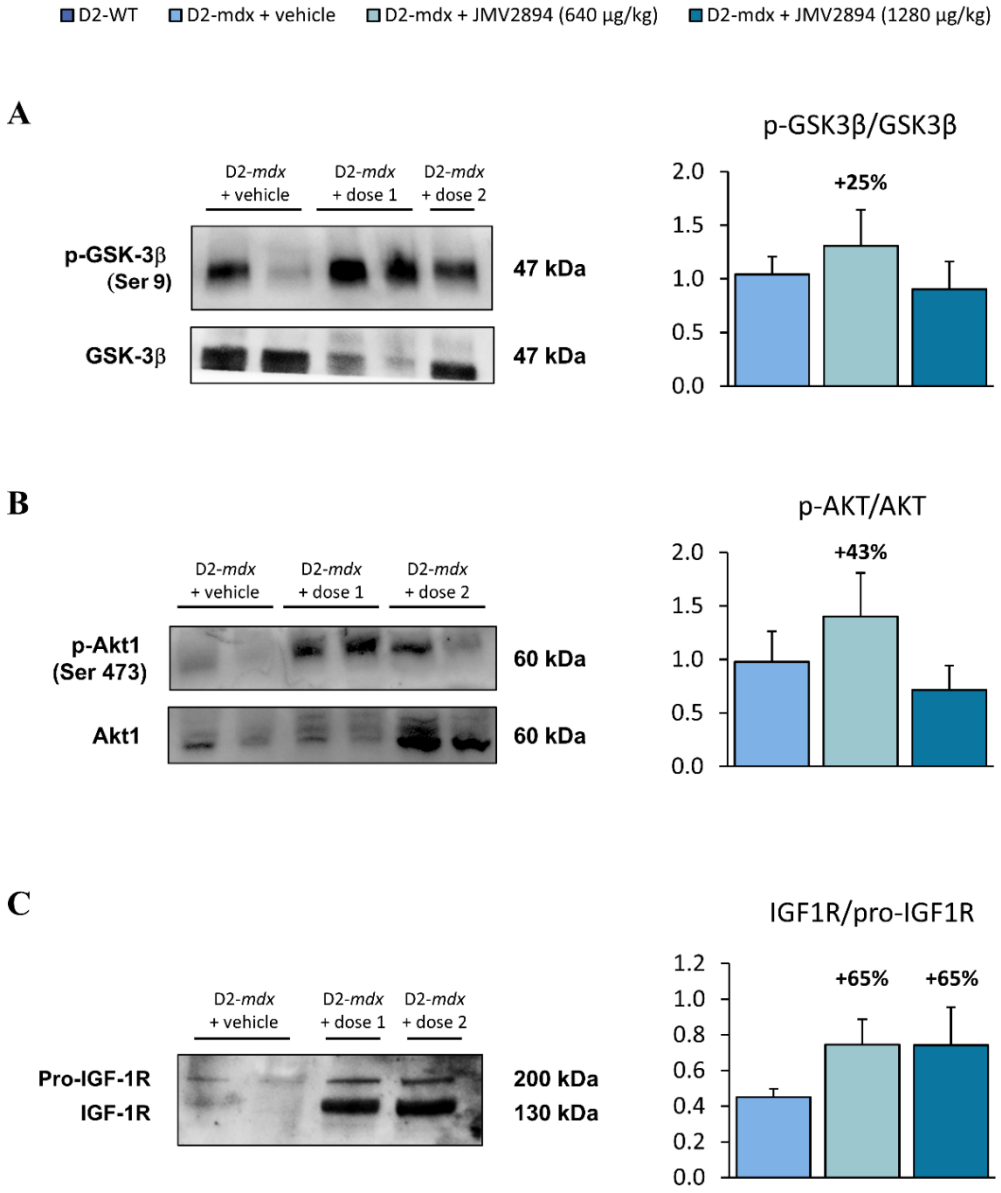

Fig 54: Western blot (WB) results showing protein expression levels of GSK-3β (**A**), Akt (**B**), and IGF-1R (**C**) in gastrocnemius (GC) muscle from D2- P Gmice treated with vehicle or JMV2894 at the dose of 640 or 1280 µg/kg. For each panel (A, B, C), representative WB images (O)are shown alongside the corresponding quantitative analysis (U L) J expressed as mean ± SEM from n = 6 – 8 mice S Hgroup. In detail, phosphorylated (inactive) GSK-3β levels were normalized to total GSK-3β and expressed as the p-GSK-3β/GSK-3β ratio, phosphorylated (active) Akt levels were normalized to total Akt and expressed as the p-Akt/Akt ratio, and mature IGF-1R (α and β subunits) levels were normalized to the immature receptor form (pro-IGF-1R) and expressed as the IGF-1R/pro-IGF-1R ratio. 2 Q DANOVA followed by Bonferroni post hoc test revealed no statistically significant differences among groups. Percentage (%) increases in protein expression levels in treated groups vs. vehicle are indicated above the bars.

4.3.1.5. IGF-1 levels in plasma

No significant variations among mice groups were found in relation to IGF-1 plasma levels assessed via ELISA, although a decreasing trend ($p = 0.88$) was observed in untreated D2-mdx mice vs. WT, with a tendency ($p = 0.58$) to increase in response to JMV2894 treatment at the lower dose.

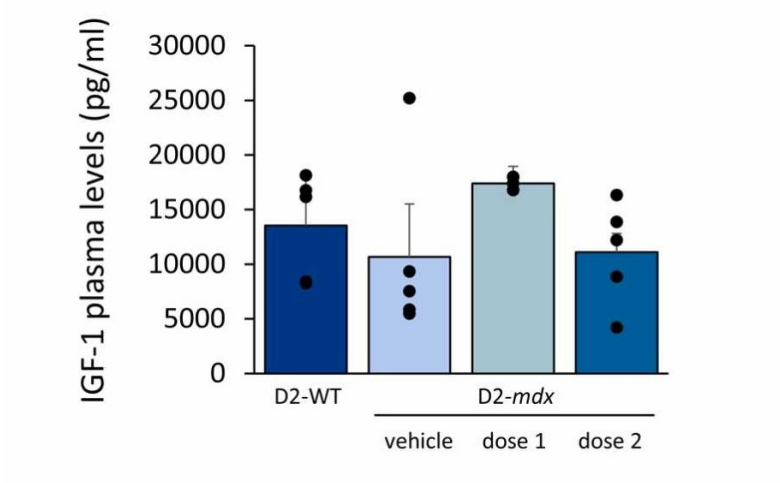

Fig. 55: Levels of IGF-1 measured in plasma samples collected from all mice cohorts. Values are expressed as mean ± SEM from 5 mice S Hgtup. No statistically significant differences were found by R QZHDANOVA followed by Bonferroni post hoc test.

4.3.2 Results Aim 3b

4.3.2.1. Renal weight modifications

In D2- P G [mice, kidney weight, normalized to body weight, was significantly increased compared to D2-WT controls (8.04 ± 0.15 mg/g vs 7.01 ± 0.18 mg/g; $p < 0.05$), suggesting a compensatory adaptation to the chronic systemic stress associated with the dystrophic phenotype, consistent with findings reported in other models of chronic injury (McFarlin et al., 2020). In JMV2894-treated groups, kidney weight showed a further increase, more pronounced at the higher dose (1280 µg/kg) compared to the lower dose (640 µg/kg) (Figure 56). While this increase may not directly reflect the impact of the peptide, it could be indicative of a compensatory renal adaptation potentially influenced by the treatment.

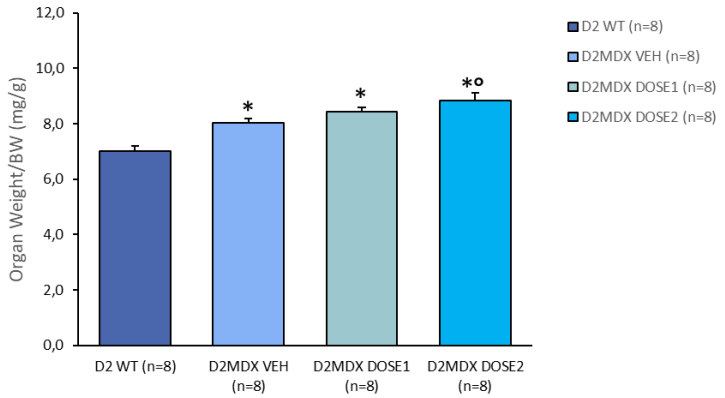

Fig. 56: Renal weight to body weight ratio in D2-WT and D2- P G [mice. Data are expressed as mean ± SEM by n = 8 mice per group. A significant difference between groups was detected by one-way ANOVA ($F = 17.6$, $p < 0.0001$). Bonferroni post hoc analysis revealed: $p < 0.03$ vs. D2-WT (*) and $p = 0.03$ vs. D2- P G([).

4.3.2.2. Histological analyses

Sirius Red/Fast Green staining was used to assess the distribution and intensity of collagen deposition within the renal parenchyma. In D2- P G[mice, a significant increase in interstitial collagen deposition was observed compared to D2-WT controls ($p < 0.0137$), characterised by irregular distribution and variable intensity, consistent with an early fibrotic process (Figure 57). In JMV2894-treated groups, staining appeared less intense and more homogeneous compared to untreated D2- P G[mice. Treatment resulted in a R.S. of 63% at the dose of 640 µg/kg and 85% at 1280 µg/kg, indicating an improvement in collagen deposition. While the response indicates a dose-dependent trend in fibrosis reduction, the overall morphological improvement was more pronounced at the lower dose (640 µg/kg) (Figure 58). Overall, the histological findings indicate that JMV2894 contributes to the preservation of renal tissue architecture, attenuating fibrotic alterations.

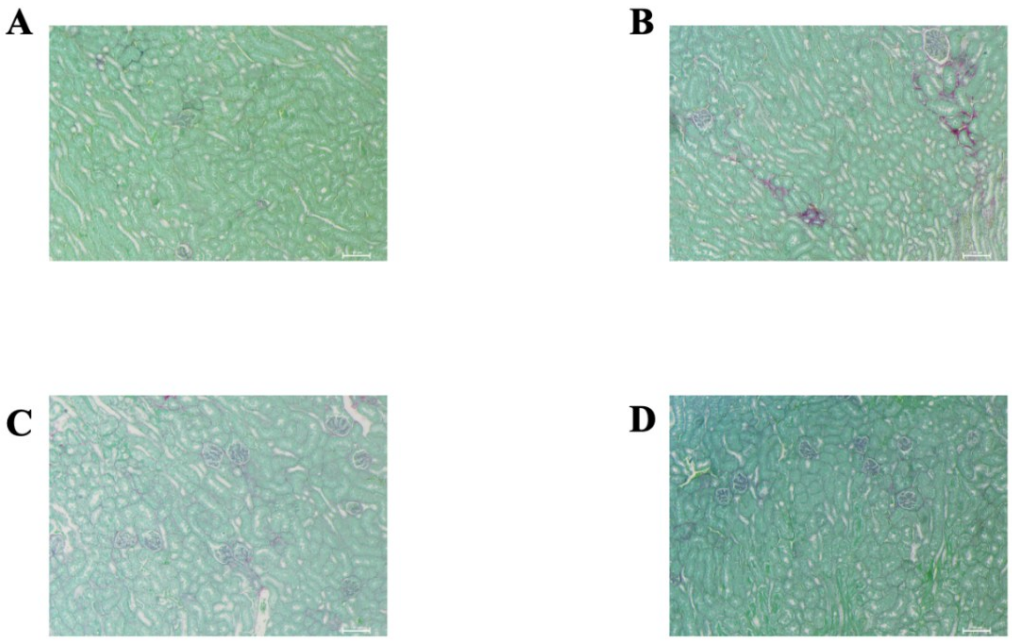

Fig. 57: Representative renal sections stained with Sirius Red/Fast Green obtained from D2-WT (A), D2- P G([B), and D2- P G[mice treated with JMV2894 at 640 µg/kg (C) and 1280 µg/kg (D). Scale bar = 100 µm. Collagen fibers, abundantly present in fibrotic areas, appear in red. Compared to D2-WT controls, D2-mdx mice exhibited a marked increase in interstitial collagen

deposition, characterized by irregular distribution and variable staining intensity, indicative of early fibrotic remodeling. In JMV2894-treated groups, collagen staining appeared less intense and more homogeneous, with a fibrosis reduction recovery score (R.S.) of 63% at 640 µg/kg and 85% at 1280 µg/kg. The images depict a representative portion of the renal cortex at the glomerular level.

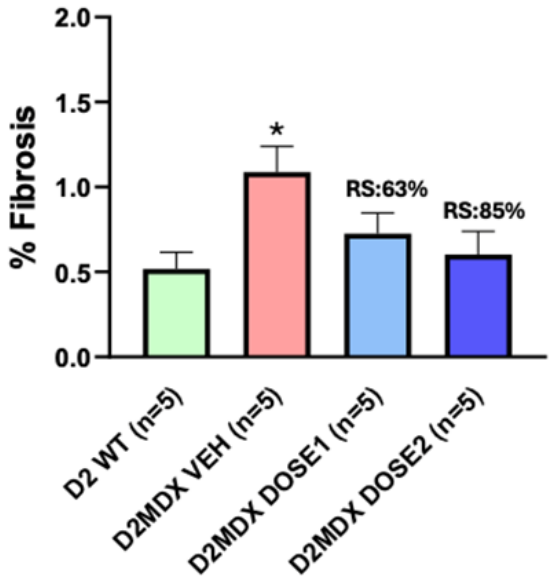

Fig. 58: Fibrotic Area Quantification. The percentage of fibrotic area was quantified in renal sections from D2-WT, D2- P G E and D2- P G mice treated with JMV2894 at 640 µg/kg and 1280 µg/kg. Data are presented as mean ± SEM from n = 5 mice per group.A significant increase in collagen deposition was detected in D2- P G mice compared to D2-WT controls ($p < 0.05$, one-way ANOVA followed by Bonferroni post hoc test). Treatment with JMV2894 reduced fibrosis by 63% at 640 µg/kg and by 85% at 1280 µg/kg, indicating a dose-dependent improvement in renal morphology.

Discussion and Conclusions

The demographic shift towards an ageing population is a pivotal challenge for healthcare systems in developed countries. The combination of increasing life expectancy and declining birth rates has resulted in a significant rise in the proportion of older individuals within the population over recent decades (Khan et al., 2024). In Italy, this phenomenon is particularly evident and has positioned the country among those with the highest average age in Europe. While increased longevity is undoubtedly an important achievement from both a healthcare and social perspective, it is also associated with a higher prevalence of chronic diseases and conditions of functional decline. These conditions have a significant impact on the quality of life of the elderly population and the sustainability of healthcare systems (Gianfredi et al., 2025b). One of the most significant biological changes associated with the ageing process is physical decline, which results in a gradual decrease in muscle mass and function. In this context, sarcopenia is now recognised as one of the most representative conditions associated with age-related physical decline (Cruz-Jentoft et al., 2019a). It is responsible for several adverse clinical outcomes, including an increased risk of falls, disability, hospitalization, and mortality. Despite the growing attention devoted to sarcopenia, its diagnosis remains challenging (Jang et al., 2025). Current diagnostic criteria are mainly based on the assessment of muscle strength, muscle mass, and physical performance, parameters that require specialised equipment and trained personnel. Consequently, there has been a marked increase in recent years in the interest of identifying circulating biomarkers that can reflect the biological processes underlying muscle decline and support the diagnostic tools currently available.

In this context, the national research programme Age-It (Ageing Well in an Ageing Society) was established as part of the Italian National Recovery and Resilience Plan (PNRR). The project's aims are to strengthen Italian research on the ageing process and to develop innovative strategies to promote healthy and sustainable ageing. The programme adopts a holistic and interdisciplinary approach, encompassing multiple research domains including the biology of ageing, clinical medicine, as well as socio-economic and technological aspects.

Within this framework, Spoke 8, in which the present PhD work is focused, is dedicated to the development and evaluation of multidimensional interventions aimed at preventing functional and cognitive decline in the elderly population. The activities of this Spoke are centred on clinical studies, biomarker analyses and the development of preventive strategies based on combined interventions. These interventions include physical activity, nutrition and technological support (Okoye et al., 2025).

As part of my PhD research, I conducted an analysis of circulating biomarkers potentially associated with sarcopenia within the IN-TeMPO clinical study (WP1, Spoke 8). In parallel with the objectives of the Age-It programme, I investigated the potential of nutritional and pharmacological therapeutic strategies to counteract the mechanisms underlying muscle atrophy. This investigation used murine models of physiological ageing and hereditary muscle degeneration at the preclinical level.

The first part of the thesis focused on analysing a panel of circulating biomarkers associated with sarcopenia in a cohort of community-dwelling older adults undergoing a multidimensional intervention. The biomarkers investigated included molecules involved in inflammatory processes, metabolic regulation, and muscle regeneration mechanisms associated with sarcopenia, such as IL-6, GDF-15, BDNF, IGF-1, irisin, and ghrelin. These factors have been described in the literature as potential indicators of the biological processes underlying muscle decline. In particular, IL-6 is considered one of the main mediators of the chronic low-grade inflammatory state typically associated with ageing, while GDF-15 has recently been proposed as a marker of mitochondrial stress and metabolic dysfunction. BDNF plays an important role in neuromuscular communication and neuronal plasticity, whereas IGF-1 represents one of the major anabolic regulators of muscle metabolism. Furthermore, irisin and ghrelin have been shown to play a regulatory role in energy metabolism and muscle adaptive responses to metabolic stress (Sala et al., 2025). During my PhD, I participated actively in all phases of the clinical study. I was also involved in a WP1 Spoke 8 task force, which conducted a systematic meta-analysis of the literature during the project's

preliminary phase. This analysis aimed to identify the most promising biomarkers to be included in the panel analysed within the IN-TeMPO study. The baseline data did not reveal any significant differences between the sexes, regardless of the biomarker considered. Therefore, sex was not a relevant confounding factor in the analysed population, and the observed variability was mainly attributable to inter-individual heterogeneity. The six-month longitudinal analysis, despite a reduction in sample size due to drop outs, showed an overall trend consistent with the physiological pattern of muscle decline already observed at baseline (T0) (Chang et al., 2017b; Chew et al., 2019; Serra-Prat et al., 2007; Webster et al., 2006). Specifically, BDNF, IGF-1, and irisin showed a trend of decrease, while GDF-15 and ghrelin increased, and IL-6 levels remained largely stable. In the overall cohort, the multidimensional intervention did not induce significant changes in the circulating biomarker profile compared with the control group. An interesting finding emerged from the sex-stratified analysis: male participants undergoing the multidimensional intervention showed an increase in BDNF levels at T6 compared with T0, whereas a decrease was observed in the control group. This observation warrants further investigation to clarify the underlying biological mechanisms. Given the potential influence of multiple factors on circulating biomarker levels, including inter-individual variability, comorbidities, dietary habits, and polypharmacy, larger sample sizes, longer follow-up periods, and the integration with functional parameters and individual clinical characteristics will be necessary to more accurately define the true diagnostic and prognostic value of these biomarkers. Therefore, the present findings should be interpreted as preliminary, also considering that the cohort is still being expanded.

At the preclinical level, I evaluated the effects of BCAAs-based formulations in a murine model of physiological ageing. BCAAs have been shown to play a key role in the regulation of muscle protein synthesis, mainly through the activation of the mTOR signaling pathway, and several studies have suggested their potential protective role in counteracting the catabolic processes associated with sarcopenia (D'Antona et al., 2010; Flati et al., 2010; Maki et al., 2012b). In line with this evidence, the results

obtained suggest that BCAAs supplementation, administered either alone or in combination with L-alanine (2ALA) or with the dipeptide Di-ALA, may modulate several molecular pathways involved in the regulation of energy metabolism and in inflammatory and tissue remodeling processes. In the ageing murine model used, the results confirmed that sarcopenic muscle is characterised by a marked alteration of Ca^{2+} homeostasis (Aim 2a). Specifically, aged muscle exhibited elevated resting intracellular Ca^{2+} levels and diminished SOCE activity, consistent with prior findings suggesting that modifications in calcium handling represent a critical pathway underlying the functional decline in skeletal muscle during the ageing process (Fraysse et al., 2006b; Michelucci et al., 2020; Thornton et al., 2011a; Zhao et al., 2008a). This condition was found to be linked with reduced expression of key sarcoplasmic reticulum proteins that play a role in Ca^{2+} regulation, particularly RyR1 and SERCA1. Reduced RyR1 expression may contribute to less efficient Ca^{2+} release from the sarcoplasmic reticulum and, consequently, to impaired activation of SOCE, whereas decreased SERCA1 levels are consistent with reduced efficiency of Ca^{2+} reuptake processes, promoting the cytosolic Ca^{2+} accumulation typically observed in aged muscle (Fodor et al., 2020; O'Connell et al., 2008). Overall, these alterations suggest that impaired Ca^{2+} homeostasis may significantly contribute to the reduced contractile capacity observed in sarcopenic muscle (Batsis et al., 2014). In this context, BCAAs-based formulations showed a significant functional effect, improving muscle contractility and counteracting the age-related shift in the Hz50 parameter. This can be interpreted as an indirect index of improved Ca^{2+} handling. Among the formulations that were tested, BCAAs + 2ALA was the most effective in restoring SOCE activity and modulating the expression of proteins involved in calcium regulation, including MG29 and MG53. MG29 has been shown to play an important role in the organisation of muscle triads and in the regulation of Ca^{2+} dynamics in skeletal muscle, and its expression has been reported to be reduced in aged muscle (O'Connell et al., 2008; Zhao et al., 2008a). Conversely, the increased expression of MG53 observed in aged muscle has been interpreted as a compensatory mechanism aimed at protecting the

muscle membrane from oxidative stress (Hord et al., 2016; Ryan et al., 2008; Wang et al., 2021). In this context, the reduction of MG53 levels observed after treatment may reflect a decreased need to activate these adaptive mechanisms, suggesting an improvement in the functional balance of skeletal muscle.

Considering the close interconnection between skeletal muscle and other organs in the context of ageing, the study was extended to evaluate the effects of the treatment also at the renal level, highlighting possible protective effects in this compartment.

At the renal level (Aim 2b), kidneys from aged mice displayed structural alterations typically associated with senescence, including thickening of the glomerular basement membrane, extracellular matrix accumulation, and features compatible with nephrosclerosis, thus confirming the validity of the experimental model (Dybiec et al., 2022). In this context, BCAAs formulations administered in combination with 2ALA or Di-ALA showed a protective effect against tubulo-interstitial fibrosis, with the greatest efficacy observed for the BCAAs + 2ALA formulation. This effect was associated with reduced expression of key mediators of fibrosis and inflammation, including TGF-β1, TNF-α, and NF-κB, suggesting modulation of molecular pathways involved in age-related renal fibrosis and chronic inflammation. With regard to the metabolic regulators AMPK and mTOR, treatment resulted in changes to total protein expression levels without causing significant alterations to their activation state. This finding suggests that the beneficial effects observed may not be directly attributable to functional modulation of this signalling axis, but rather to the activation of alternative or compensatory pathways.

Overall, these results indicate that BCAAs-based formulations may exert protective effects not only at the muscular level but also at the renal level, likely through the modulation of key processes such as Ca^{2+} homeostasis and inflammatory and fibrotic mechanisms. Among the formulations that were tested, BCAAs + 2ALA was the most effective in restoring muscle functional balance and counteracting structural and molecular alterations associated with renal ageing. These findings suggest that targeted

nutritional strategies may contribute to the coordinated modulation of processes of functional decline affecting multiple organs during the ageing process.

Finally, the last part of this PhD work explored the therapeutic potential of the growth hormone secretagogue JMV2894 in the D2- P Gmurine model, which is currently used a model for the study DMD. However, given its background, we are using it as a model of hereditary atrophy. JMV2894 has garnered interest due to previous evidence demonstrating its ability to modulate several pathological processes associated with muscle wasting, including mitochondrial dysfunction, pro-atrophic signaling, and extracellular matrix remodeling (Boccanegra et al., 2023b; E. Conte et al., 2017b). In previous studies conducted in the Pharmacology laboratories of the University of Bari using the BL10- P Gmodel, this molecule demonstrated a significant antifibrotic effect, likely mediated through the modulation of metalloproteases involved in tissue remodelling, such as ADAMTS-5 and MMP-9 (Boccanegra et al., 2023b). In light of these findings, the present study aimed to verify whether these effects could also be confirmed in the presence of a more severe genetic and pathological background. Overall, the results obtained indicate that treatment with JMV2894 is able to modulate several pathways involved in muscle degeneration and tissue remodelling processes. Specifically, a decrease in the expression of genes linked to fibrosis and extracellular matrix remodelling, including TGF-β1, MMP-9, and ADAMTS-5, was noted. This suggests a possible modulation of the molecular mechanisms driving fibrotic progression in dystrophic muscle. At the same time, the treatment also had an impact on processes linked to muscle atrophy. Specifically, there was a reduction in the expression of the main pro-atrophic markers Atrogin-1 and MuRF-1, suggesting a possible involvement of the ubiquitin–proteasome system in the response to treatment. These effects were accompanied by an increase in muscle fibre size and an increase in muscle volume, as assessed by ultrasound analysis. This indicates a potential anti-atrophic effect of the molecule. From a mechanistic perspective, the data obtained suggests that the pharmacological action of JMV2894 may be mediated, at least in part, through activation of the GH/IGF-1 axis. Treatment induced an increase in IGF-1

expression and enhanced activation of the Akt/GSK-3β pathway, a signalling axis known to play a key role in the regulation of muscle growth, protection against atrophy, and muscle regeneration processes (Marcella et al., 2024). In this context, the functional inhibition of GSK-3β, associated with Akt activation, may contribute to the observed anti-atrophic effects, in agreement with recent studies showing that modulation of this pathway can improve the histopathological and functional profile in the D2- P Grmodel (Marcella et al., 2024). Despite the morphological and molecular improvements observed, the functional effects of the treatment were relatively modest, likely due to the severity of the experimental model and the marked fibrotic component characterising D2- P Grmuscle. It is also possible that greater tissue exposure to the drug or longer treatment durations may be required to achieve more pronounced functional effects.

Also in this second preclinical study, the evaluation of the antifibrotic effects of the treatment was expanded to include the renal compartment, highlighting potential protective effects at this level.

In D2- P Grmice, an increase in normalized kidney weight was observed, suggesting a possible adaptive response to systemic stress or increased metabolic demand (McArdle et al., 2020; Oliveira et al., 2016). This increase was more pronounced in the JMV2894-treated groups and may be associated with modulation of the GH/IGF-1 axis. These findings are consistent with literature evidence indicating that elevated GH levels may be associated with renal and glomerular hypertrophy and progressive alterations in renal function. In addition, transgenic mouse models overexpressing IGF-1 have been observed to display increased body size, accompanied by proportionally enlarged kidneys and glomerular hypertrophy in the absence of glomerulosclerosis (Gurevich et al., 2021). Histological analysis using Sirius Red/Fast Green staining revealed a significant increase in collagen deposition in the kidneys of D2- P Grmice compared with controls, confirming that the pro-fibrotic background characteristic of this model is not limited to skeletal muscle but may extend to other organs. In line with the

observations made at the muscular level, JMV2894 treatment showed a tendency to reduce fibrotic processes in the renal compartment. However, further molecular biology studies will be necessary to more thoroughly characterise the intracellular mechanisms underlying the antifibrotic activity observed at the renal level. Overall, these results suggest that JMV2894 may exert effects not only on skeletal muscle but also potentially on other organs involved in disease progression, opening new perspectives for systemic and multi-organ therapeutic strategies.

In conclusion, this PhD thesis allowed the investigation of muscle decline from multiple perspectives. The findings suggest that muscle decline should not be considered a phenomenon confined exclusively to skeletal muscle, but rather a more complex condition involving multiple organs and biological systems, reflecting the continuous cross-talk between muscle and other tissues. While clinical data highlighted the need for longitudinal studies in larger cohorts and for the integration of biological parameters to obtain more robust results less influenced by inter-individual variability, preclinical investigations provided relevant insights into the molecular mechanisms underlying muscle dysfunction, fibrotic processes and alterations in calcium homeostasis. In particular, BCAAs supplementation, especially when combined with 2ALA, showed promising effects in modulating pathways involved in muscle contractility, inflammation and tissue remodelling, with antifibrotic actions also observed at the renal level. Furthermore, in the D2- P Grodel, the growth hormone secretagogue JMV2894 was found to influence key processes involved in muscle degeneration, including pro-atrophic signalling, extracellular matrix remodelling and fibrosis. The activation of the GH/IGF-1 - Akt/GSK-3β axis and the reduction of fibrosis-related markers support the potential of this compound as a candidate for multi-target therapeutic strategies. The effects observed in the renal compartment further reinforce the concept of systemic involvement in muscle decline and highlight the relevance of multi-organ pharmacological approaches. Collectively, these findings emphasise the importance of the translational approach supported by the Age-It programme, which integrates clinical evidence and preclinical research. This approach

is crucial for understanding the mechanisms behind muscle decline and for developing innovative therapeutic and diagnostic strategies that promote healthy ageing.

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