STUDY PROTOCOL
Bed rest SBI 2023

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Study financing:

- ARIS project (head: prof. dr. Boštjan Šimunič; J5-4593 Physical inactivity induced neuromuscular impairment: comparison of younger and older adults),
- PRIN project (head: prof. dr. Marco Narici; InactivAge – Inactivity induced neuromuscular impairment through different ages: from children, to young and middle age adults) and
- INTERREG SLO-ITA project (head: prof. dr. Gianni Biolo, MD and study coordinator prof. dr. Rado Pišot; X-BRAIN.net – Mreža čezmejnega sodelovanja rehabilitacije bolnikov po možganski kapi z uporabo inovativnih tehnologij).

Upgrade of MEMORI.net clinical protocol:

MEMORI.net clinical protocol will be upgraded based on new scientific findings on the effect of short-term physical inactivity on our health. We will scientifically evaluate health deterioration during 10-days of physical inactivity with and without interventions. Interventions will be based on cognitive stimuli as well as on diet supplementation.

Short (<10 days) periods of physical inactivity are very common after stroke. In that period a detailed diagnosis take place as well as psychological stress of patients are faced. However, it seem that this period is crucial for secondary health deterioration and delays treatment of primary stroke that might have impact on rehabilitation prognosis and results. Even very short

periods of physical inactivity have a negative impact on our health, most notably in older people who are ill. This has the additional, secondary effect of markedly worsening our health, which can lead to irreversible deterioration from which many people do not find a way out. Based on a review of the latest scientific literature, we will revisit and select the best exercise and cognitive interventions that we have jointly developed in the MEMORI.net project and complement them with nutritional interventions that can reduce the pathophysiological changes due to physical inactivity in the first phase of functional rehabilitation after stroke. To assess the effectiveness of functional rehabilitation, we will define a protocol for a clinical study of physical inactivity in participants of the same age group in which stroke most frequently occurs. In addition, we will define all health assessment procedures to be performed before and after the physical inactivity study.

Short description, motivation, scientific background:

Although physical inactivity (PI) progresses to the 2nd risk factor of overall mortality (WHO report 2019), from 7th in 2006, it should be noted that it is the most easily modifiable health factor in all age groups. Impacts of PI (a silent killer) may go undetected for years or decades before a preventable disease develops from it. PI is associated with the early onset of noncommunicable chronic diseases that lead to health problems and to all-cause mortality. Furthermore, **PI is an independent factor of our health** as it shows weak correlation with moderate to vigorous physical activity (MVPA) **and represents the greatest threat to our health**. We are witnessing increasingly long periods of PI in both active and inactive populations. A recent study of young college athletes and their inactive peers highlighted high daily values with no difference in mean values of sitting time (11.0 ± 3.0 hours) between both groups [1]. Athletes can be highly active and highly sedentary at the same time, so we must consider net effect of both factors on their health. Important here is to know that sitting time is >20-times more prevalent than MVPA, the latter being only 22 ± 15 daily minutes in adults (WHO report 2019). And indeed, the interventions might produce better results if they focus on reducing PI/sedentary time, rather than solely promoting MVPA.

Many scientific publications consider PI a pandemic, supporting the publication of the WHO Global Plan of Action for Physical Activity 2018–2030. This document aims to provoke a relative reduction in PI of 10% by 2025 and of 15% by 2030, which will contribute to a longer life expectancy and higher quality of life of the population. To support this, numerous studies demonstrated that the consequences of PI on physical and mental health are also severe, and the mechanisms of deterioration are very rapid, even more when PI is coupled with ageing or comorbidities. **PI has a negative impact on most subsystems of the human body**, among which negative consequences were reported on:

- muscle mass and architecture [2–5];
- muscle function [2,6];

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- bones [7,8];
- protein metabolism [9,10];
- cellular oxidative metabolism [11,12];
- neural processing efficiency [13] and cognitive functions [14,15] and
- cardiovascular and respiratory functions [16].

Additionally, recently **COVID-19 pandemic, public health recommendations and governmental measures have enforced lockdowns and movement restrictions**. While these restrictions help to abate the rate of infection, such limitations result in negative effects by limiting participation in normal daily activities, PA, travel, and access to many forms of exercise. Globally, a multicentric 22-country study revealed that the COVID-19 home confinement has had a medium to large (23–43%) negative effect on all levels of PA (vigorous, moderate, walking and overall) and a large increase in daily sitting time (28%) in adults [17]. Such sudden increases of PI lead to rapid muscle wasting, being detectable within only 2 days, and being associated with fibre denervation, neuromuscular junction (NMJ) damage and upregulation of protein breakdown, but is mostly explained by the suppression of muscle protein synthesis. PI also affects glucose homeostasis, reduce insulin sensitivity, principally in muscle. Additionally, aerobic capacity is impaired at all levels of the O_2 cascade, from the cardiovascular system, including peripheral circulation, to skeletal muscle oxidative function. Positive energy balance during PI is associated with fat deposition, associated with systemic inflammation and activation of antioxidant defences, exacerbating muscle loss [18].

Problem identification:

- The project activities **will focus on neuromuscular and metabolic deterioration during PI and restoration afterwards**. Effects of PI are studied using different models, with **bed rest (BR)** being a gold standard that few institutions have been able to develop in a complex hospital setting. The latter is intended to not only evaluate the effects of inactivity per se, but also to examine the efficacy of methods, including training countermeasures and rehabilitation, to address muscle atrophy and dysfunction caused by inactivity and disuse [19,20].
- **Skeletal muscle disuse is accompanied by morphological and functional decline** [21–25]. The decrease of muscle mass is very rapid: about 2% of quadriceps cross-sectional area (CSA) is lost in just 2 days of immobilization by knee bracing [26] or after 3 days of dry immersion [27], and about 5% is lost after 14 days of unilateral lower limb suspension [21]. Importantly, the associated loss of muscle strength is considerably greater than that of muscle size, about 9% after 3-day dry immersion [27] or 5-day immobilization [28] and 15-27% after 14 or more days of unloading [21,23,29–31]. In turn, such loss of muscle force is associated with an even greater loss of mechanical power [32–34], which is the most relevant parameter for an effective performance of most daily-life motor activities. At the single fibre level, the reduction in specific force (Po), resulting from a greater decrement in isometric

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force, Fo, than CSA) is a major determinant of the loss of peak power, while maximum shortening velocity seems less affected by disuse [24,35,36].

- Since **short-term (7-15 days) disuse is a common condition experienced by both young and older people**, when bedridden following either injury [37] or surgery [38], and by critically ill patients [39], the investigation of the possible determinants of the disproportionate reduction in muscle force compared to that of muscle size is of considerable relevance. Furthermore, few studies addressed the interactions between muscle and neural control, through sensorimotor loops and NMJ, in causing exercise intolerance and whole-body metabolic impairment and the underlying mechanisms. PI affects all ages. Little is known on the impact of PI in older adults (55-65 years). The recent COVID-19 outbreak has induced PI in millions of people, of all ages worldwide [17,18,40] and stressed its detrimental consequences.
- However, a clear and **exhaustive explanation of why contractile force declines faster than muscle mass is still lacking**. Only partial answers have been obtained from the analysis of the myofibrillar force generation process [25,41–44] and from the force transmission through extracellular matrix and tendons [21,45,46] to bones. A possible clue has been given by a recent study [27] which showed that dry immersion, **an extreme form of disuse, is able to induce in only 3 days a very initial and partial progression of fibre denervation**. This finding is in line with previous observations on the impact of 14-day BR in a cluster of men and women in their sixth decade, thus prone to age-related alterations of the neuro-muscular connection [47]. Based on these considerations, we will investigate further the stability of the NMJ, representing the connection between muscles and nerves, linked to myofiber innervation. Additionally, we will aim to extend the analysis upstream to the central nervous system voluntary capacity of muscle activation and down-stream to the intracellular calcium signals, linking the action potential triggered at the NMJ to the myofibrillar motors.

Although **PI affects most organ systems, deterioration of muscle mass and strength, neuromuscular control and energy metabolism are the major phenomena** [54]. Furthermore, PI causes a series of responses involving nervous system, metabolism, and cardiovascular system. Several issues are still incompletely understood especially in humans:

a) the mechanisms of disproportionate loss of force compared to muscle mass [55,56].
b) the relative role of somatomotor control, NMJ, muscle and energy metabolism in impairing movement [54,57,58] and muscle work [59].
c) the crosstalk between nerve and muscle in NMJ instability [60].
d) the mechanisms responsible for impairment of oxidative metabolism, and the relationship with the development of neuromuscular alterations [61] and insulin resistance [62].

Although aims and tasks are written only to the disuse part (the main purpose of the project), we will study equally important also the recovery afterwards. To tackle such issues, we will

pursue 3 specific aims through 10 intertwined tasks in young and middle-aged adults in PI model of BR:

Thus, we will design a **21-day horizontal BR (with 21-day recovery) in 10 healthy younger men** (18-30 years) and **10-day horizontal BR (with 21-day recovery) in 10 healthy older adult men** (65-75 years) to gain further insights into the early determinants of marked unloading-related muscle volume, strength, and power loss. Accordingly, we will investigate neuromuscular system morphology, integrity, and function before, during and at the end of the BR period. We will test the hypotheses that, (i) early impairment of the neuromuscular transmission, possibly based on NMJ instability, might develop shortly after unloading exposure, (ii) impaired neuromuscular transmission might be accompanied by alterations of intra-fibre calcium signalling, (iii) such changes might be accompanied by impairments in activation capacity, and (iv) there are differences in decline and re-training capacity between younger and older adults.

Aims and expected results:

Task 1 *muscle volume, architecture, contractability, strength, and power*
We will evaluate: (i) whole body and quadriceps muscle volume change; (ii) change in muscle architecture in 4 leg skeletal muscles; (iii) mechanical twitch contractile properties in 4 leg skeletal muscles; (iv) changes in voluntary quadriceps muscle strength and power.

Expected results. We expect up to 50% higher deterioration in older adults, when compared to younger adults in BR exposure of the same length (10 days). Further, older adults will have lower retraining capacity than younger adults.

Task 2 *muscle fibre size, force, tension, velocity, extracellular matrix with cytoskeleton and MHC composition*
We will evaluate: (i) muscle fibre cross sectional area; (ii) velocity, force and specific tension; (iii) calcium dynamics based on caffeine-response (iv) force-pCa curve; (v) myosin heavy chain isoform distribution; (vi) components of extracellular matrix and cytoskeleton.

Expected results. As in Task 1, we expect similar findings also on muscle fibre levels, i.e, greater impact of BR on muscle fiber function in older adults. We do not expect changes in myosin heavy chain proportion after BR and SR exposures, but we expect changes in extracellular matrix and cytoskeleton composition.

Task 3 *Supraspinal and spinal excitability*
We will: (i) use the interpolated twitch doublet technique [64] to assess central activation, an estimate of the integrity of somatomotor control from motor cortex to α-motoneurons; (ii) study the rate of force development and relaxation time by electrically evoked twitch contractions to estimate alterations in muscle contractility, an indirect estimate of E-C coupling efficiency; (iii) study H-reflex, V-wave and M-wave [65] to assess plastic changes in spinal neuronal circuitries responsible for pre/post-synaptic control to the spinal cord [66].

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Task 4 *Neuromuscular activation patterns and spinal maps*
We will record multimuscle surface EMG from 16 lower limb muscles, compute the temporal patterns of muscle activations, the muscle synergies, and the spatiotemporal spinal maps to describe the output of alpha-motoneurons [67].

Task 5 *Motor unit behaviour and NMJ function*
We will investigate the time course of adaptation to reduced activity of several individual MUs (discharge times (MUDR), recruitment thresholds (RT) and conduction velocity (MUCV)) employing the latest technical advances in EMG recordings (e.g., high-density surface EMG (HD-EMG)) and decomposition [68]. Adaptations in NMJ function will be estimated using quantitative needle electromyographic techniques that reflect properties of the MU potentials (MUNE technique) to assess the jitter and jiggle parameters, accepted indices of NMJ transmission efficiency [69].

Expected results. It is expected that the disuse will correspond to an increase in MU recruitment thresholds and that this will be associated to a decrease in both the MUDR and MUCV. In association with task 5, a comprehensive picture of the adaptations of somatomotor control to disuse will emerge.

Aim 2: define the NMJ alterations and their functional role
Based on very recent findings on NMJ in mice **we hypothesize** that very early adaptations could bring, on a different time base, to: NMJ instability, alteration in neuromuscular control, insulin resistance and muscle wasting.

Task 6 establish the occurrence and extent of NMJ alteration
We will evaluate the presence of NMJ stability by analysing: (a) AChR subunits expression [70]; (b) signaling components of agrin/MuSK/Lrp4 pathway; (c) Homer 2 content (a molecular player in translating synaptic input to skeletal muscle fibers [71] and in controlling skeletal muscle trophism [72]); (d) expression of muscle specific miRNAs influenced by inactivity which affect NMJ stability [73]; (e) c-terminal agrin fragment (CAF) in serum samples, a biomarker of NMJ damage [74]; (f) serum creatinine as a biomarker of muscle denervation and atrophy [75].

Expected results: complex adaptations at multiple levels of NMJ integrity such as altered AChRs expression and clustering, lower Lrp4 and MuSK expression in muscle together with increased CAF levels in blood indicating NMJ damage.

Task 7 *define the relationship between NMJ alterations and integrity of muscle structure and function*
The presence of denervation will be studied through the expression level of specific genes associated with inactivity and NMJ denervation and occurrence of NCAM positive fibres.

The presence of skeletal muscle alterations will be determined in terms of: (1) myofibers size; (2) intracellular pathways balancing protein synthesis and degradation; (3) molecular and

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functional mitochondrial adaptation; (4) redox status and oxidative stress; (5) neurotrophins levels in muscle.

Expected results: we expect to verify the working hypothesis on the role of the AMPK/PGC1alpha axis in bridging muscular, metabolic and somatomotor adaptations.

Aim 3: define metabolic impairment: energy and glucose metabolism

Oxidative metabolism limits exercise tolerance and work capacity during everyday life, and its impairment is a major risk factors for chronic diseases. Central (cardiovascular) as well as peripheral factors (peripheral blood flow, endothelial function, intramuscular matching between O_2 delivery and O_2 uptake, peripheral O_2 diffusion, mitochondrial respiration) contribute to the impairment of oxidative metabolism following inactivity. Among peripheral factors, mitochondrial dysfunction and redox imbalance can contribute to (i) development of maladaptive changes within motor neurons, the NMJ and muscle fibres [76] and (ii) insulin resistance[62].

Task 8 *define metabolic impairment at muscle and whole-body level*
During exercise on a cycloergometer at different intensities, we will determine: (i) peak VO_2; peak cardiac output; peak fractional O_2 extraction by skeletal muscle [77]; respiratory function and respiratory muscle function (maximal inspiratory and expiratory pressures) at rest and after maximal exercise "thresholds" [77], work rate decrease at a heart rate slightly above GET [78]; (ii) vascular peripheral - endothelial functions [79]; (iii) intramuscular matching between O_2 delivery and O_2 uptake [80]; (iv) mitochondrial respiration by the analysis of muscle VO_2 recovery kinetics [81].

On muscle samples we will study: (v) mitochondrial respiration by high-resolution respirometry on permeabilized muscle fibers [82] and mitochondrial sensitivity to submaximal [ADP] [83], (vi) estimates of mitochondrial mass; (vii) reactive oxygen species (ROS) production [83]; (viii) susceptibility to permeability transition in the inner mitochondrial membrane [84]; (ix) assembly status of mitochondrial ATP synthase (complex V) and its interaction with the matrix protein Cyclophilin D, master regulator of permeability transition [84].

Expected results: characterization of subjects in terms of exercise capacity and tolerance; comparison of central *vs.* peripheral impairments of oxidative metabolism and of impairments related to O_2 delivery *vs.* those related to mitochondrial dysfunction; extent, time-courses and individual behaviors of mitochondrial dysfunction markers and relationship with alterations in NMJ and the development of insulin resistance. Formation of vestigial ATP synthase complexes potentially responsible for altered respiration and/or increased propensity of ATP synthase to switch from an energy-conserving to an energy dissipating enzyme, forming the permeability transition pore, and possible role for the AMPK/PGC1alpha axis in counteracting these alterations.

Task 9 *investigate insulin resistance & reactive oxygen species (ROS) role*

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To assess the time course of redox state and insulin resistance we will determine the erythrocyte concentration of whole gamma-glutamyl cycle [Qi], the GSH-to-GSSG ratio [85], and the glutamyl cysteine-synthetase expression [86]. Insulin resistance through hyperinsulinemic euglycemic clamp and HOMA Index [87]. Moreover, we will explore the mechanisms downhill the modification of the expression and activity of matrix metalloproteases and their inhibitors [88].

Expected results: Determination of the extent of oxidative stress, insulin resistance and MMP/TIMP levels, and correlation of these findings with changes in muscle mass, performance, and other metabolic modifications.

Estimated sample size of study participants: The study will be done in 30 participants:

- YOUNG: 10 young males, aged 18-30 years;
- OLDER: 20 older males, aged 65-75 years.

Research power estimation: We have calculated and decided to enroll 10 participants in both BR studies. The power analysis was performed for the most relevant part of the studies – disuse, on several assumptions: $\alpha \leq 0.05$; 1-sided tests, $\beta=0.3$; $P=0.7$, for main outcomes of muscle strength, size and anaerobic power:

Outcome	Pre	Post	Required N	References
10-day BR in old				
Quadriceps volume / cm^3	1667±234	1525±211	13	[51]
Quadriceps strength / N	546±110	475±109	11	[51]
Anaerobic power / W/kg	35±5.7	30±5.7	10	[51]
Aerobic power / $mlO_2/kg/min$	27±4.9	22±3.4	5	[51]
21-day BR in young				
Aerobic power / $mlO_2/kg/min$	38±6	31±5	4	**[99]**
Knee extensors volume / cm^3	270±30	247±31	10	[100]
Quadriceps strength / kg	48±8	40±8	6	[101]

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Procedures: **After ethical approval, a 21-day BR in 10 young males (18-30 years) and 10-day BR in 10 older adults (55-65 years), both with 21-day recovery, will be organized in a hospital setting** with 24-hour medical supervision (as in our 5 previous studies, ranging from 10-35 days of BR/recovery). We will recruit healthy participants (+2 for a reserve and a pilot testing) with exclusion criteria (smoking, regular alcohol consumption, ferromagnetic implants; history of deep vein thrombosis with D-dimer >500 μg·L−1; acute or chronic skeletal, neuromuscular, metabolic and cardiovascular disease conditions; pulmonary embolism; 21>body mass index>25 kg·m^{-2}, athletes, <90 minutes daily MVPA). All participants will pass kinesiological (VO_2max, leg power test) and medical examination (resting and exercising ECG, blood pressure, medical interview). After pilot tests in 2 reserve participants, 2 by 2 participants/day, will enter the study with 2 days of accommodation to hospital activities and diet, being followed by 2 days of baseline assessment (BDC), 10/21 days of BR and 2 days of reambulation before leaving the hospital (BR10/BR21). Afterwards, they will follow a recovery protocol (see below) and return for the assessment on 21st day of recovery (REC21). All tests will be performed in the same schedule (BDC, BR10, BR21 and REC21). During the BR they will spend 10/21 days in a horizontal position (horizontal movements allowed), with 1 pillow under the head. No further elevation or drop in vertical position will be allowed to minimize cardiovascular stress. All daily activities and tests during the BR will be performed in such a position. A nurse and students will help with food distribution, with personal hygiene, bed transport, supervision. Physician will do daily monitoring of their general health. Physiotherapist will do passive stretching every second day of BR. Diet during the BR will be energetically neutral, being 1.2-times resting metabolic rate [9]. The macronutrient food content will be det at 60% carbohydrate, 25% fat and 15% protein. The sleeping time will be between 22:00 and 07:00.

Interventions:

Twenty older participants will be randomly split into two groups per 10. Only experimental group will have 3 interventions:

a) 8-week pre-exercise supervised program with 3 weekly 60-minute sessions. Aiming at strengthening leg muscles and cardiovascular conditioning.
b) During bed rest period: daily 45-minute cognitive exercise using VR systems (motor imaginary) targeting motor control in knee extensors.
c) During bed rest period: additional protein supplements 1,6 g/kg/day with amino acids (11.4 g/day free leucin, 1.33 mmol/kg/day glycine, 0.81 mmol/kg/day N-Acetyl-Cysteine).

Assessment methods

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All assessments will be done at 4 time points (BDC, BR10, BR21 and REC21):

- Physical activity will be measured with hip-worn accelerometers.
- Body composition (fat) will be measured by standard anthropometry and whole-body DXA.
- Leg muscle mass will be measured by continuous MRI scan. The turbo spin-echo T1-weighted data sets of both thighs will be acquired in the axial (transverse) orientation. Acquisition parameters will be set as follows. Sequence: vibe, TR/TE: 7.8/3.69 ms, flip angle: 20°, field of view: 450 × 337.5 mm, voxel size: 0.9×0.9×6 mm, no inter-slice gap, slice thickness: 6 mm, readout-bandwidth 320 Hz/pixel (278 kHz). In the corresponding images, contours of the quadriceps femoris (QF) and vastus lateralis (VL) will be digitized in randomized order using the OsiriX DICOM image analysis software (Version 11). Within the scan, QF and VL muscle CSA contours will be carried out every two axials; VL and QF volumes will be derived by summing a series of truncated cones between two axial images (~20–25 images evaluated per scan).
- VL, gastrocnemius medialis, tibialis anterior and biceps femoris architecture will be measured by linear ultrasound with extended field of view (EFOV) also at BR1, BR2, BR5, BR10 and REC2. Muscle CSA of the QF will be measured using ultrasonography imaging (Mylab70). The ultrasound transducer will be moved along the skin surface and along the plane of interest (in our case, the transverse plane). While moving the transducer, successive frames will be recorded and stitched together by the algorithm, to obtain a panoramic image. Different regions of interest will be identified at 30%, 50% (CSA50) and 70% of the femur length (measured from greater trochanter to the mid-patellar point) and marked on the skin.
- Contractile properties will be assessed in parallel to architecture, in the same muscles by TMG. Shortly, a maximal mechanical response to 1 millisecond electrical impulse will be assessed in each muscle with displacement sensor to measure contraction time to estimate myosin heavy chain I composition [103] and early time point of muscle atrophy [4].
- QF maximal voluntary contractions (MVC), rate of force development (RFD) and activation capacity will be measured in isometric conditions at knee angle set at 30 deg flexion and hip fixed at 90 deg flexion using a custom-made knee dynamometer fitted with a load cell (RS 206–0290). Prior to the testing a warm-up and familiarization will be assured. The load-cell output will be acquired (BIOPAC MP100, Biopac Inc.) at 2 kHz and analyzed using Ackqnowledge software. The contraction with the highest force value will be considered as the MVC. Knee extensor MVC will be normalized to QF CSA to obtain QF specific force. RFD will be measured as the time required to reach the 63% of the MVC. Activation capacity will be assessed using the double interpolated twitch technique.

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- Muscle power and function will be assessed by a series of 3 separate counter-movement jumps. These tests will be performed on a Kistler ground force platform, with the best jump based on peak power selected. Arms will be fixed on hips. Peak relative (i.e. normalized to body mass) power and force, as well as peak jump height will be recorded.
- Muscle VL biopsy samples (about 150 mg) will be obtained by percutaneous biopsy from right VL at the beginning and end bed rest, as well as at BR5, from the left VL by an experienced surgeon. Biopsy sampling will be done after anaesthesia of the skin, subcutaneous fat tissue, and muscle fascia with 2 ml of lidocaine (2%). A small incision will be made to penetrate skin and fascia, and the tissue sample will be collected with a Weil–Blakesley conchotome. After the biopsy, muscle samples will be divided into three parts. The first will be used for dissection of single muscle fibres for determination of CSA, force (Fo) specific tension (Po), caffeine-responsiveness and force-pCa curve. The second part of the biopsy will be frozen in isopentane, cooled down on liquid nitrogen and stored at −80°C for subsequent immunohistochemical analysis, and the third part will be frozen in liquid nitrogen and stored at -80°C for protein and mRNA analysis.
- Single fibre experiments will be done after skinning, activating, loading, and washing fibres. Muscle fibre bundles, pinned straight on small sylgard platforms, will be kept at −20 °C in skinning solution mixed with 50% glycerol. Single fibres will be manually dissected to 1−2 mm long segments and light aluminium clips will be applied at both ends. The segments will then be transferred to the experimental setup, placed on the stage of an inverted Zeiss Axiovert 25 microscope (Zeiss, Milano, Italia) and mounted between the force transducer (AME-801 SensorOne, Sausalito) and the electromagnetic puller (SI, Heidelberg) equipped with a displacement transducer. The segments will be mounted in relaxing solution at slack length and then stretched by ~20% to reach a sarcomere length close to 2.8 μm, i.e. at the upper edge of the plateau of the tension–length relationship. Sarcomere length and 3 diameters on the horizontal plane equally spaced along the segment will be measured on the images collected at high (320x) magnification with a digital camera (Optikam B5, Optika), CSA calculated assuming a circular shape and averaged. Once mounted in the setup each fibre will undergo a protocol including caffeine-induced calcium releases and maximal activations after shortening–relengthening maneuver. We will register isometric force (Fo) and velocity (vo), the rate constant KTR of tension redevelopment. Caffeine-induced calcium release will test the function of the sarcoplasmic reticulum. Therefore, the fibres will be immersed for 5 min in the specific loading solution with 4 mM to 20 mM of caffein to induce a maximal release.
- Neural cell adhesion molecule (NCAM) will be detected by immunohistochemistry from muscle samples. Frozen samples will be cut to 8 μm thick sections. Detection and quantification of denervated myofibres were performed by NCAM immunolabelling. Shortly, cryosections will be fixed in cold methanol for 10 min at

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20°C, washed, labelled by antibody directed against NCAM and rinsed. NCAM-positive fibres will be counted on captured images, using Image software and expressed as the number of positive myofibres per total number of myofibres detected in the biopsy area by laminin staining.

- Blood samples will be taken from each participant's medial cubital vein at 07:00 on BR0, BR5, and REC21. After blood being collected samples will be centrifuged (CENTRIC 400, Tehtnica Železniki) at 2480 g for 10 min to separate serum and plasma. After this procedure, 500 µl aliquots of both serum and plasma will be prepared, cooled and stored at -80°C until analysis.
- Serum C-terminal agrin factor (CAF) will be determined using enzyme-linked immunosorbent assay (ELISA) kit (Human Agrin SimpleStep ELISA, Ab216945) following the manufacturer's instructions. The obtained CAF concentrations will be determined through a microplate ELISA reader (Tecan, Infinite M200). To assess CAF serum concentrations, a standard curve (loaded with known and increasing CAF concentrations) will be prepared and read at 450 nm. Serum CAF concentrations will be interpolated from the CAF standard curve and corrected for sample dilution.

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