The Trajectories of Neuromuscular Aging (TRAJECTORAGE Clinical Trial): Study Rationale and Methodological Protocol

Title

The trajectories of neuromuscular aging (TRAJECTOR-AGE clinical trial): study rationale and methodological protocol

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Key Points

- Neuromuscular aging is a multifaceted process that involves structural, metabolic, and neural factors.
- TRAJECTOR-AGE takes a prospective, longitudinal approach to identify biomarkers of age-related decline.
- Both middle-aged and older adults are assessed regularly over two years, with integrative measurements at multiple levels (clinical, molecular, physiological).

Why Does This Paper Matter?

Understanding the distinct trajectories of neuromuscular aging can help clinicians identify at-risk individuals early and design preventive strategies. Our findings may ultimately guide interventions to preserve functional independence and quality of life in older populations.

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ABSTRACT

Objectives
To examine the structural, metabolic, and functional trajectories of neuromuscular decline in aging, and to identify key mechanisms and early biomarkers that may inform interventions aimed at preserving function and independence.

Design
The TRAJECTOR-AGE project is a prospective, longitudinal cohort study conducted over two years at multiple centers in Italy.

Setting and Participants
Community-dwelling, physically and cognitively healthy middle-aged (50–60 years) and older (>70 years) adults are recruited. Individuals with significant comorbidities (e.g., diabetes, neurological disorders, severe heart failure) are excluded.

Measurements
Every six months, participants undergo comprehensive geriatric and anthropometric evaluations, including bioimpedance analysis, cardiopulmonary exercise testing, echocardiography, vascular assessments, neuromuscular examinations, and ultrasound imaging of the quadriceps. Yearly, multiparametric magnetic resonance imaging (MRI) is performed to quantify muscle volume and fat infiltration. Biological samples (blood draws) are collected periodically to assess inflammatory and neuromuscular biomarkers. Muscle biopsies of the Vastus Lateralis are performed annually in a subset of participants to evaluate markers of mitochondrial function, muscle fiber composition, and neuromuscular junction stability.

Expected Results
By integrating clinical, functional, and molecular data over time, the TRAJECTOR-AGE study aims to clarify the pathophysiological mechanisms underlying neuromuscular decline, capture inter-individual variability, and explore the influence of habitual physical activity on aging trajectories.

Conclusions
A multidimensional understanding of neuromuscular aging may lead to earlier identification of at-risk individuals and inform preventive or therapeutic interventions to sustain functional independence in later life.

Keywords: Motor function, muscle aging, neuromuscular decline, physical frailty, cognitive frailty

Introduction

Between the years of 2000 and 2019, global life expectancy increased from 66.8 to 73.4 years, implying that people live approximately 7 years longer (and consequently age more) (1). Unfortunately, this trend has also been accompanied by a decline in quality of life among older individuals, even in the absence of overt diseases (1). Among the potential causes, the age-related decline in neuromuscular control, loss of muscle mass, and metabolic alterations are of primary importance (2). For example, impaired muscle function slows gait speed and adversely affects mobility, leading to increased risk of falls, loss of independence, and higher morbidity (3,4). Concomitantly, alterations in skeletal muscle oxidative capacity play a central role in reducing energy production and impairing physical performance, ultimately increasing mortality risk (5,6).

Cross-sectional data suggest that neuromuscular impairments in older adults follow different time-courses. Muscle mass begins to decline in midlife at ~1% per year, leading to a loss of ~50% by the 8th–9th decade, while muscle strength/power declines more rapidly (2–4% per year) (7,8). Similarly, cardiorespiratory fitness, strictly related to cardiovascular and mitochondrial function, shows a mean yearly change of −0.2 to −0.4 mL/kg/min, resulting in ~40% reduction by age 85 compared to age 25 (9). Recent longitudinal findings, however, indicate these physiological changes do not always occur uniformly, and may accelerate or vary by decade (10). Moreover, reductions in muscle strength/power—along with cycles of denervation and reinnervation—tend to become more pronounced after age 50 (11), and older adults of the same chronological age can differ widely in neuromuscular decline (12). Cardiorespiratory fitness and muscle oxidative function also exhibit non-uniform declines, reportedly accelerating from 3–6% per decade in younger adults to beyond 20% in the very old (13).

A growing body of literature indicates that chronological age alone does not fully explain this heterogeneity in structural, functional, and metabolic changes (14). Potential contributing factors include lifelong physical activity (15). Morphological and neural alterations are closely linked, yet it remains unclear to what extent neural phenomena drive muscle structural changes or vice versa in older adults, particularly with varying levels of habitual physical activity (16). Likewise, skeletal muscle oxidative function has been reported to be either impaired or preserved in older adults (17,18), and these contrasting results could arise from secondary factors such as physical activity and lifestyle (19). Thus, further longitudinal studies are needed to clarify how neuromuscular and metabolic systems evolve over time across different activity levels.

In summary, most current knowledge on aging derives from cross-sectional research, potentially confounded by large inter-subject variability. Longitudinal insights are crucial to better characterize the nonlinear deterioration and to anticipate future health trajectories. Accordingly, the TRAJECTOR-AGE study aims to (i) describe the temporal evolution of neuromuscular, structural, and metabolic parameters with aging, (ii) identify the mechanistic bases of aging-related functional losses by elucidating neural-structural-metabolic interactions, and (iii) examine how habitual physical activity might modulate these trajectories. By integrating in vivo measurements (clinical, physiological) with ex vivo assays (muscle bundles, single-fiber analyses), the project seeks to delineate the timeline of neuromuscular decline and to guide targeted interventions to preserve or improve healthy life expectancy and quality of life.

Methods and Analysis

Study Design

The TRAJECTOR-AGE Study is a prospective, longitudinal cohort investigation funded by the PRIN2020477RW5 grant (Principal Investigators: Porcelli, Franchi, Botter, Re,

Lauretani). Six research institutions participate: University of Pavia (UNIPV), University of Padua (UNIPD), Politecnico di Torino (POLITO), Politecnico di Milano (POLIMI), National Research Council (CNR), and University of Parma (UNIPR). Data collection commenced in September 2022, and baseline visits were completed in August 2023. Follow-up visits will continue until August 2025, with plans to extend if additional funding becomes available. Figure 1 outlines the study design, structured in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) statement (20). After the initial screening (Figure 1A), participants meeting inclusion and exclusion criteria were assigned to one of two age groups (Figure 1B): MIDDLE-AGED (55–60 years) or OLD (75–80 years). To account for differences in physical activity, participants were also classified as ACTIVE or SEDENTARY based on current public health guidelines and accelerometry data (21). Following baseline evaluations at T0, each participant undergoes follow-up every 6 months (T6, T12, T18, T24) (Figure 1C). Evaluations include comprehensive clinical assessments, blood sampling, neuromuscular tests, muscle morphology imaging, and exercise tolerance measurements (Table 1). To avoid confounding effects, these are conducted on non-consecutive days. Once per year (T0, T12, T24), eligible and willing participants undergo a Vastus Lateralis muscle biopsy and Magnetic Resonance Imaging (MRI) (Figure 1C).

Participants and Recruitment Procedures

MIDDLE-AGED and OLD participants of both sexes were recruited among individuals visiting the Geriatric Clinic Unit of the Medical Geriatric Rehabilitative Department (UNIPR). Community-dwelling, cognitively intact, and ambulatory adults were considered eligible if they had a Mini Mental State Examination (MMSE) ≥24, a 4-m gait speed >0.8 m/s, Short Physical Performance Battery (SPPB) >9, Timed-Up and Go <20 seconds, and handgrip strength >27 kg (men) or >16 kg (women). Screening for malnutrition and dysphagia was performed as previously described (22). Exclusion criteria included:

Parkinson's disease or other major neurological conditions (e.g., stroke), diabetes, advanced cancer, severe chronic kidney or liver insufficiency, major cardiac disease or recent cardiac surgery, and severe osteoarthritis.

Recruitment Results

A total of 190 volunteers were screened, of whom 125 met eligibility criteria. Among these, 102 participants completed baseline measures (T0), and were allocated to either MIDDLE-AGED (n=57; age 57.9±3.1 years, weight 72.9±15.2 kg, height 168±9 cm) or OLD (n=45; age 76.9±4.4 years, weight 74.5±13.0 kg, height 166±11 cm). Both women (38 in MIDDLE, 15 in OLD) and men (19 in MIDDLE, 22 in OLD) are included. Table 1 presents the baseline anthropometric and clinical characteristics. Among enrolled participants, 32 (31%) underwent muscle biopsy and provided ≥100 mg of tissue, sufficient for full planned analyses and biobanking.

Project Schedule and Completed Assessments

Table 2 summarizes the timetable for each assessment and sample collection at baseline and follow-ups, indicating the measures already completed.

Clinical Assessments

At baseline and annually, participants undergo blood pressure measurement, electrocardiogram (ECG), and echocardiography. A Comprehensive Geriatric Assessment is conducted via validated questionnaires assessing cognition, mood, activities of daily living, and nutritional status (23–25). Physical activity is quantified by the IPAQ survey and 7-day accelerometry. Physical performance is evaluated using SPPB components (timed 4-m walk, chair stand, balance tests) plus handgrip strength with a Jamar dynamometer (26). Total-body and appendicular skeletal muscle mass are estimated by bioimpedance (27).

Dual Energy X-ray Absorptiometry (DXA)

Fan-beam DXA (Hologic or GE-Lunar systems) is used to determine total-body and regional

lean mass, fat mass, and appendicular lean mass (28). Standardized positioning and daily calibration are performed according to manufacturer guidelines.

Blood Collection

Five milliliters of fasting venous blood are drawn and processed to obtain serum and plasma, then stored at −80°C. Subsequent analyses will measure inflammation-related markers (e.g., IL-1, IL-6), indices of neuromuscular damage (CK, troponin T), and markers of neuromuscular junction instability (C-terminal agrin fragment).

Neuromuscular Evaluation

Age-related neuromuscular decline involves muscle fiber type shifts, denervation/reinnervation, and altered motor unit firing patterns. To characterize these, high-density EMG (HD-EMG; MEACS system, LISiN, Politecnico di Torino) is recorded from the Vastus Lateralis using a 64-electrode array. Simultaneous ultrasound imaging tracks muscle architecture changes.

Isometric Test

Maximal voluntary isometric knee extension is measured on a custom apparatus. Participants perform ramped contractions at ~30% and ~50% of maximum force. HD-EMG decomposition yields recruitment thresholds, mean firing rates, and spatial features of individual motor units. Concurrent ultrasound captures fascicle length and pennation angle in the Vastus Lateralis.

Fatigability Test

A dynamic fatiguing protocol consists of 80 maximal knee extensions at 20% of isometric peak load. Progressive decreases in power output across repetitions are related to HD-EMG changes in root mean square amplitude, frequency content, and activation patterns.

Muscle Morphology and Architecture

Ultrasound assessments of quadriceps volume, muscle thickness, fascicle length, pennation

angle, and echo intensity are performed to detect architectural modifications associated with aging. During isometric ramps, real-time ultrasound videos are also acquired to evaluate changes in fascicle geometry under load.

Magnetic Resonance Imaging (MRI)

MRI provides detailed, noninvasive evaluation of muscle volume, cross-sectional area, and intramuscular fat infiltration. A multiparametric MRI protocol (T1-weighted turbo spin-echo, Dixon method) quantifies muscle tissue composition. Elevated intramuscular fat correlates with diminished muscle function and increased fracture risk.

Functional Evaluation of Muscle Oxidative Metabolism

Progressive declines in VO_2peak and related oxidative indices are central features of aging. Cardiopulmonary exercise testing (CPET; COSMED Quark CPET) measures breath-by-breath gas exchange. Bioimpedance cardiography (PhysioFlow) tracks cardiac output. Near-infrared spectroscopy (NIRS) and diffuse correlation spectroscopy (DCS) quantify local oxygenation and blood flow in the Vastus Lateralis.

Cardiopulmonary Exercise Testing

Participants complete a ramp-incremental cycling test to exhaustion. Test endpoints include respiratory exchange ratio >1.05 and perceived exertion ≥17 (Borg scale). Peak oxygen consumption ($\dot{V}O_2$peak), cardiac output ($\dot{Q}$peak), and peripheral O_2 extraction [(a–v)O_2] are estimated.

Submaximal Constant Work Rate Test

O_2 kinetics are assessed at intensities below the gas exchange threshold. Slower on-kinetics in older adults indicates greater reliance on anaerobic metabolism. Concurrent NIRS evaluates muscle oxygen delivery/utilization in the Vastus Lateralis.

In-vivo Muscle Oxidative Capacity and Oxygen Diffusion

After moderate cycling, repeated transient arterial occlusions estimate maximal oxidative

capacity and O_2 diffusion constraints. Tissue saturation is monitored at different oxygenation levels, enabling calculation of diffusion-limited kinetics during recovery.

Peripheral Vascular and Endothelial Function

Endothelial dysfunction is a hallmark of vascular aging. To measure endothelial-dependent vasodilation, we combine passive leg movement (PLM) and a vascular occlusion test. Ultrasound quantifies changes in femoral artery flow during 60 seconds of passive knee flexion–extension. NIRS-based reactive hyperemia (5-minute occlusion) gauges microvascular reactivity.

Muscle Biopsy

At T0, T12, and T24, percutaneous Vastus Lateralis biopsies (~150–200 mg) are obtained from a subset of participants. Samples are partitioned for molecular assays (Western blot, qPCR), high-resolution respirometry (Oroboros O2k), and histology. Figure 2 illustrates the percutaneous biopsy procedure, showing the incision site and the use of a Weil-Blakesley rongeur to obtain the muscle specimen under local anesthesia.Data Management and Analysis

The Principal Investigators and the TRAJECTOR-AGE research team authored the study manual and oversee data handling, storage, and analysis. Each site stores anonymized participant data on password-protected drives. Only authorized team members can access the data, under the responsibility of the PIs.

Ethical Considerations

The study is registered at ClinicalTrials.gov (NCT06168591) and was approved by the AVEN Ethical Committee (Emilia Romagna region, Italy) on July 5, 2022 (protocol #28022; study ID 283/2022/SPER/UNIPR). Monitoring or audits by the committee may occur at any time. Any significant protocol amendments will be submitted for review and approval prior to implementation. All participants provide written informed consent, receiving a copy for their records.

Patient Safety

Participant safety is paramount. All assessments are conducted under medical supervision. CPET and muscle biopsies are performed by experienced clinicians. Any adverse events are documented; tests are halted if a risk arises. At baseline, seven adverse events and one serious adverse event were recorded. Most minor events were biopsy-related (pain or minor local issues). The serious event involved severe hypotension during CPET requiring overnight hospitalization.

The TRAJECTOR-AGE project investigates the integrative mechanisms driving the nonlinear progression of muscle aging, ranging from midlife to advanced age, and explores how to preserve skeletal muscle function in older populations. Innovative features include the longitudinal design, comparison across distinct age cohorts, and a multidimensional, translational approach. By merging diverse data streams—clinical, physiological, and molecular—this initiative aims to expand knowledge in geriatric research and to inform potential preventative or therapeutic strategies.

TRAJECTOR-AGE is poised to yield novel insights into aging processes, including factors linked to longevity, disability, and disease. The ongoing collection of clinical, biochemical, and physiological measures, combined with advanced technologies (e.g., HD-EMG, MRI, muscle biopsies), establishes a robust framework for large-scale longitudinal studies of aging in Italy.

Neuromuscular function shows minimal decline in younger adults but accelerates in midlife and older age (29). Inter-individual variability becomes more prominent in those ≥80 years old (12). The longitudinal monitoring employed here is therefore essential for capturing the uneven decay in neuromuscular parameters, recognizing early deficits, and potentially predicting adverse health trajectories.

Aging is characterized by progressive loss of muscle mass and strength, contributing to

mobility limitations and physical frailty (30,31). Neurophysiological changes—including diminished motor drive, motoneuron loss, and neuromuscular junction (NMJ) alterations—interact with muscle fiber atrophy and shifts in oxidative metabolism (32). Whether structural muscle deficits precede or follow neural alterations, and how these changes relate to mitochondrial function, remains unresolved. By simultaneously measuring parameters across multiple levels, TRAJECTOR-AGE seeks to uncover the primary drivers of functional decline.

Age-related deficits in neuromuscular function are shaped by several lifestyle factors. Physical activity throughout adulthood appears to delay functional disability and potentially extend healthy lifespan (15,33,34). Elite masters athletes in their 80s show higher motor unit numbers and less collateral reinnervation compared to sedentary age-matched controls (35). Exercise programs maintain NMJ stability and mitigate motor unit losses (36), while VO_2max and mitochondrial capacity are higher in active older individuals (37). However, longitudinal data on how physical activity alters the rate and mechanisms of neuromuscular decline remain limited. TRAJECTOR-AGE will address this gap by examining muscle denervation, NMJ degeneration, and mitochondrial biogenesis in ACTIVE vs SEDENTARY older adults.

Worldwide, the population is aging rapidly, with over 2 billion individuals >60 years estimated by 2050. Consequently, the prevalence of physical disability is escalating, already impacting ~2 million older adults in Italy alone, of whom ~700,000 experience severe loss of independence. This poses growing socioeconomic demands. Identifying early predictors (i.e., “red flags”) and effective interventions is thus a public health priority. By mapping longitudinal changes in neuromuscular function, TRAJECTOR-AGE aims to facilitate timely strategies to avert frailty and disability. Novel biomarkers and intervention approaches may ultimately yield broad social and economic benefits.

Study findings will be communicated to stakeholders via press releases, conference

presentations, and publications in peer-reviewed open-access journals. A dedicated project webpage will share objectives, activities, and outcomes. Authorship on TRAJECTOR-AGE publications will follow International Committee of Medical Journal Editors (ICMJE) guidelines (37). All resulting manuscripts will comply with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement or related guidelines (38). While a key strength is the longitudinal design (in contrast to previous cross-sectional studies), the multi-year follow-up demands considerable commitment from both researchers and participants, potentially clashing with other obligations. In older individuals, health conditions may change quickly due to unexpected events (e.g., new comorbidities, injuries, hospitalizations), complicating follow-up. To mitigate attrition, assessments are scheduled well in advance, with reminder calls and contingency planning (e.g., maintaining a "waiting list" participant available on short notice). Partnerships with additional recruitment centers also help reduce dropout risk and foster enrollment of both active and non-active populations.

Conclusions

By combining clinical, physiological, and molecular evaluations over two years, the TRAJECTOR-AGE study is expected to provide a more nuanced understanding of how neuromuscular aging progresses and how it may be modified by habitual physical activity. This knowledge may lead to earlier identification of those at elevated risk for functional decline, ultimately enabling targeted interventions to preserve independence and quality of life in later years.

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Author Contributions

- **Conceptualization/Methodology/Supervision**: FL, SP, MVF, AB, RR, MM
- **Data Acquisition/Investigation**: AM, MA, CA, NM, CB, CM, IZ, MS, AM, ReR, MC
- **Analysis/Interpretation**: CT, AC, NS, and all co-authors
- **Writing – Original Draft**: FL, SP, MVF, and co-authors
- **Writing – Review & Editing**: All authors contributed and approved the final manuscript

Conflict of Interest Statement

The authors declare no conflicts of interest.

Sponsor's Role

Funding bodies played no role in study design, data collection, analysis, interpretation, or manuscript preparation.

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Tables

Table 1.

Baseline anthropometric and clinical characteristics of the participants (MIDDLE-AGED vs. OLD).

	OVERALL	**MIDDLE-AGE**	**OLD**
Age (years)	66± 4	58 ± 3	77 ± 4
Sex			
Men	45	19	26
Women	57	38	19
HR (b/min)	70 ± 9	71 ± 9	69 ± 10
SAP (mmHg)	132 ± 13	129 ± 14	136 ± 13
DAP (mmHg)	75 ± 8	77 ± 8	74 ± 8
NRS	0.67 ± 1.54	0.92 ± 1.68	0.51 ± 1.42
Height (cm)	166.2 ± 9.8	167.6 ± 8.8	165.1 ± 10.6
Weigth (Kg)	71.8 ± 15.1	71.3 ± 15.1	72.4 ± 13.4
BMI (kg/m2)	25.77 ± 3.92	25.15 ± 4.04	26.41 ± 3.48
CC Dx (cm)	36.73 ± 3.25	36.75 ± 2.85	36.71 ± 3.59
CC Sin (cm)	36.59 ± 3.18	36.51 ± 2.84	36.71 ± 3.57
TC Dx (cm)	46.76 ± 4.27	48.45 ± 4.50	45.57 ± 4.03
TC Sin (cm)	47.03 ± 4.14	48.38 ± 4.67	45.64 ± 4.04
WC (cm)	89.97 ± 15.25	88.63 ± 12.74	92.31 ± 17.29
HC (cm)	103.70 ± 8.16	103.27 ± 8.98	104.56 ± 6.59
Hand Grip Dx (kg)	31.9 ± 12.7	32.7 ± 12.7	31.0 ± 12.8
Hand Grip Sin (kg)	29.8 ± 13.5	31.0 ± 13.1	29.00 ± 13.8
SPPB	11.55 ± 0.59	11.84 ± 0.37	11.35 ± 0.86
No ADL disability (%)	96.88	99.74	95.55
IADL	7.81 ± 0.63	7.95 ± 0.22	7.77 ± 0.88
MMSEc	28.18 ± 1.46	28.99 ± 1.33	27.86 ± 1.53
CDT	2.69 ± 0.62	2.77 ± 0.50	2.61 ± 0.65
GDS	2.15 ± 2.41	2.02 ± 2.19	2.21 ± 2.56
MNA-SF	13.04 ± 1.27	12.88 ± 1.35	13.37 ± 1.23

Table 2.
Schedule of baseline and follow-up assessments for TRAJECTOR-AGE.

ASSESSMENTS		T_0	T_0	T_6	T_{12}	T_{18}	T_{24}
Clinical assessment							
Initial screening and recruitment		X					
Monitoring of clinical conditions and functional performance			X				
Blood sample collection and analysis			X				
Neuromuscular evaluation							
Isometric contractions HD-EMG and ultrasound			X	X	X	X	X
Fatigability test			X	X	X	X	X
Muscle morphology and architecture							
Muscle ultrasound assessment			X	X	X	X	X
Muscle fascicle behavior tracking during isometric tasks			X	X	X	X	X
Multiparametric MRI			X		X		X
DXA			X		X		X
Functional evaluation of muscle oxidative metabolism							
Central and peripheral determinants of exercise tolerance			X	X	X	X	X
Muscle oxidative capacity by TD-NIRS			X	X	X	X	X
Peripheral blood flow and endothelial function			X	X	X	X	X
Ex-vivo measurements	Muscle biopsies collection		X		X		X
	Single fibres work and NMJ stability		X		X		X
	Mitochondrial function and respiration		X		X		X

Figure Legends

Figure 1.

Overview of the TRAJECTOR-AGE study design, including participant enrollment (A), age-group classification (B), follow-up timeline (C), and evaluations (D).

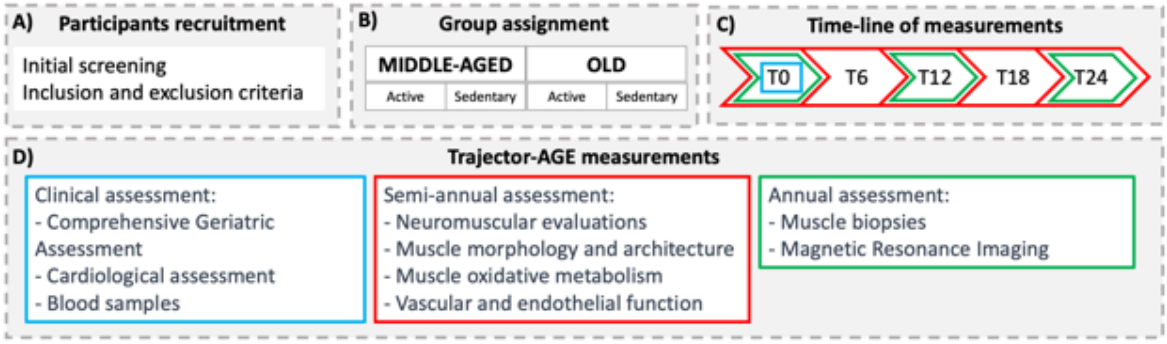
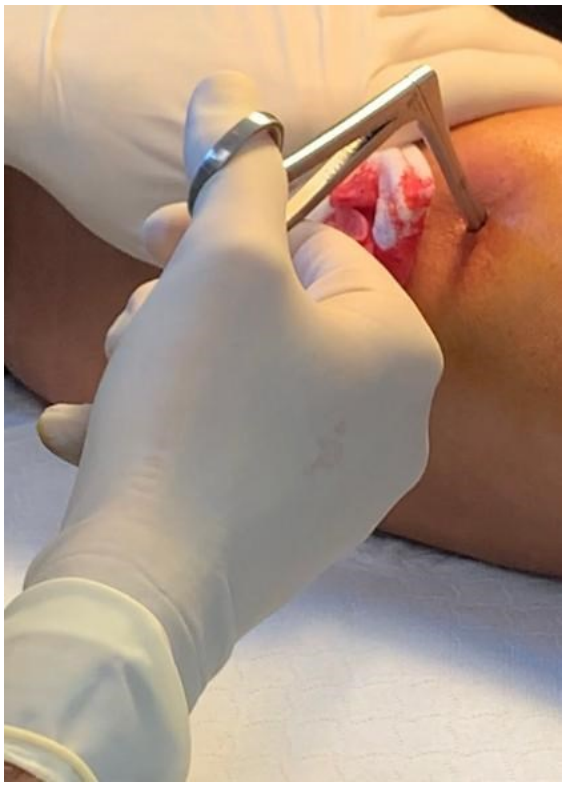


Figure 2. Representative image of the percutaneous Vastus Lateralis biopsy procedure.

A photograph demonstrating the sterile field setup, local anesthesia, and incision site for the Weil-Blakesley rongeur insertion. Approximately 150–200 mg of muscle tissue is collected under local anesthesia and immediately processed for downstream analyses.



Supplementary Materials (Appendix S1)

Extended Protocol Details

Clinical assessments

At baseline and annual follow-up visits, participants undergo to blood pressure monitoring, resting electrocardiogram recording and echocardiography screening. Then, a complete Comprehensive Geriatric Assessment and questionnaires are administered for the assessment of cognitive, social and neuropsychological status. Basic Activity of Daily Living (ADL) and Instrumental Activity of Daily Living (IADL) scales are administered to verify the autonomy of the subjects. Levels of physical activity are assessed by IPAQ questionnaire and accelerometric data recorded over a 7-day window (GT9X, Actigraph, FL, USA). Physical performance is evaluated by the Summary Performance Physical battery, and the Short Physical Performance Battery (SPPB), which includes a timed 4 m walk, chair stands, balance test, and short narrow walk. Grip strength is measured with a Jamar hand-held dynamometer. Total body Skeletal Muscle Mass (SMM) and Appendicular Skeletal Muscle Mass (ASM) are estimated by bioimpedance analysis (BIA) (1).

Dual energy X ray Absorptiometry (DXA)

Total body fat mass and total bone-free lean mass (in kg) will be acquired from total body scans using fan-beamed DXA using standardized protocols (2). Appendicular lean mass will be derived from the sum of lean mass in both upper and lower extremities.

Blood collection

All participants underwent a blood sampling of 5 mL from the median cubital vein, and serum and plasma were obtained. Blood samples were collected after a 12-hour fast, with the participant sitting or lying supine for at least 15 minutes. The samples were stored at -80° Celsius and will be used to evaluate circulating biomolecules linked to muscle re-modelling (type III procollagen peptide), systemic inflammation and neuronal apoptosis (IL1, IL6, eHsp72, etc.), sarcomere dysfunction and muscle wasting (creatin phosphokinase, troponin T, etc.) and neuromuscular junction instability (for example, C-terminal agrin fragment).

Neuromuscular evaluation

The age-related decrease in muscle strength and power is underpinned by changes in muscles structure and neural control (3). Type 2 fast muscle fibers are preferentially denervated and are reinnervated by slow-twitch motor neurons, entailing in lower motor unit discharge rates, and increased variability of motor unit discharges in older adults (4). For this purpose, HD-EMG (MEACS, LISiN, Politecnico di Torino, and ReC Bioengineering Laboratories, Turin, Italy (5) signals from the Vastus Lateralis are recorded during ramped isometric and dynamic knee extensions (4) with a grid of 64 surface-EMG electrodes, specifically developed by POLITO (8 rows with 10 mm inter-electrode distance along the medio-lateral direction and 8 columns with 20 mm distance along the longitudinal direction), to assess muscle excitation and motor unit properties together with the muscle movements assessed with ultrasound (6). The electrodes grid placed over the muscle belly, with the 40-mm ultrasound transducer positioned at 50% of the femur length, longitudinally with respect to the muscle fibers' direction, between the first and the fourth column and fourth and fifth row.

Isometric test

Maximal strength is estimated with isometric knee extension test performed on a modified knee extension machine. The value obtained is also used to determine the target strength for the ramp contractions (30% and 50% of maximal isometric knee extension). Other than global EMG variables, single motor units characteristics are extracted (7-8) at different force levels of ramp isometric contractions to describe central (i.e. recruitment and firing properties), and peripheral (i.e. motor unit spatial dimensions, and innervation patterns) changes in single motor units (6). The main analyzed variables concerning peripheral aspects are: amplitude, size, and barycenter of the motor unit action potential distribution. The variables related to central aspects are: motor unit recruitment threshold, motor unit firing rate, and coefficient of variation of the inter-spike interval (4). In addition, ultrasound images sequences are also simultaneously recorded during the ramped isometric contraction to assess Vastus Lateralis muscle architecture parameters (see "Muscle morphology and architecture").

Fatigability test

Neuromuscular function is assessed during a fatiguing task consisting in eighty consecutive knee extensions at maximum velocity against a submaximal load (20% of the maximal isometric knee extension test). Contraction-by-contraction power outputs is calculated and analyzed with respect to HD-EMG variables. Fatigability is quantified as the relative percentage decline of power output of the last contractions with respect to the baseline power

output (first contractions) (9). The power output decline is then correlated with changes in temporal and spatial variables derived from HD-EMG such as the magnitude of the signals (i.e. root mean square), frequency bandwidth and median frequency of the signals, and activation pattern map (10).

Muscle morphology and architecture

Muscle architecture is a major determinant of muscle force, velocity and excursion (11-13). Structural adaptations have been assessed in-vivo using ultrasonography, during resting and during muscle contraction. Age-related loss of muscle entails an alteration in muscle cross-sectional area (CSA), volume and architecture (spatial arrangement of muscle fibres) (14). Quadriceps femoris muscle size across muscle length for volumetric reconstruction (15-17), Vastus Lateralis architecture (fascicle length, fascicle angle, muscle thickness) (18-19) and muscle quality using Echo-intensity (20-21) in the relaxed muscle are assessed.

When contracted, pennate muscles, such as the Vastus Lateralis, change in shape promoting alterations is muscle architecture parameters (22). Changes in fascicle length, fascicle angle, muscle thickness are expected to occur in the aged muscle with concomitant alterations in connective tissue (11, 23), to understand whether age affects muscle shape change during contraction, ultrasound image sequences are recorded concurrently to the HD-EMG signals during the isometric ramped contractions (see previous "Isometric contractions assessment"). The developed grids for the HD-EMG, allow simultaneous ultrasonography recording in the same muscle area, giving the possibility to combine mechanical and electrical analysis. The combined analysis of EMG and ultrasound allows to correlate muscle activation patterns with muscle structural changes, giving information of the mechanisms behind functional decline occurring with aging.

Magnetic Resonance Imaging assessment

The utilization of multiparametric muscle magnetic resonance imaging (MRI) has emerged as a powerful tool in understanding and quantifying age-related changes in muscle tissue. This comprehensive approach involves various MRI-based measurements and techniques aimed at characterizing muscle health and function in older adults (24). MRI assessments provide valuable insights into the aging-related changes in muscle tissue, including muscle volume measurements that quantify the loss of muscle mass (25). Reduced muscle mass has been linked to increased fracture risk (26) and is a primary diagnostic criterion for sarcopenia, a condition associated with muscle weakness and frailty (27). Additionally, MRI allows for the evaluation

of intramuscular adipose tissue, which can compromise muscle function and mobility (28). Research has revealed significant disparities in muscle fat infiltration among older individuals, with frail individuals showing higher levels compared to non-frail counterparts (29). Furthermore, in women over 50, MRI assessments revealed a link between muscle fat infiltration and an elevated risk of fractures (30). Moreover, the accumulation of fat within the muscles of the lower extremities has been inversely related to physical function measurements (31) emphasizing the critical role of adipose tissue infiltration in impacting muscle health and fracture risk in the old people. Thus, Multiparametric MRI protocols are employed to acquire a non-invasive assessment of muscle properties and their age-related changes, as outlined by previous works (32). In particular, morphological images, obtained using a T1-weighted (T1-w) turbo spin-echo sequence, are acquired to derive measurements of total thigh muscle volume, individual muscle volumes, and cross-sectional areas. Additionally, the study includes "quantitative fat-water imaging" as per Dixon's method (33), which leverages the chemical shift between proton resonance frequencies in water and fat to evaluate muscle fat infiltration accurately.

Functional evaluation of muscle oxidative metabolism

Exercise intolerance, defined as the incapacity to produce/maintain adequate muscle force or power to accomplish a task commensurate to the needs of everyday life (34), represents one of the clinical hallmarks of aging (35). Several functional biomarkers related to the muscle oxidative metabolism, such as peak work-rate and peak O_2, decline on average of 8-10% per decade in healthy adults (36). Consequently, non-invasive methods and tools capable to identify and quantify the metabolic and functional impairments of the muscle oxidative metabolism are utilized in this study. Pulmonary gas exchange and ventilatory variables are determined breath-by-breath using a metabolic cart (Quark CPET, COSMED, Italy). Central physiological parameters are investigated using cardiovascular biomarkers detected through bioimpedance cardiography (PhysioFlow, Manatec, Paris, France). Oxygenation changes and microvascular perfusion parameters of the Vastus Lateralis are measured by a wireless, portable, continuous-wave (CW), spatially resolved, near-infrared spectroscopy (NIRS) device (Train.Red PLUS, Artinis, The Netherlands) together with a hybrid device developed by Politecnico di Milano (POLIMI) combining Time-Domain (TD) NIRS (37) and Diffuse Correlation Spectroscopy (38-39). Briefly, light in near infrared region can penetrate for few centimeters inside biological tissues, and by exploiting the different absorption coefficient spectra of tissue chromophores (e.g., oxygenated and deoxygenated hemoglobin) information

on concentration of its main constituents can be retrieved (40). On the other hand, DCS allows to estimate blood flow by measuring variations in intensity correlation of coherent light source due to red blood cells movement in biological tissues. For more specific details about NIRS and DCS, please refer to Amendola et al (39).

Cardiopulmonary exercise testing

The functional responses of oxidative metabolism to peak exercise are evaluated using cardiopulmonary exercise testing (CPET), which provides an assessment of the integrated responses of the muscular, pulmonary, cardiovascular, and skeletal systems (41) to measure maximal values of oxygen consumption ($\dot{V}O_2$peak), cardiac output ($\dot{Q}$peak) and peripheral oxygen extraction [(a-v)O_2]. Using a ramp incremental maximal test on cycle-ergometer (E100, COSMED, Italy), participants are asked to cycle until exhaustion, defined when the respiratory exchange ratio (RER) is higher than 1.05 and the self-reported perceived exertional fatigue (RPE) is equal or higher than 17 (range of scale 6–20) (42).

Submaximal constant work rate test

It has been shown that during the transition from rest to moderate intensity exercise the rate of adjustment of the pulmonary oxygen uptake, namely VO_2 kinetics, closely reflects the adjustments of oxidative metabolism at the skeletal muscle level (43). VO_2 kinetics is typically slower in older compared with young healthy men (44); thus, older individuals rely more on anaerobic sources for energy production during exercise on-transients, which could lead to higher intracellular metabolite accumulation during exercise and premature fatigue. To investigate this issue, VO_2 kinetics are evaluated during the on-transient of constant work-rate exercise below gas exchange threshold (45). Moreover, intramuscular matching between O_2 delivery and O_2 utilization is evaluated by NIRS measuring oxygenation changes at Vastus Lateralis (46).

In-vivo muscle oxidative capacity and oxygen diffusion limitation

In-vivo maximal oxidative capacity and limitation in oxygen diffusion of the VL muscle are also evaluated by NIRS using a specific protocol of repeated intermittent arterial occlusions (47). More specifically, at rest a prolonged arterial occlusion is performed by a cuff positioned on the thigh in correspondence to the femoral artery to identify the physiological normalization (PN) of the tissue saturation index of VL muscle when participants are seated on the cycle ergometer (48). Then, 10–20 intermittent arterial occlusions are performed immediately after 5 min of moderate-intensity cycling. Duration and timing of the repeated occlusions are

controlled by the investigator to maintain the tissue saturation index in two different ranges: from 10% to 20% of PN (LOW) and from 50% to 60% of PN (HIGH). The HIGH range is selected to ensure that occlusions are performed under well oxygenated conditions (49-50). The LOW range is selected as the lowest boundary to evaluate muscle oxygen recovery in poorly oxygenated conditions. The rate of muscle desaturation during each intermittent arterial occlusion is fitted to estimate the exponential muscle oxygen consumption recovery as described previously (51). The difference between HIGH and LOW conditions as calculated to estimate oxygen diffusion limitation at skeletal muscle level.

Peripheral evaluation of vascular and endothelial function

Peripheral vascular and endothelial function serve as crucial indicators of the overall health of the vascular system since endothelium plays an important role in maintaining vascular homeostasis and regulating blood vessel function whereas endothelial dysfunction precedes cardiovascular disease including hypertension, atherosclerosis, and coronary artery disease (52). Several studies have been conducted over the years in older individuals and it has been demonstrated that aging is characterized by endothelial dysfunction, vascular remodeling, and loss of arterial distensibility (53). While endothelial function is commonly assessed by flow-mediated dilation following circulatory occlusion, recent studies suggest novel approaches to assess NO-dependent endothelial function as passive leg movement (54) and vascular occlusion test (55).

Passive leg movement test

Vascular and endothelial function is assessed using passive lower limb movement-induced hyperemia (PLM) protocol whereby femoral artery blood flow is measured by ultrasonography technique. This method has been recently recognized as a novel and noninvasive technique in aging able to distinguish differences in vascular function across the spectrum of cardiovascular health and disease (56). Blood flow in the common femoral artery is estimated by measurements of blood flow velocity and vessel diameter proximally to the bifurcation of the superficial and deep femoral artery, by using an ultrasound system (MyLab30Gold, Esaote, Italy). Blood flow measurements are performed during 60s of cyclical passive knee extension and flexion movements of the right leg across a 90° range of motion (180°–90°–180°) at 1 Hz (56).

Vascular occlusion test

Reactive hyperemia is a well-established technique for noninvasive assessment of peripheral microvascular function and a powerful predictor of all-cause and cardiovascular morbidity and mortality (57). Peripheral microvascular function is assessed by vascular occlusion test (VOT) evaluating tissue oxygenation changes at the Vastus Lateralis muscle at rest, during and after prolonged (5 minutes) occlusion of the femoral artery.

Muscle biopsy

Once per year (T0, T12 and T24), percutaneous muscle biopsies are performed on the Vastus Lateralis of a subset of volunteers as previously described (58). Briefly, participants considered eligible for muscle biopsy after medical screening underwent local anesthesia (1% lidocaine HCL) before the incision, and biopsy samples was collected using a 130 mm Weil-Blakesley rongeur (NDB-2; Fehling Instruments, GmbH&Co, Karlstein am Main, Germany). In order to obtain 150-200 mg of muscle tissue, if the yield of a single insertion is not sufficient, the biopsy's needle is inserted 2 or 3 times ensuring that the participant is comfortable and provides verbal consent. The entire procedure takes about 15 minutes and following the biopsy adhesive sterile strips are applied to close the wound, the area is covered, and ice is applied to it. Once collected, samples are quickly dissected to remove excess blood, fat and connective tissue, and divided into several parts to perform molecular protein and gene expression, DNA methylation, mitochondrial respiration and histological analysis.

Molecular investigations

A portion of muscle specimens at each time point has been dedicated to molecular investigations to assess the primary pathways governing the regulation of mitochondrial biogenesis, content, and dynamics and pathways related to protein synthesis and breakdown (59). As previously described (47), a portion of the biopsy was flash-frozen in liquid nitrogen and stored in cryovials at -80°C. Frozen samples was then pulverized in a steel mortar using a pestle previously treated with RNase Zap (Merck, Germany) to remove any trace of RNase presence, being careful to constantly add liquid nitrogen to avoid the loss of specimen properties. The powder obtained was then divided into two different cryovials either designed to western blot, for quantifying the expression of specific proteins, or to RT-PCR to detect and quantify *expression* profiles of desired genes.

Western-Blot

For protein expression analysis, muscle powder was homogenized with a specific liquid buffer and centrifuged to separate the supernatant and pellet. The supernatant was then transferred to clean Eppendorf tubes and stored at -80° until protein concentration determination was carried out. Protein concentration was determined using the reducing agent and detergent compatible (RC-DC) protein assay (Bio-Rad, CA, USA). After that, equal amounts of muscle samples have been loaded on pre-cast gels, electrophoresis was run and then proteins were electro-transferred to PVDF membranes, subsequently stained with Ponceau Red. Membranes were primarily incubated in specific primary antibodies and then in peroxidase (HRP)-conjugated secondary antibody. Protein detection was made by using an enhanced chemiluminescence advanced detection system (GE Healthcare Life Sciences, UK). The content of each investigated protein was evaluated by determining the brightness-area product of the protein band then normalized to total proteins in lysates by Ponceau stain or evaluated as the ratio between phosphorylated and unphosphorylated total forms of the same protein.

RT-PCR

For protein expression analysis, muscle powder was used for RNA extraction using a specific kit (SV Total RNA Isolation System, Promega, Italia). RNA concentration was assessed by spectrophotometer (NanoDrop instrument, Thermo Scientific, MA, USA). Muscle samples were then reverse transcribed by using a mixed solution composed of the Superscript III enzyme (Life Technologies, CA, USA), random primers, deoxyribonucleosides and RNase inhibitors as previously described (58). The obtained cDNA has been then analyzed by real-time PCR (Applied Biosystems, MA, USA) with the SYBR Green PCR kit (Applied Biosystems, MA, USA). Briefly, a mixture of SYBR Green PCR Master Mix, oligo primers specific to each gene, cDNA mold of each sample and sterile H_2O, were prepared. Then, cDNA was amplified using a protocol consisting of forty cycles of amplification, each one divided into a first phase at 95° for denaturation, a second phase at 60° for allowing primer annealing, a third one at 72° to allow DNA elongation. The ΔCt was calculated by subtracting the threshold cycles (Ct) of each sample from the referenced Ct sample for both target genes and housekeeping genes. Values obtained by each target gene has been then normalized on the values obtained by the housekeeping gene.

Mitochondrial function evaluation by High-resolution respirometry

For each subject at each time point, a portion of the muscle sample has been dedicated to ex-vivo evaluation of mitochondrial function by high-resolution respirometry (HRR). As

previously described (47), after trimming from connective and adipose tissue in excess, about 10 mg of muscle were placed into cryovials filled with ice-cold BioPS. Within the following 24 hours, fiber bundles underwent mechanical and chemical preparation to permeabilize muscle fibers, and mitochondrial respiration was assessed by an O2k-respirometer (Oroboros Instruments, Austria). For each sample, the analysis has been performed in duplicate. At the end of each experiment, muscle samples were removed from the instrument, washed and centrifuged with PBS, and stored at -80°C for subsequent measurement of citrate synthase (CS) activity for evaluation of mitochondrial content. Mitochondrial respiration was expressed normalized to both muscle specimen mass and specific mitochondrial content.

Muscle histology

A portion of the muscle sample at each time-point has been dedicated to the investigation of histological features as previously described (47). Briefly, muscle specimens were oriented under the microscope, fixed in OTC (Tissue-Tek; Sakura Finetek Europe, Netherlands), quickly frozen in liquid nitrogen, and then stored at –80°C. Several sections of each sample have been obtained using a cryostat and subsequently used for several histological investigations including myosin heavy chain immunostaining to determine fiber type proportion and cross-sectional area, and CD31 immunostaining for investigation of capillarization parameters. Other protocols included the immunostaining of N-CAM as a marker of denervation, Perilipin and Sirius red staining for the investigation of fat and connective tissue infiltration.

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