Protein Supplementation in Older Adults with Sarcopenic Obesity Undergoing Caloric Restriction and Exercise.

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SUMMARY

PROTOCOL

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SUMMARY OF STUDY

Sarcopenic obesity increases the risks of mortality, comorbidities, and the development of various geriatric syndromes, making it a growing concern for the older adults. However, treatment options are controversial and lack sufficient data. Combining exercise training, caloric restriction, and increased dietary protein is a potential strategy, but the long-term effects on muscle, bone health, cardiovascular function, and aging are still unclear. Therefore, this study aimed to investigate the impact of combining protein supplementation with exercise training and caloric restriction in older adults with sarcopenic obesity.

This will be a single-center, double-blinded, parallel-group, randomized, placebo-controlled trial. Older adults with sarcopenic obesity (n= 105) will be allocated to one of three groups: protein supplementation (PTN, n=35), placebo supplementation (PLA, n=35), or control (CON, n=35). The PTN and PLA groups will receive dietary supplements in addition to an exercise training and calorie restriction intervention, while the CON group will receive no intervention. Body composition, bone health, cardiorespiratory fitness, pulmonary function, functional capacity, muscle cross-sectional area, upper and lower-limb maximal dynamic strength, endothelial function, biochemical parameters, health-related quality of life, cognitive function, sleep quality, symptoms of anxiety and depression, and nutritional status will be assessed before and after a 16-week intervention period. A linear mixed model with repeated measures analysis will be conducted to assess within- and between-group effects of the intervention, using baseline values of the outcome as covariates. Whenever significant group-by-time interactions will be found, post-hoc tests on the adjusted predictions with Sidak's adjustment will be performed for multiple comparisons. Significance will be set at $p<0.05$. Data will be reported as the mean and standard deviation, along with the estimated mean difference (EMD) between groups and its 95% confidence interval (95% CI) when a main effect or interaction is observed.

BACKGROUND AND SIGNIFICANCE

The prevalence of obesity among older adults is growing worldwide (1-3). Excessive adipose tissue is linked to insulin resistance, high blood pressure, endothelial dysfunction, and dyslipidemia, obesity offers a heightened risk for developing cardiovascular and metabolic diseases, particularly among older individuals (4-6). Concurrently, aging is associated with declines in skeletal muscle mass and function (i.e., sarcopenia) (7), which can be further exacerbated by obesity (8-10). The interplay of these conditions poses an emergent public health issue termed sarcopenic obesity (11-13), a condition associated with greater risk of morbimortality and worse health-related quality of life (14, 15).

The optimal treatment approach for sarcopenic obesity in older adults is still a subject of debate. Numerous studies have highlighted the potential benefits of caloric restriction for managing obesity and mitigating its associated negative effects (16-21). However, weight loss resulting from caloric restriction may further worsen age-related declines in skeletal muscle and bone mass in older adults (20). For this reason, exercise training has been frequently recommended as a countermeasure to offset skeletal muscle and bone mass losses while potentiating weight loss (22, 23). Although previous studies partly support this idea, exercise training alone has been shown unable to fully preserve skeletal muscle and bone tissue (17, 24, 25), warranting additional strategies to counteract the adverse effects of caloric restriction on these tissues.

For instance, acute protein intake can activate anabolic pathways in skeletal muscle and stimulate the release of growth factors essential for maintaining bone structure (26, 27). Over the long term, high-protein diets may help support muscle and bone turnover, potentially reducing caloric restriction-associated adverse effects on body composition, even in older populations (28-34). Importantly, increased dietary protein intake have been demonstrated to positively impact functional capacity in older adults when combined with exercise training interventions, possibly preserving/enhancing their autonomy (35, 36).

Protein supplementation may also confer metabolic benefits on lipid metabolism, insulin resistance, endothelial function, and immune function (37-40). Indeed, protein supplementation, especially whey protein, modulates incretin hormones and is highly insulinotropic (38, 39), which may improve insulin sensitivity, glycemic variability (41, 42) and lipid profile (43). Furthermore, immunoglobins present in whey protein are

thought to possess anti-inflammatory properties (37-39), which may help reduce inflammatory status in these patients (44-46). Also, the use of whey protein has been recommended to improve endothelial function. Significant improvements in flow-mediated dilation (FMD) response, a prognostic marker of cardiovascular events (47), have been demonstrated following whey protein supplementation in different populations (40, 48, 49).

Cardiorespiratory fitness, a clinical prognostic marker that is highly responsive to exercise training, is also likely to respond positively to dietary interventions when combined with exercise training (50). Previous small-scale studies have demonstrated the benefits of protein supplementation during endurance training on cardiorespiratory fitness in both healthy individuals and clinical populations (51-53). It can be speculated that the positive effects of protein supplementation on skeletal muscle mass and mitochondrial adaptations (54) may enhance mechanical efficiency and bioenergetic metabolism, further contributing to improvements in cardiorespiratory fitness. Moreover, ventilatory inefficiency and exertional dyspnea, which are often associated with obesity, may also improve with changes in body composition and weight loss, particularly through reductions in fat mass (55, 56).

Finally, recent studies have also shown a positive association between skeletal muscle mass and leukocyte telomere length, a marker of biological aging (57, 58). A similar negative association is observed between obesity-related parameters (e.g., body mass index, fat mass, waist-to-hip ratio, etc.) and leukocyte telomere length (59-62). It is plausible to speculate that improvements in body composition (e.g., maintaining muscle mass while reducing fat mass) through interventions combining both protein supplementation and exercise training may influence the rate of telomere shortening, potentially impacting overall health.

Collectively, these positive effects of protein may be of significant clinical relevance, considering that these conditions are highly prevalent in older adults, especially among those with sarcopenic obesity (63-67). Therefore, this study aimed at investigating the impact of protein supplementation in addition to exercise training and caloric restriction on fat-free mass (as primary outcome) in older adults with sarcopenic obesity. Secondary outcomes include skeletal muscle fiber area, muscle strength and functionality, fat mass (total, visceral and subcutaneous), bone health parameters (bone mineral density, microarchitecture and bone metabolism markers). Other outcomes included cardiopulmonary capacity (cardiorespiratory fitness and pulmonary function),

glycemic control and insulin sensitivity, endothelial function, inflammation, and related cellular and molecular mechanisms (telomere length, oxidative stress, and lipid profile). We hypothesized that this integrated approach could provide substantial benefits for body composition, and various health-related outcomes crucial to managing sarcopenic obesity in this population.

STUDY HYPOTHESIS

We hypothesized that protein supplementation can provide additional benefits on body composition and bone health more than only caloric restriction and exercise training in this population. Additionally, we hypothesized that these improvements would enhance muscle function. Furthermore, we also expected that protein supplementation would enhance the effects of caloric restriction and exercise training on glycemic control, insulin sensitivity, endothelial function, inflammatory markers, lipid profile, telomere length and oxidative stress markers.

AIM

This study aimed at investigating the impact of protein supplementation in addition to exercise training and caloric restriction on fat-free mass (as primary outcome) in older adults with sarcopenic obesity. Secondary outcomes include skeletal muscle fiber area, muscle strength and functionality, fat mass (total, visceral and subcutaneous), bone health parameters (bone mineral density, microarchitecture and bone metabolism markers). In addition, this study will aim the effects of protein supplementation in other outcomes including cardiopulmonary capacity (cardiorespiratory fitness and pulmonary function), glycemic control and insulin sensitivity, endothelial function, inflammation, and related cellular and molecular mechanisms (telomere length, oxidative stress, and lipid profile).

RESEARCH DESIGN AND METHODS

This 16-week study will be a single-center, double-blinded, parallel-group, randomized, placebo-controlled trial.

STUDY POPULATION

Older adults will be recruited from the Division of Geriatrics at the School of Medicine of the University of São Paulo, as well as through advertisements on social media and in community-based centers for older adults. All participants will provide written informed consent before enrollment in the study.

Inclusion criteria

Men and women aged 65 years or older, with central obesity (waist circumference >102 cm for men and >88 cm for women), body mass index (BMI) of ≥30 kg/m², sarcopenia (according to the criteria set by the Foundation for the National Institutes of Health (FNIH) (68)), not engaged in an exercise training program, and with stable weight within the past 6 months will be considered eligible to participate. Reference cut-off values for sarcopenia will include a handgrip strength-to-BMI ratio of less than 1.0 kg/BMI and an appendicular fat-free mass-to-BMI ratio of less than 0.789 kg/BMI for men, and a handgrip strength-to-BMI ratio of less than 0.56 kg/BMI and an appendicular fat-free mass-to-BMI ratio of less than 0.512 kg/BMI for women (68).

Exclusion criteria

Exclusion criteria will include a history of recent malignancies (within the past 5 years); cognitive deficits or dementia that prevent the participant from reading and signing the informed consent form; and medical conditions in which physical testing or engaging in exercise training would be contraindicated (e.g., locomotor disabilities or cardiopulmonary diseases, including unstable angina, heart failure, myocardial infarction, or chronic obstructive pulmonary disease).

Follow-up procedure:

In case of drop-out, the participant will be asked to complete a full set of examinations at the time of drop out.

STUDY DESIGN

The present study will be a single-center, double-blinded, parallel-group, randomized, placebo-controlled trial. Figure 1 illustrates the study design. Initially, participants will be pre-screened through phone calls or personal interviews. Those who meet the preliminary criteria will then be further assessed for their medical condition and eligibility criteria. Eligible participants will be matched based on sex and then randomly allocated in a 1:1:1 ratio into one of the following experimental groups: (a) protein supplementation (PTN), (b) placebo supplementation (PLA), or (c) control (CON).
Both the PTN and PLA groups will receive dietary supplementation, will be caloric-restricted, and will engage in an exercise training program. Participants in the CON group will receive no intervention other than general guidance on healthy eating and physical activity. All outcomes of interest will be assessed immediately before participants' randomization at baseline (PRE) and after 16 weeks of intervention (POST). Anthropometric and dietary parameters will be reassessed at weeks 4, 8, and 12. Participants will also meet with a registered dietitian from the research team every two weeks in order to ensure compliance with the dietary intervention.

RANDOMIZATION

Eligible participants will be sex-matched and then randomly allocated using a computer-generated block design in a 1:1:1 ratio into one of the following experimental groups through a specific software (https://randomizer.org): (a) protein supplementation (PTN), (b) placebo supplementation (PLA), or (c) control (CON). The allocation sequence will be concealed from the research staff.

EXAMINATIONS

The primary outcome will be the change from baseline in fat-free mass (total, appendicular, appendicular relative to BMI and leg). Secondary outcomes will include other measures of body composition (i.e. fat and bone density), bone microarchitecture, bone metabolism, and specific muscle functions. Other outcomes, including cardiopulmonary capacity (cardiorespiratory fitness and pulmonary function), glycemic control, insulin sensitivity, endothelial function, inflammation, cognitive function and

related cellular and molecular mechanisms (telomere length, oxidative stress, and lipid profile) will also be assessed.

Anthropometry

Body weight and height will be measured on a calibrated digital scale with a stadiometer, respectively. Also, a trained researcher will carefully measure anthropometric data, including waist (WC) and hip circumferences (HC). WC will be determined at the midpoint between the lowest rib and top of the iliac crest on the axial plane, while the HC will be measured at the widest part of the hips. All assessments will be performed with the participant standing; a plastic tape will be used for these measurements.

Body composition

Total and segmental fat-free, and fat mass parameters, and areal bone mineral density (aBMD) of the whole body, lumbar spine, femoral neck, and total hip parameters will be assessed by a whole-body dual-energy X-ray absorptiometry (DXA) using Hologic QDR 4500A densitometry equipment (Discovery Densitometer, Hologic Inc., Bedford, MA, USA). The DXA will be calibrated with phantoms as per the manufacturer's guidelines each day before measurements are taken. Assessments will be carried out in the morning, using a consistent positioning of participants on the scanning bed of the DXA equipment (36). All exams will be conducted by an experienced technologist blinded to the study protocol.

The precision errors for aBMD assessments will be determined following recommendations from the International Society for Clinical Densitometry (69).

Skeletal muscle size

Skeletal muscle size will be assessed using computed tomography (CT) and B-mode ultrasound. For both CT and ultrasound imaging exams, participants will first be positioned supine with their shoulders extended, hands placed above their heads, and knees fully extended. All muscular cross-sectional area (mCSA) images will be acquired in the right leg. Before assessments, participants will lay down for 20 minutes to allow

fluid distribution (70). During assessments, the legs will be restrained with Velcro straps to prevent internal or external hip rotation and participants will be instructed to relax as much as possible their lower limb muscles.

Quadriceps muscular cross-sectional area

Quadriceps mCSA will be evaluated through computed tomography (CT) scans (Discovery CT 750HD, GE Healthcare, Chicago, IL). PRE and POST images will be acquired by the same blinded specialized radiology technician, under medical supervision, and processed using GE Healthcare workstation. To avoid bias, both will be blinded to the participants' group assignment. Initially, an image will be captured to determine the thigh length, which will be defined as the perpendicular distance from the greater trochanter to the inferior border of the lateral epicondyle of the femur. Quadriceps mCSA images will be acquired at 50% of the segment and processed using GE Healthcare workstation. Image acquisition parameters will be as follows: slice thickness = 10 mm; table increment = 10 mm, 0.70 rev/s, 120 kV, 68 mAs per slice, and field of view = 500. Density values used will range from 30–100 HU. Quadriceps mCSA will be determined using computerized planimetry through the 'freehand region of interest (ROI)' tool of the image analysis software Isite PACS 4.1 (Philips Healthcare Informatics, 4100 East Third Ave., Suite 101 Foster City, CA, USA).

Rectus femoris and vastus lateralis muscular cross-sectional area

Rectus femoris and vastus lateralis mCSA will be assessed by a B-mode ultrasound with a 7.5-MHz linear-array probe (LOGIQ e PRO – GE Healthcare, Chicago, IL, US) as previously described (71). Similarly to CT scans, images will be obtained at 50% of the segment. This anatomical point will be transversally marked every 2 cm with semipermanent ink and used as a reference for the probe displacement. Sequential images will be acquired, aligning the superior edge of the probe with each mark on the skin following a middle-to-lateral direction. To avoid pressure on the muscle tissue, which could result in image distortion, a generous amount of conductive gel will be used. After the data collection, the muscle images will be reconstructed in PowerPoint (Microsoft, Redmond, WA, USA). mCSA will then be measured using computerized planimetry (i.e., by contouring following the muscle fascia using an 800-dpi mouse) (Madena 3.2.5; Eye-

Physics, Los Alamitos, CA, USA). The planimetry software (ImageJ, NIH, USA) will be calibrated with fixed distance scales displayed in the US images. A single investigator, blinded to the participants' group assignment, will perform the analyses.

Visceral and subcutaneous fat areas

Visceral (VAT) and subcutaneous adipose tissue (SAT) areas will be measured by CT scans (Discovery CT 750HD, GE Healthcare, Chicago, IL). Similarly, the same blinded specialized radiology technician will acquire the images at PRE and POST under medical supervision and process them using the GE Healthcare workstation. First, an image will be initially obtained to determine the umbilical scar, which will allow the delineation of total and visceral adipose tissue areas (72).

VAT will be assessed using the following procedures: (i) the technician will manually draw a line below the skin to obtain the total adipose tissue, which will be defined as the sum of adipose tissue hounsfield units (HU) inside this line, and (ii) VAT will be segmented by drawing a line, which begins at the abdominal linea alba, bisects the rectus abdominis, the internal oblique, the iliacus, and laterally around the peritoneum surrounding the vertebral body to join at the midline anterior to the vertebral body. All HU inside this line that meet the criteria for adipose tissue X-ray density will be counted as visceral adipose tissue (73).

Abdominal SAT area will be determined by the difference between VAT and total abdominal fat, by using computerized planimetry through the 'freehand ROI' tool of the image analysis software Isite PACS 4.1 (Philips Healthcare Informatics, 4100 East Third Ave., Suite 101 Foster City, CA, USA). Image acquisition parameters will be as follows: slice thickness = 10 mm; table increment = 10 mm, 0.70 rev/s, 120 kV, 68 mAs per slice and field of view = 500. Density values used will range from −30 to −190 HU.

Muscle biopsies

Prior to tissue extraction, the area will be cleansed with an antiseptic solution. Subsequently, the selected site will be anesthetized with a subcutaneous injection of 3 mL of 2% lidocaine. Following local anesthesia, a small incision (~5 cm) will be made down to the muscle fascia using a surgical scalpel. A Bergström biopsy needle will then be inserted into the lateral portion of the vastus lateralis at a depth of approximately 4 cm, adjusted based on the thickness of subcutaneous fat (74). Suction will be applied to the

outer end of the needle using a 120 mL syringe, allowing a small muscle tissue sample to be excised by the needle's internal blade (75). After the needle is withdrawn, pressure will be applied to the incision site to prevent bleeding, and the incision will be closed and covered with sterile bandages (76).

Immediately after extraction (~150 mg), visible adipose or connective tissue fragments will be carefully removed under a standard dissection microscope (Quimis Q7740SZ, Quimis, Brazil). Aliquots will then be separated for subsequent analyses. Following separation, the samples will be rapidly frozen in liquid nitrogen and stored at −80 °C until further examination. For immunofluorescence assays, a small fragment (~20 mg) will be embedded in optimal cutting temperature (OCT) medium (Tissue-Tek, Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) with muscle fibers oriented transversely. These fragments will be rapidly frozen, initially in isopentane cooled with liquid nitrogen, and subsequently immersed in liquid nitrogen.

Assessment of cross-sectional area, myonuclei count, myonuclear domain, capillarization, and satellite cell content

Immunofluorescence techniques will be employed to assess the muscle fiber cross-sectional area (CSA), the number of myonuclei, satellite cells, capillaries, and myonuclear domain. Seven-micrometer transverse sections will be prepared from OCT-embedded samples, brought to room temperature, and fixed in chilled methanol for 10 minutes. The sections will then be washed and blocked for 60 minutes in phosphate-buffered saline containing 5% goat serum and 0.3% Triton X-100.

For quantification, the sections will be incubated with primary antibodies against MHCI, Laminin, Pax7, and CD31, followed by appropriate secondary antibodies. Nuclei will be visualized using a fluorescent mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Abcam, Mounting Medium With DAPI - Aqueous, Fluoroshield, ab104139), and coverslips will be applied to the slides.

Images will be captured with an Olympus BX51 Fluorescence microscope with a magnification of ×200. A minimum of 120 fibers will be analyzed for CSA, myonuclei count, myonuclear domain, and satellite cells, as recommended by Mackey et al. (2009) (77). For capillarization assessment, a minimum of 50 fibers will be evaluated following Hepple et al. (1997) (78). Parameters analyzed will include the capillary count (CC, the number of capillaries surrounding a fiber), the capillary-to-fiber ratio (C/Fi), and the

capillary index per fiber perimeter. All images will be analyzed by a single researcher in a blinded manner.

Bone health

Bone microarchitecture

Bone microarchitecture [volumetric bone mineral density (vBMD) and structural parameters of trabecular and cortical bones] will be assessed at the distal region of the radius of the non-dominant limb using High-Resolution Peripheral Quantitative Computed Tomography (HR-pQCT), in accordance with previously established guidelines (79). Specifically, the following parameters will be obtained: total vBMD; trabecular vBMD, cortical vBMD, trabecular bone volume fraction, trabecular number, trabecular thickness, trabecular separation, cortical thickness (Ct.Th), diameter and relative cortical porosity (Ct. Po), and stiffness and failure load of the finite element (FE).

During the scan, the participant's limb will be immobilized in a carbon fiber shell. The procedure allows the simultaneous acquisition of approximately 110 parallel slices with a resolution of 82 μm, corresponding to a 9.02 mm section along the axial direction, starting 9.5 mm proximal to the reference line, which will be defined as the region of interest (ROI) for data evaluation. All HR-pQCT measurements and analysis will be performed by the same experienced evaluator, blinded to the participants' experimental group. All images will be acquired using the following settings: effective energy of 60 kVp, X-ray, tube current of 95 mAs, and matrix of 1536 x 1536. Quality control will be monitored through daily examinations of a Phantom standard, which contains hydroxyapatite sticks (HA) embedded in a resin fabric (QRM, Moehrendorf, Germany). Test-retest coefficient of variation (CV) in our laboratory is 0.93%-1.41% for volumetric bone mineral density measurements, and 1.49-7.59% for structural parameters (80).

For morphological analyses, except for cortical parameters, the standard analysis method will be used. The technician will perform a semi-automatic contouring process slice by slice to identify the periosteal limit of the bone, thus extracting the bone region of the surrounding soft tissue. The bone region will be extracted using a Laplace-Hamming filter (ε 0.5, cut-off 0.4, respectively) and a global cut-off threshold of 400 permille (‰) to generate the segmented bone volume. This method will be used to perform the morphological analysis [81]. Due to limitations in the standard method (e.g.,

insufficient extraction of the cortex, which may be thin and/or highly porous, or when the trabecular structure is rich and well connected to the cortex), the advanced analysis method, also known as the extended analysis, will be used for the determination of cortical parameters (cortical vBMD, Ct.Th, and Ct. Po) (79).

For the analyses of volumetric density and structural parameters, the analysis of FE models of the distal radius will be carried out based on images obtained directly from HR-pQCT, with the aim of evaluating the biomechanical parameters of the bone and, therefore, estimate tissue susceptibility to fractures. For FE analysis, the software provided by the equipment manufacturer will be used (Finite Element Software, v.1.13, Scanco Medica AG, Switzerland, January 2009, Manufacturer's Guide). The vector information obtained from the models will be converted into blocks called voxels, which comprise the smallest unit that makes up the image of the analyzed material and have a cubic format. In FE analysis, the software will estimate and analyze the behavior of bone tissue when it is subjected to a force along its axis.

Bone mineral content and areal bone mineral density

Bone mineral content (BMC) and areal bone mineral density (aBMD) will be assessed by whole-body DXA using a Hologic QDR 4500A densitometry equipment (Discovery Densitometer, Hologic Inc., Bedford, MA, USA). The aBMD will be measured at the following sites: lumbar spine (L1 to L4), femoral neck, total hip, and whole body in accordance with the recommendations from the International Society for Clinical Densitometry (81).

Strength and functionality

Isometric strength

Isometric handgrip strength will be obtained using a handgrip dynamometer (Jamar®, Sammons Preston Rolyan, USA). The test will be conducted with the participant seated, shoulders adducted, and in a neutral rotation with their elbow bent at a 90° angle (82). They will perform three maximum effort attempts, each lasting 5 seconds, with a 1-minute rest interval between each attempt. The best result will be recorded for analysis.

Isoinertial strength

The maximal amount of weight that can be lifted in a single repetition will be measured using the one-repetition maximum (1RM) strength test, following widely used and well-established recommendations (83). The test will be performed in the inclined leg-press 45º (Nakagym, São Paulo, Brazil) and bench-press machines (Nakagym, São Paulo, Brazil) for lower body and upper body strength assessments, respectively. The same testing protocol will be followed for both machines. Briefly, participants will complete a 5-minute general warm-up on a treadmill at 3 km·h-1, followed by a specific warm-up consisting of one set of eight repetitions at 50% of their estimated 1RM and one set of three repetitions at 70% of their estimated 1RM, with a 1-minute rest between sets. After that, participants will have up to five attempts to achieve their 1RM, with a 3-minute rest between each attempt. The 1RM will be considered the higher load at which the participant can perform a full eccentric-concentric movement through a 90° range of motion. Importantly, before the baseline assessments, participants will perform two familiarization sessions spaced at least 72 hours apart. If the variation between familiarization and testing sessions is higher than 5%, which is acknowledged as the normal interday variation in strength assessments (84, 85), an additional testing session will be performed respecting the same time interval between sessions.

Functional capacity

Functional capacity will be measured through the 30-s sit-to-stand test, the timed-up-and-go test, and the short physical performance battery (SPPB) (86-88). These tests are widely used to evaluate functional status across various clinical (89-91), including older adults and frail participants (86, 92), due to its ease of application. They are considered valid instruments and present excellent reliability (93).

The 30-s sit-to-stand test will evaluate the maximum number of stands that an individual can perform from a standard-height armless-chair (i.e., 45 cm) in 30 seconds, whereas the timed-up-and-go test will register the minimal time (in seconds) required to rise from a standard chair, walk to a line on the floor 3 meters away, turn around, and sit back down again. Before the test, the investigator will verbally explain and demonstrate how to perform each test correctly. Participants will then practice each test once to

familiarize themselves. A 1-minute rest will be provided between familiarization and testing. Then, two new attempts will be allowed for each test. All tests will be conducted by the same investigator to avoid bias.

The SPPB consists of a static standing balance test, habitual gait speed, and lower limb muscle strength (assessed through an adapted timed-stands test) (92). For the balance test, the participant must be able to maintain each position (i.e., side-by-side, semi-tandem stand, tandem stand) for 10 s. The score is zero for participant who are unable to maintain balance in the first position for 10 s. If the participant can stay in the first position for 10 s but cannot maintain the second position for 10 s, the score is 1 point. A score of 2 is assigned to participants who can remain in the second position for 10 s but cannot sustain the third position for more than 3 s. A score of 3 is assigned to participants who can remain in the third position for 3 to 9 s, and the maximum score (4 points) is given if they can stay in the third position for 10 s. For the gait speed test over a distance of 4 m, a score of zero is assigned to participants unable to complete the test. A score of 1 is given to participants who complete the course in more than 8.70 s (i.e., 0.46 m/s); a score of 2 for a time between 6.21 to 8.70 s (i.e., 0.47 to 0.64 m/s); a score of 3 for a time between 4.82 to 6.20 s (i.e., 0.65 to 0.82 m/s); and the maximum score, 4 points, for a time less than 4.82 s (i.e., 0.83 m/s). In the timed-stands test, the participant must sit and stand from a chair 5 times consecutively as quickly as possible. A score of zero is given when the participant cannot perform 5 repetitions. A score of 1 is given for a time greater than 16.7 s; a score of 2 for a time between 13.7 to 16.6 s; a score of 3 for a time between 11.2 to 13.6 s; and the maximum score, 4 points, for a time less than 11.1 s (92). The total SPPB score is calculated by adding the scores from each test, with individual test scores ranging from zero (worst performance) to four points (best performance); the highest possible SPPB total score is 12 points.

Cardiopulmonary assessments

Pulmonary function

Forced vital capacity (FVC), forced expiratory volume in the first second (FEV1), FEV1/FVC ratio, forced mid-expiratory flow, and both peak inspiratory and expiratory flow will be evaluated through a pulmonary function test (PFT). The PFT will be conducted in the upright position, without the use of bronchodilators, following

established recommendations (94, 95). The system (Cortex, Germany) will be calibrated immediately before each test following the manufacturer's specifications.

Cardiorespiratory fitness

Classical cardiorespiratory fitness-related variables, including measured and percent-predicted values for oxygen uptake at peak (V̇O2peak) and at ventilatory threshold (V̇O2VT), oxygen pulse (V̇O2/HR), respiratory exchange ratio (RER), and HR responses will be assessed during a maximal graded cardiopulmonary exercise test (CPX) and recovery. Pulmonary ventilation (V̇E), breathing reserve [V̇E-to-maximum voluntary ventilation ratio (V̇E/MVV)], ventilatory equivalent for carbon dioxide (V̇E/VCO2), and the end-tidal carbon dioxide pressure (PETCO2) will also be evaluated.

CPX will be performed on a treadmill (Centurion C200, Micromed, Brazil) using an individualized modified Balke protocol to the limit of tolerance (95). All tests will be conducted in the same intrahospital laboratory under controlled room temperature (20-23°C). The system will be calibrated immediately before each test using standard calibration gases (12% O2, 5% CO2, and balanced in N2) and a 3-L calibration gas syringe (Cortex, Germany) following the manufacturer's specifications. Gas exchange will be assessed breath-by-breath by continuous sampling using a facemask coupled to a gas analyzer (Metalyzer 3B, Cortex, Germany). Ventilatory parameters will be measured by a turbine (Cortex, Germany) with a volume transducer (flow sensor). Outlying values (greater than ± 2 SD from the mean rolling averages) will be automatically identified and excluded by the analytic software (MetaSoft Studio, Cortex, Germany). Background noise will be smoothed from the breath-by-breath data by using a 30-second rolling average. HR will be continuously recorded beat-by-beat from the R-R interval using a 12-lead electrocardiograph. (ErgoPC Elite, Micromed, Brazil).

Dietary assessments

Dietary parameters, including caloric intake and macronutrient and micronutrient content, will be determined using a specific software based on the USDA (United States Department of Agriculture) food database (Nutritionist Pro® v.7.3, Axxya Systems, Woodinville, WO, USA). Initially, a trained dietitian will provide oral and written

instructions on how to complete 3-day food records (two from non-consecutive weekdays and one from a weekend day), including guidance on timing and estimation of portion sizes using household measures. All 3-day food records will be individually reviewed in-person through structured interviews with the same trained dietitian using the USDA Automated Multiple-Pass Method, which is a standardized validated method that includes five memory cues (1: quick list; 2: forgotten foods list; 3: time and occasion; 4: detail and review; and 5: final probe) to avoid recording errors and ensure comprehensive recall of all possible foods consumed. (96). This double-checking strategy will be implemented in order to reduce recall bias and enhance data collection accuracy. During interviews, portion size aids will be further clarified using food booklets with household measures (97, 98) and real-size food pictures (99).

Biochemical analyses

All blood-related biochemical analyses will be carried out in an accredited laboratory at our Clinical Hospital. Initially, blood samples (15 mL) will be collected between 08:00 and 10:00 hours, after a 12-hour overnight fast from the median or cephalic basilic vein. Samples will be processed and stored at −80° C until analysis.

Lipid profile

Lipid profile, including triglycerides, total cholesterol and its subfractions (i.e., VLDL-C, LDL-C, HDL-C, non-HDL-C), will be evaluated via colorimetric enzymatic methods.

Glycated hemoglobin

Glycated hemoglobin will be assessed via high-performance liquid chromatography performance by ion exchange as a surrogate marker of long-term blood glucose levels.

Oxidative Stress markers

Enzymatic antioxidant assays for catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST) will be performed on plasma samples centrifuged at 10,000 g for 10min at 4°C. CAT activity will be measured using Fossati et al.'s modified method, where H_2O_2 is dismutated into water and oxygen, followed by a peroxidase-catalyzed reaction with o-dianisidine. SOD activity will be evaluated using a colorimetric assay kit (Sigma Aldrich). GPx and GR activities will be determined by tracking NADPH oxidation at 340nm, as previously described (100). GST activity was measured by GSH catalysis and CDNB complex formation. Lipid peroxidation was assessed through malondialdehyde levels, following the TBARS method described by Conti et al. (101)..

Gut hormones and inflammatory markers

Gut hormones [ghrelin, gastric inhibitory peptide (GIP), pancreatic polypeptide (PP) and peptide YY (PYY)], inflammatory markers, including pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-10, IL-1ra, IL-1B, and IL-4) will be assessed using Luminex xMAP technology, except for C-reactive protein which will be assessed via an immunoturbidimetric assay.

Bon-related parameters

Bone turnover markers, including Collagen type I C-telopeptide (CTX) and procollagen type I N-propeptide (P1NP), will be measured by automated electrochemiluminescence methods following recommendations of the International Osteoporosis Foundation and International Federation of Clinical Chemistry (102). Serum levels of 25-hydroxyvitamin D, intact PTH, total and ionized calcium, will be determined using chemiluminescent immunoassay, electrochemiluminescence, enzymatic colorimetric assay, and ion-selective electrodes methods, respectively.

Oral glucose tolerance test (OGTT)

To assess insulin sensitivity and glucose metabolism, a 2-h oral glucose tolerance test will be conducted. Blood samples will be collected at 0 (i.e., after a 12-h overnight fast), 30, 60, 90, and 120 minutes following the ingestion of a 75 g glucose bolus.Serum

glucose levels will be measured using a colorimetric enzymatic assay, insulin levels will be quantified using a human-specific radioimmunoassay, and C-peptide levels will be determined through a chemiluminescent immunometric assay. Area under the curve (AUC), along with the Matsuda index (103), homeostatic model assessment-insulin resistance (HOMA-IR) and homeostatic model assessment of β-cell function cell function (HOMA-B) (104) will be calculated.

Telomere length

Particularly for telomere length (TL) analysis, blood samples will be collected using ethylenediaminetetraacetic acid (EDTA) tubes and stored at -80ºC until analysis. DNA will be extracted using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's instructions. DNA will be quantified using the NanoVue Plus spectrophotometer (General Electric), and its integrity will be evaluated via gel electrophoresis technique (105, 106). TL analysis will be measured in accordance with the modified protocol described by Cawthon and colleagues (107). The relative average of telomeres will be quantified via Real-Time Polymerase Chain Reaction (RT-qPCR) technique. RT-qPCR reactions will be conducted in 96-well plates using the StepOnePlus™ Real-Time PCR System (Applied Biosystems®). All samples and reference DNA will be analyzed in duplicates using 25 ng of DNA. Three wells for internal control will be included in each run. For the reaction, the SYBR® Green PCR Mastermix kit (PureLink™) will be used with a final volume of 25 µL. The specific primer sequences used for telomeres are presented in Table 2. The 36B4 reaction will include 12.5 µL of SYBR Green (PureLink™), 0.03 µL of forward primer, 0.05 µL of reverse primer, and 11.42 µL of ultrapure water. For the telomere reaction, 12.5 µL of SYBR Green, 0.03 µL of forward primer, 0.03 µL of reverse primer, and 11.44 µL of ultrapure water will be used. The RT-qPCR run will consist of four distinct steps: first, an initial step at 50°C for 2 minutes. Second, 40 amplification cycles at 95°C for 2 minutes each. Third, a step at 55°C for 15 seconds. Finally, a step at 60°C for 1 minute.

Table 2. Primer sequences and concentrations for telomere and 36B4 gene amplification in RT-qPCR Assay

Primer	Sequence (5' -> 3')	Concentration (nM)
Tel 1 Forward	GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T	300
Tel 2 Reverse	TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA	300
36B4u Forward	CAG CAA GTG GGA AGG TGT AAT CC	300
36B4d Reverse	CCC ATT CTA TCA TCA ACG GGT ACA A	500

The relative quantification of TL will be determined using the telomere to single-copy gene ratio (T/S) by calculating the delta values (Δ) of cycle threshold (Ct) [Ct (telomeres) - Ct (reference gene)]. Following the parameters previously described (108), the ratio of T/S for each sample will be calculated using the equation:

$$2 - \Delta\Delta CT \;=\; 2 - \Delta CT(a\ target\ sample) - \Delta CT(a\ reference\ sample)$$

Health-related quality of life

Health-related quality of life (HRQoL) will be evaluated through the Medical Outcomes Study 36 – Item Short-Form Health Survey (SF-36) (109). The SF-36 provides scores across eight domains: physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health. Additionally, physical and mental component summary scores will be calculated. Scores will range from 0 to 100, with lower scores indicating worse HRQoL.

Sleep quality

Sleep quality will be assessed using the Portuguese version of the Pittsburgh Sleep Quality Index (PSQI) (110). In brief, the PSQI assesses sleep quality over a 1-month period by means of a questionnaire involving 19 self-rated questions and 5 questions answered by bedmates/roommates. The latter questions are used only for clinical information, and thus, will not be used in the current study. The 19 questions are categorized into 7 components, graded on a score that ranges from 0 to 3. The PSQI

components are as follows: subjective sleep quality (C1), sleep latency (C2), sleep duration (C3), habitual sleep efficiency (C4), sleep disturbances (C5), use of sleeping medication (C6) and daytime dysfunction (C7). The sum of scores for these 7 components will yield one global score, which will range from 0 to 21, in which the highest score indicates worst sleep quality. Participants will be classified as good sleepers in case of PSQI ≤ 5 and poor sleepers for PSQI > 5.

Depression and anxiety

Depression will be assessed using the short form of the Geriatric Depression Scale (GDS) with 15 items (GDS-15), which is specifically designed for screening depression in older adults (111). This shortened version offers very similar sensitivity and specificity compared to the original 30-item scale (112). Each item on the abbreviated scale will be scored as either 0 or 1, resulting in a total score range from 0 to 15. A cutoff score between 5 and 6 will be used to differentiate between non-cases and cases of depression.

Anxiety will be assessed using the Geriatric Anxiety Inventory (GAI), a self-administered tool comprising 20 questions with dichotomous responses (agree/disagree) (113). This brief questionnaire will be easy to use even in cases of fatigue, lower education levels, or mild cognitive impairment. The GAI will employ a cutoff score system where scores between 0 and 10 will indicate absence of anxiety, scores between 11 to 15 will denote mild to moderate anxiety, and scores between 16 to 20 will be suggestive of severe anxiety.

Ccognitive processing speed

Cognitive processing speed will be assessed utilizing the Trail Making Test (TMT). The most widely employed version of the TMT comprises parts A and B. In part A, the subject utilizes a pencil to connect a series of 25 encircled numbers in numerical order. In part B, the subject connects 25 encircled numbers and letters in numerical and alphabetical order, alternating between the numbers and letters. For instance, the first number "1" is followed by the first letter "A," followed by the second number "2" then second letter "B" and so forth. The numbers and letters are positioned in a semi-random fixed order, in such a manner as to prevent overlapping lines being drawn by the

examinee. The primary variables of interest are the total time to completion for parts A and B. A cutoff time of 300 s is generally employed to discontinue test administration and is therefore the typical maximum score (114).

Cognitive executive function

Cognitive executive function will be assessed by Stroop Test (ST) (Victoria version). The ST will follow the guideline specifications suggested by Duncan 2006 (115). Three cards, each containing 24 stimuli against a white background, will be used. Card A will consist of rectangles printed randomly in green, pink, blue, and brown. Card B will be organized similarly to Card A but will have rectangles replaced by unrelated words to concepts of color (each, never, today, and all) printed in uppercase in the four colors mentioned. Card C will also be organized similarly to Card A, representing the interference card where the written stimuli will be the names of the colors (brown, blue, pink, and green) printed in the same colors, ensuring that the ink color and the color name never match (e.g., the word "brown" printed in pink, green, or blue). For the first card, participants will state the colors of the rectangles as quickly as possible. For cards B and C, subjects will state the color of the printed words instead of reading the words themselves. The criterion score will be the time taken to perform the task for each card, and all errors will be corrected promptly without stopping the chronometer (116).

EXPERIMENTAL INTERVENTIONS

Caloric restriction and dietary supplementation

Participants allocated to the intervention groups (PTN and PLA) received individual meal plans to induce a caloric deficit of 300-500 kcal/day from their habitual energy intake assessed at baseline. Habitual intake values were contrasted with total energy requirements individually estimated by FAO/WHO equation for older adults, applying a sedentary activity factor at baseline and an active factor for later time points (differences between calculation methods were <1% throughout the study). The caloric deficit was maintained based on regular reassessment of dietary parameters every four weeks. In addition, participants met biweekly with registered dietitians for individual

behavioral therapy involving nutritional education in order to enhance adherence to the caloric restriction protocol.

Participants from PTN and PLA will be provided, on a weekly basis, with unlabeled dietary supplement bottles to be consumed daily at breakfast. The PTN group will receive 40g of whey protein (Nutrata®, São Paulo, Brazil), while the PLA group will receive an isocaloric non-nitrogenous placebo (Nutrata®, São Paulo, Brazil), consisting of a carbohydrate blend (isomaltulose, waxy maize, maltodextrin), with the same flavor (natural chocolate) and texture. They will be asked to record their supplement intake (time and day) and return the bottles (empty or not) weekly to monitor adherence. To ensure a daily intake of 1500mg of calcium and 1000IU of vitamin D, calcium and vitamin D supplements will also be provided to prevent deficiencies associated with caloric restriction, as previously recommended (117).

The research team will be available to answer any questions about the supplementation protocol throughout the trial.

Exercise training intervention

Participants from both PTN and PLA groups will engage in a three-times-a-week exercise training program for 16 weeks. The program will include resistance and endurance exercises. Resistance training will consist of six exercises targeting major muscle groups: incline leg press 45°, leg extension, leg curl, calf raise, horizontal bench press, and lat pull-down. Endurance training will involve walking and/or running. The exercise training protocol's periodization will follow recommendations from widely accepted position stands for older adults (118, 119) (Table 3). For each exercise, resistance training will progress from 1 set of 15-20 repetition maximum (RM) in the first week to 3 sets of 10-12 RM in the final four weeks of training. Whenever a participant exceeds the upper limit of the repetition range for two consecutive sets, the intensity will be adjusted by increasing the exercise load. Similarly, endurance exercise will progress from 30 minutes at 55–65% of heart rate maximum (HRmax) during the first four weeks to 40 minutes at 70–80% HRmax during the last four weeks of training. Borg's Rating of Perceived Exertion (RPE) scale (120) will be used as an auxiliary tool for monitoring exercise intensity and training progression. In these cases, the targeted intensity will range from 'very light'/'fairly light' (Borg's RPE score 9–11 a.u.) to 'somewhat hard'/'hard'

(Borg's RPE score 13–15 a.u.). All training sessions will be supervised by an experienced exercise physiologist blinded to the participants' group assignment. A training log will be maintained for each session to monitor adherence and control the training load.

Table 3. Exercise training prescription.

Type of exercise	Weeks	Volume	Intensity	Borg's RPE
Resistance training	1	1 set	15-20 RM	NA
	2-4	2 sets	15-20 RM	NA
	5-8	3 sets	15-20 RM	NA
	9-12	3 sets	12-15 RM	NA
	13-16	3 sets	10-12 RM	NA
Endurance training	1-4	30 min	55-65% HR_{max}	9-11 a.u.
	5-8	35 min	55-65% HR_{max}	9-11 a.u.
	9-12	40 min	60-70% HR_{max}	11-13 a.u.
	13-16	40 min	70-80% HR_{max}	13-15 a.u.

Abbreviations: RM: repetition maximum, HR_{max}: maximum heart rate, RPE: rating of perceived exertion, a.u.: arbitrary units, NA: Not applicable.

To address the higher prevalence of comorbidities in this population, additional precautions will be implemented. All participants will have their resting blood pressure measured by an automatic monitor (SPACELABS, São Paulo, Brazil) immediately before each training session, and sessions will be suspended if systolic or diastolic blood pressure readings are ≥160 mmHg or ≥105 mmHg, respectively (121, 122). Likewise, blood glucose levels will be measured before training sessions for participants with type-2 diabetes, with acceptable values considered to be between 90 and 250 mg/dL (123). Additionally, heart rate (HR) and peripheral oxygen saturation (SpO2) will be continuously monitored during endurance exercise. Whenever necessary, participants will receive outpatient care and consultation with our medical staff.

The CON group will receive one-on-one guidance and behavioral orientations focusing on a healthy lifestyle, including nutritional education and physical activity at PRE, and every four weeks throughout the trial.

SAMPLE SIZE CALCULATION

Sample size was determined a priori using the G-Power software (Version 3.1.9.2, University of Kiel, Germany). The analysis was conducted by inputting α error (0.05), power (1 - β error = 0.95), and effect size (Hedges' g = 0.61), considering previous data indicating a lower loss of fat-free mass in individuals undergoing caloric restriction who had an increase in protein intake compared to their counterparts who maintained their usual protein intake (31). Calculations were based on an ANOVA with repeated measures (within-between interactions) and the total sample size was determined to be 81 individuals. To account for midtrial withdrawals, the sample will be increased by ~30% (i.e. 105 participants).

DATA ANALYSIS

Data will be presented as mean ± standard deviation (SD), estimated mean difference between groups (EMD) at POST (only in the presence of group x time interaction), 95% confidence interval (95%CI), unless otherwise indicated. Baseline characteristics will be compared between groups using either Fisher exact test or generalized linear model with Sidak post hoc adjustment whenever necessary. Data will be analyzed using an intention-to-treat (ITT) approach to preserve the integrity of randomization. Before inferential analysis, outliers will be identified, and the data will be tested for homogeneity of variance and sphericity. Dependent variables will be analyzed by a linear mixed model analysis for repeated measures (assuming time [PRE and POST] and group [PTN, and PLA] with baseline values as covariate. The restricted maximum likelihood method, compound symmetry covariance structure, and Kenward-Roger degrees of freedom approximation will be applied for all models. In order to maintain the overall significance level at 0.05, the primary endpoints will be assessed using Hochberg's procedure (124). For all secondary outcomes the significance level will be set at $p \leq 0.05$. Whenever significant group-by-time interactions are identified, post-hoc tests on the adjusted predictions with Sidak's adjustment will be performed for multiple comparisons.

All analyses will be performed in the statistical environment R (version 3.5.3; R Core Team 2020).

STATISTICAL ANAYSIS PLAN

All analyses will be performed in the statistical environment R (version 3.5.3; R Core Team 2020). We will perform all the comparisons using two-sided significance tests. Intention-to-treat analyses will be performed by analyzing data from all participants originally randomized to preserve the integrity of randomization. The longitudinal analysis of all outcomes will be performed using a linear mixed model for repeated measures, assuming 'group' (PTN, and PLA) and 'time' (pre-intervention and post-intervention) as fixed factors, and 'subjects' as a random factor, with an interaction between 'group' and 'time' added to the model; baseline values of the outcome will be used as covariates. For all models, maximum restricted likelihood and Kenward-Roger degrees of freedom will be used for model fit and estimation. We will adjust for testing of the four primary outcomes, total fat-free mass, appendicular fat-free mass, appendicular fat-free mass relative to body mass index, and leg fat-free mass, using Hochberg's procedure in order to maintain the overall significance level at 0.05. Since the other hypothesis tests (for the secondary outcomes) will be more exploratory in nature, the Hochberg's correction will not be used, and two-sided p-values less than 0.05 will be considered to indicate statistical significance. For both primary and secondary outcomes, whenever significant group-by-time interactions are identified, post-hoc tests on the adjusted predictions with Sidak's adjustment will be performed for multiple comparisons.

Mixed model analyses will be used instead of standard repeated measures analysis of variance because of the likelihood that there will be dropouts and missed visits that preclude the use of the classical approach (125, 126). The mixed model approach we will employ fits a linear regression model to each subject and assumes that both error terms and regression coefficients are normally distributed in these lines. It then uses the EM algorithm to combine the individual lines and to generate empirical Bayes, maximum likelihood, and restricted maximum likelihood estimates of model parameters. Because lines are generated for individuals, the model is more general than standard repeated measures analysis of variance in that it has no requirements about common measurement times between subjects and can deal easily with missing data. In all analyses, careful attention will be given to ensuring that the conditions that are required of a particular statistical method are satisfied. We will assess the normality of regression residuals and ensure the homogeneity of variances when analyzes of variance and covariance are performed. When required assumptions are not satisfied, data transformations will be explored and utilized as appropriate.

STUDY TIME LINE

July 2021 – August 2023

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