EFFECTS OF 12 WEEKS OF ESSENTIAL AMINO ACIDS (EAA)-BASED MULTI-INGREDIENT NUTRITIONAL SUPPLEMENTATION ON MUSCLE MASS, MUSCLE STRENGTH, MUSCLE POWER AND FATIGUE IN HEALTHY ELDERLY SUBJECTS: A RANDOMIZED CONTROLLED DOUBLE-BLIND STUDY

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Abstract: *Objective:* To counteract muscle mass, muscle strength and power loss during aging, and to study age-related change of neuromuscular manifestation of fatigue in relation to nutritional supplementation. *Design:* randomized controlled double-blind study. *Setting:* Twice-daily consumption for 12 weeks of an Essential Amino Acids (EAA)-based multi-ingredient nutritional supplement containing EAA, creatine, vitamin D and Muscle Restore Complex®. *Participants:* 38 healthy elderly subjects (8 male, 30 female; age: 68.91±4.60 years; body weight: 69.40±15.58 kg; height: 1.60±0.09 m) were randomized and allocated in supplement (SUPP) or placebo (PLA) group. *Mean Measurements:* Vitamin D blood level; Appendicular Lean Mass (ALM); Visceral Adipose Tissue (VAT); Maximal Voluntary Contraction (MVC) and Peak Power (PP); myoelectric descriptors of fatigue: Fractal Dimension and Conduction Velocity initial values (FD iv, CV iv), their rates of change (FD slopes, CV slopes) and the Time to perform the Task (TtT). *Mean Results:* Significant changes were found in SUPP compared to baseline: Vitamin D (+8.73 ng/ml; p<0.001); ALM (+0.34 kg; p<0.001); VAT (-76.25 g; p<0.001); MVC (+0.52 kg; p<0.001); PP (+4.82 W; p<0.001). Between group analysis (SUPP Vs. PLA) showed improvements: vitamin D blood levels (+11,72 ng/ml; p<0.001); Legs FFM (+443.7 g; p<0.05); ALM (+0.53 kg; p<0.05); MVC (+1.38 kg; p<0.05); PP (+9.87 W; p<0.05). No statistical changes were found for FD iv, CV iv, FD and CV slopes and TtT, either compared to baseline or between groups. Significant correlations between mean differences in SUPP group were also found. *Conclusion:* The study demonstrates that in healthy elderly subjects an EAA-based multi-ingredient nutritional supplementation of 12 weeks is not effective to change myoelectric manifestation of fatigue and TtT failure but can positively affect muscle mass, muscle strength, muscle power and VAT, counterbalancing more than one year of age-related loss of muscle mass and strength.

Key words: Alpha lipoic acid, coenzyme Q10, resveratrol, sarcopenia, muscle function.

Introduction

In order to attenuate muscle aging and prevent the development of sarcopenia's adverse consequences (1), studies have extensively explored physical activity interventions and nutrition strategies with interesting and promising data (2, 3). In particular, studies have evaluated the combined effects of resistance exercise training and dietary supplementation, such as protein/amino acids (4) and creatine (5), suggesting a potential additional effect to counter muscle mass and strength loss. However, during aging, medical conditions often prevent subjects from carrying out physical activity and the need for effective supplements *per se* becomes essential to slow down the progression of muscle mass and function loss. In this direction, some authors, using a single nutritional supplement (6-8), have shown a beneficial effect on neuromuscular performance and muscle protein synthesis in older adults, independently of exercise, but others could not observe

positive results (9, 10). To overcome this discrepancy, which is likely to be related to the heterogeneity of the response to supplementation in older subjects (11), several trials were conducted by employing a multi-ingredient approach (11-24), based on the rationale that a combination of ingredients could be more effective in regulating multiple aging-related relevant mechanisms than the use of single compounds (11). However, almost all the multi-ingredient studies included physical activity programs (11-21), while only few data (11, 22-24) come from protocols that investigated the effect of targeted nutritional supplements independently of combined physical intervention.

Therefore, in an effort to produce further advancement of knowledge regarding sarcopenia prevention by means of a multi-ingredient supplementation without physical exercise, we hypothesized that a twice-daily consumption of a mix containing Essential Amino Acids (EAA), creatine, vitamin D and Muscle Restore Complex® (MRC®: Alpha Lipoic Acid (ALA), Coenzyme Q10 (CoQ10), resveratrol)

for 12 weeks would result in the improvement of primary outcomes including Fat Free Mass (FFM), Appendicular Lean Mass (ALM), ALM index (ALM/H^2), muscle strength (Maximal Voluntary Contraction, MVC) and muscle power (Peak Power, PP), in non-sarcopenic well-nourished elderly subjects. Furthermore, since data on the possible effects of multi-ingredient supplementation on myoelectric descriptors of fatigue (peripheral and/or central) are completely lacking in aging literature, we evaluated whether the treatment can affect the surface electromyography (sEMG)-derived TtT failure, as a measure of endurance, and CV, FD (initial values and slopes), as a measure of peripheral and central myoelectric manifestations of fatigue, respectively, during submaximal isometric contractions (60% MVC) to exhaustion. Secondary outcomes we considered including vitamin D serum levels, Resting Metabolic Rate (RMR), Respiratory quotients of different substrates (R) and their fasting utilization rates (CHO%; FAT%), Fat Mass (FM) and Visceral Adipose Tissue (VAT).

Some of the ingredients we used have been shown to independently affect aspects of sarcopenia in elderly and thus have a rational basis for inclusion in a mixture: notably, protein/amino acids enhance lean mass and strength (25-27); creatine improves muscle strength and power (28, 29); vitamin D stimulates muscle function and reduce the risk of falls (30, 31). For ALA, CoQ10 and resveratrol, although there is a bulk of references of their use to counteract oxidative stress and inflammation in skeletal muscle *in vitro* models and animal studies (32-36), their therapeutic potential on muscle mass, muscle functions and metabolic outcomes during aging in humans is not well documented and needs to be further clarified.

Based on the above, the aim of this study was to evaluate the efficacy of an EAA-based multi-ingredient supplement on primary and secondary outcomes in the elderly, independently of exercise, comparing results to the few available studies and to increase the overall knowledge of how nutrients can affect muscle aging and sEMG-derived fatigue expression.

Materials and Methods

Study design and trial organization

A total of 50 healthy elderly individuals (aged 65-80 years) were initially identified, following which 38 eligible subjects were recruited in a randomized controlled design study and received either a multi-ingredient nutritional supplement (SUPP; 3 men and 16 women) or a placebo (PLA; 5 men and 14 women). Recruitment phase took place between November 2016 and January 2017. Potential participants were contacted first by enrollment meetings and then involved in a one-to-one interview at our medical facility. All potential participants completed a medical screening in February 2017, to determine the inclusion/exclusion criteria of enrollment. Habits regarding diet and physical activity were assessed through food and physical activity diaries.

All participants were informed of the nature and possible risks of the experimental procedures before their written informed consent was obtained. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Department of Internal Medicine and Medical Therapy at the University of Pavia with the code 001A0012.

The enrolled subjects ingested the supplement twice daily (two sachets) before lunch and dinner for 12 weeks: from the beginning of April 2017 to the end of June 2017. In all subjects experimental procedures and measurements were performed at the baseline (March 2017) and at the end of the study (July 2017). An accurate simulation of the experimental procedures to measure muscle strength, muscle power and fatigue was performed before the baseline. The simulation was conducted to allow the volunteers to familiarize themselves with all the procedures and to avoid an impairment of results caused by a "learning effect." Full details concerning the flow of participants through this study can be found in Figure 1.

In the previous 6 months, potential participants had not participated in any structured high-level resistance or aerobic training; they were instructed not to begin any exercise program and not to change their physical activity habits (based on daily life) for the duration of the study. Subjects were also instructed to avoid any other supplements or remedies to counteract sarcopenia during the entire duration of the study and until the end of the measurements (July 2017).

For each subject (SUPP and PLA) an appropriate diet plan was prepared to guarantee an average intake of protein of 1.2 g/kg of bodyweight/day, in accordance with the recommended amount of protein intake for healthy elderly people (37).

The equivalent amount of protein introduced by the supplement (~20 g/day) in SUPP was excluded from the average intake of protein recommended per day.

During treatment, weekly meetings based on self-report and questionnaires were held with the aim to ascertain compliance to treatment and to suggested nutrition and physical activity instructions. The study was conducted at CRIAMS-Sport Medicine Centre laboratory of the University of Pavia, located in Voghera.

Selection of population

Eligible subjects were aged 65 years or older. Subjects included in the study were not affected by acute illness, severe liver disease, heart disease, respiratory or kidney dysfunction, or severe dementia, and had a body weight that had been stable for 6 months. Moreover, subjects with uncontrolled diabetes, disthyroidism and other endocrinopathies, neoplasia, neuromuscular conditions, as well as patients treated with steroids, statins or other anti-sarcopenic supplements in the 6 months before the trial were excluded.

Sample size and random assignment

We based our sample size calculation on the findings of Bell et al. (11) and considered an expected mean of 0.4 kg increase of ALM in the SUPP group, and 0 kg in the PLA group, with a power of 80% and a level (2-tailed) of 5%, as well as 10% attrition. This gave a sample size of 40 patients (20/group). A random-blocks 1:1 random assignment list was prepared by a statistician.

Nutritional supplement

The supplement (a product of Laborest Italia S.r.l., Italy and manufactured by S.I.I.T. S.r.l., Italy) was composed of EAA, creatine, vitamin D and MRC® (ALA, CoQ10, resveratrol). All compounds in powder form were packaged in individual sachets and administrated to SUPP group. SUPP daily ingested two sachets prepared at home by mixing the contents of each sachet with 200 mL water and consuming as follows: first beverage during the morning/before lunch, and second beverage late in the afternoon/before dinner. The before-meal rationale for the supplement-feeding pattern was based on preliminary tests, targeted to avoid dislike of taste and gastrointestinal discomfort, which revealed that assumption of the mix during meals was generally not appreciated by the subjects.

The placebo administrated to PLA was composed of maltodextrine. Compared to the active forms, the placebo powder was isocaloric and undistinguishable for flavor, color and odor. All the sachets were labeled in a blinded manner and double blindness was accurately maintained during each step of the experimental design. The composition of the supplement is described in Table 1.

Table 1
EAA-based multi-ingredient supplement composition

Compounds	Mean quantity for single-dose/sachet
EAA	5000 mg
L-Leucine	1400 mg
L-Phenylalanine	600 mg
L-Lysine	700 mg
L-Isoleucine	670 mg
L-Valine	700 mg
L-Threonine	450 mg
L-Methionine	290 mg
L-Tryptophan	190 mg
Creatine (from creatine citrate)	1500 mg
Vitamin D	1000 UI
MRC®	
ALA	300 mg
CoQ10	50 mg
Resveratrol	50 mg

Notes: EAA, Essential Amino Acids; MRC®, Muscle Restore Complex®; ALA, Alpha Lipoic Acid; CoQ10, Coenzyme Q10.

Anthropometric and body composition assessment

Body weight and height were measured using a periodically calibrated scale equipped with a statimeter (SECA 700, SECA GmbH & co, Germany). Subjects were measured in light clothes (underwear) and without shoes, feet joined and parallel to each other with the head horizontally aligned to the Frankfurt plane. Body Mass Index (BMI) was calculated by the ratio between body weight and the square of height in meters.

Fat Free Mass (FFM), Fat Mass (FM), gynoid and android fat distribution (%) were measured with the use of Dual Energy X-Ray Absorptiometry (DXA) equipped with Lunar Prodigy DXA technology (GE Healthcare Medical Systems, USA). The *in vivo* coefficients of variations were 0.89% and 0.48% for FM and FFM, respectively (38).

Diagnosis of Sarcopenia

Appendicular Lean Mass (ALM) was taken as the sum of the fat-free soft tissue mass of arms plus legs and Appendicular Lean Mass index (ALM/H^2) was obtained by dividing ALM by height squared. ALM/H^2 cutoffs for men and women were then used to assess the condition of sarcopenia (39).

Diagnosis of Visceral Adipose Tissue (VAT) with Core Scan

Diagnosis of VAT were estimated within the android region. FM data from DXA core Scan was transformed into X-ray computed tomography (CT) adipose tissue volume using a constant correction factor (0.94 g/cm^3). FM, android fat and visceral fat data were derived from DXA using the DXA Prodigy enCORE software (version 17; GE Heathcare, USA). The software automatically places a quadrilateral box, that represents android region, outlined by the iliac crest and with a superior height equivalent to 20% of the distance from the top of the iliac crest to the base of the skull (40).

Blood sample measurement (Vitamin D)

For the assessment of 25-hydroxyvitamin D, fasting venous blood samples were drawn between 8 am and 10 am. Subjects were placed in a sitting position and the median cubital vein was used as a selected venipuncture site. Blood handling and collection were carried out under strictly standardized conditions. For the quantitative determination of vitamin D the chemiluminescent immunoassay technology was used.

Metabolic evaluation

Resting Metabolic Rate (RMR), Respiratory quotients of different substrates (R) and their fasting utilization rates (CHO%; FAT%) were measured by using a respiratory gas analyzer (Quark PFT, Cosmed, Italy). Ambient conditions were standardized (25 °C) and the analyzer was gas- and volume-calibrated each morning prior to the measurements, according to the recommendations stated in the manufacturer's user manual. Gas exchange and metabolic variables were measured continuously using the breath-by-breath method. After an

overnight fast, participants were instructed to lie down quietly for 10 min, wearing a two-way breathing mask covering their nose and mouth (V2 MaskTM, Hans Rudolph Inc, USA). Thereafter, the measurement period started by connecting the mask to the gas analyzer and data collection continued for a total of 20 min.

Isometric muscle strength and fatigue assessment with sEMG technique

sEMG recording procedure was carried out as follows: subjects' dominant upper limb was fastened in a isometric-ergometer (MUC1, OT Bioelettronica, Turin, Italy) fitted with a load cell (CCT Transducer, linear, full scale 100 kg), in order to isolate the action of the biceps brachii. Participants were sitting, with the elbow at 120 degrees.

A 64-channel bidimensional array (10 mm IED, 8 lines, 8 columns) was positioned between the distal tendon and the innervation zone of the biceps brachii, with electrode columns parallel to the orientation of the muscle fibers in order to have a pure propagation of motor unit action potentials. Biceps brachii was selected primarily to obtain high-quality sEMG signals due to the isolation of the muscle contraction, fluency of movement, and fiber orientation. The adhesive array was applied following muscle fiber leanings in correspondence to the muscle belly previously localized by ultrasound scan (Phillips CX-30). The sEMG signals were amplified (EMG-USB2+, OT Bioelettronica, Turin, Italy) and sampled at 2048 Hz.

Following 5 min rest, two isometric Maximal Voluntary Contractions (MVCs) were completed, separated by 2 min rest. Two contractions were performed in order to consider the highest MVC value. Participants were instructed to increase the force as maximum as they can, and to hold it as steady as possible, for 2–3 s. Participants were given verbal stimulation.

Following 2 min rest, a low intensity sustained contraction (20% MVC) was performed for 90 s.

Following 4 min rest, subjects were asked to execute a high level sustained contraction (60% MVC) until exhaustion, during which they were verbally stimulated to keep the force level as long as possible, until the force value decreased to below 5% of the target (41). At 60% of MVC, CV iv and FD iv (initial values), their slopes and the time to perform the task (TtT) were registered.

The sEMG signals were processed based on methodology recently described by our lab (42).

Muscle power assessment

Muscle power was measured by a force-velocity device analysis (MusclelabTM 4000, Ergotest Innovation A.S., Norway). Participants performed 3 tests of biceps curling (3 sets each, to choose the best execution) with a dumbbell (Technogym S.p.A., Italy) loaded at 30%, 40% and 50% of the 1 Repetition Maximum (1RM), respectively, connected to the device by a cable. A rest of 90 s between sets and

180 s between tests was held. Indirect 1RM tests to establish the dumbbell load (with the use of Brzycki's equation) were performed one week before the muscle power assessment. A week was considered appropriate to exclude any individual variability in relation to the time required for a complete muscle recovery and to resolve the Delayed Onset Muscle Soreness (DOMS) that could compromise the maximum speed of muscle contraction. Participants were instructed to execute each contraction as fast as they could and were given verbal stimulation. Muscle power values registered from each test were computed to create a force-velocity curve. Based on this curve the peak power (PP) value was obtained. Muscle power assessment was performed on dominant upper limb.

Statistical analyses

All analyses were performed using statistical package SPSS, version 21.0 (SPSS Inc., USA). Descriptive statistics representing raw data for each of the three categories and the full sample were provided, including means, standard deviations (sd), and frequencies, where appropriate. After the verification of the normal distribution of the continuous variables, data were analyzed as descriptive statistics. We carried out a paired t-tests and 95% Confidence Intervals (CI) to evaluate statistical significance on model parameters at baseline (supplement versus placebo). P-values <0.05 were considered significant.

Linear Mixed Model (LMM) for repeated measures (43) was applied to assess all differences for the variables considered among individuals at pre- and post-treatment (post-pre). These data were presented as mean differences with 95% CI.

Non-normally distributed data were checked by Shapiro-Wilk test and log transformed for parametric statistics.

For each outcome we fitted a LMM where age, sex, BMI and time (pre=0, post=1) were the explanatory variables. A random effect was used to adjust the models for intra-subject variability produced by two different measurements carried out on same patients. The time LMM parameters were interpreted as adjusted mean changes from baseline.

To compare changes between groups, a general linear regression model was fitted with FFM as the dependent variable, and treatment, time, and the interaction of treatment with time were used as independent variables.

A Pearson's correlation analysis was used to assess the relationships between mean differences in all markers investigated.

Results

Participants

38 elderly subjects were randomized: 31 completed the study and 7 dropped out (n=2 in SUPP: 1 for dislike of taste of the supplement drink, 1 for gastrointestinal discomfort probably related to supplement intake; n=5 in PLA: 3 for medical conditions which occurred over the course of the study, 1 who

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Table 2
Baseline characteristics and descriptive statistics of the 38 participants (mean ± sd)

Variables	PLA	SUPP	Total	P-value
General data				
Gender (men, women)*	19 (5, 14)	19 (3, 16)	38 (8, 30)	0.26
Age (years)	70.09±4.22	68.34±4.75	68.91±4.60	0.29
Blood analysis				
Vitamin D (ng/ml)	23.92±9.28	25.59±8.73	25.05±8.80	0.62
Anthropometric measures				
Height (m)	1.60±0.11	1.60±0.09	1.60±0.09	0.94
BMI (kg/m^2)	28.79±3.72	25.72±4.80	26.70±4.64	0.09
DXA measures				
DXA weight (kg)	74.42±13.73	67.04±16.21	69.40±15.58	0.25
FFM (g)	44046.00±10589.38	41915.78±9538.50	42561.30±9750.74	0.32
Arms FFM (g)	4795.90±1642.47	4533.56±1511.04	4618.44±1534.66	0.66
Legs FFM (g)	16453.09±3727.66	15736.00±3443.33	15968.00±3497.30	0.59
ALM (kg)	20.87±5.44	20.26±4.80	20.45±4.92	0.76
ALM/H^2 (kg/m^2)	8.06±1.20	7.82±1.09	7.89±1.11	0.60
FM (g)	25672.60±8320.66	22614.13±9756.25	23540.94±9324.62	0.37
Gynoid FM %	36.61±9.58	36.93±9.60	36.83±9.44	0.93
Android FM %	43.50±8.15	38.99±13.53	40.36±12.20	0.25
VAT (g)	1522.57±824.75	960.76±915.61	1124.62±910.32	0.16
VAT/FM	0.05±0.02	0.04±0.02	0.04±0.02	0.11
Indirect calorimetry				
RMR (kcal)	1292.73±264.19	1235.41±244.60	1254.51±248.65	0.55
R	0.84±0.07	0.85±0.05	0.85±0.06	0.79
FAT (%)	51.42±25.11	48.84±18.91	49.70±20.81	0.77
CHO (%)	48.97±25.12	51.57±18.88	50.70±20.80	0.76
Strength and Power assessment				
MVC (kg)	9.97±4.11	9.06±3.46	9.51±3.78	0.53
PP (W)	41.04±26.40	29.72±17.45	35.38±21.92	0.21
sEMG fatigue assessment				
FD iv	1.60±0.50	1.62±0.40	1.62±0.05	0.32
FD slopes (%/s)	-0.05±0.02	-0.04±0.03	-0.05±0.03	0.78
CV iv (m/s)	4.27±0.93	4.07±0.61	4.15±0.77	0.44
CV slopes (%/s)	-0.38±0.32	-0.18±0.19	-0.24±0.25	0.15
TtT (s)	66.50±17.81	61.00±17.38	62.67±17.42	0.42

Notes: BMI, Body Mass Index; DXA, Dual Energy X-Ray Absorptiometry; FFM, Fat Free Mass; ALM, Appendicular Lean Mass; FM, Fat Mass; VAT, Visceral Adipose Tissue; RMR, Resting Metabolic Rate; R, Respiratory quotient; MVC, Maximal Voluntary Contraction; PP, Peak Power; sEMG, surface electromyography; FD iv, Fractal Dimension initial value; CV iv, Conduction Velocity initial value; TtT, Time to perform the Task; * X^2: 1.27

moved, 1 for hospitalization). The main details on participants' baseline characteristics are shown in Table 2.

Baseline outcomes observed showed an inadequate level of vitamin D (<30 ng/ml is considered insufficient) and a slightly high BMI (>24.9 kg/m^2). SUPP and PLA were similar on all counts and this means that randomization was correctly carried out. Based on the score obtained from the compliance questionnaires, for the subjects that completed the study we reached a compliance percentage which was close to 100%.

Treatment effect compared to baseline

The complete results for all variables considered are presented in Table 3. The main variations observed are described as follows.

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Table 3
Treatment effect from baseline and between groups refers to subjects that completed the study

Variables	Mean changes from baseline	P-value	95% CI	Mean changes from baseline	P-value	95% CI	Mean difference between groups and (CI 95%)	P-value
	PLA			SUPP				
Blood analysis								
Vitamin D (ng/ml)	-2.98	<0.001	-5.39;-0.58	8.73	<0.001	7.12; 10.35	11.72 (8.74; 14.70)	<0.001
Anthropometric measures								
BMI (kg/m²)	-0.12	ns	-0.77; 0.53	-0.01	ns	-0.46; 0.42	0.12 (-0.69; 0.90)	ns
DXA measures								
DXA weight (kg)	0.25	ns	-0.79; 1.30	0.18	ns	-0.48; 0.84	-0.07 (-1.34; 1.19)	ns
FFM (g)	58.30	ns	-809.67; 926.27	232.76	ns	-313.72; 779.24	174.45 (-870.23; 1219.15)	ns
Arms FFM (g)	37.63	ns	-203.45; 278.71	148.23	ns	-13.33; 309.80	110.60 (-187.61; 408.82)	ns
Legs FFM (g)	-235.37	ns	-604.28; 133.54	208.33	ns	-23.94; 440.60	443.70 (0.326; 887.72)	<0.05
ALM (kg)	-0.18	ns	-0.58; 0.21	0.34	<0.001	0.09; 0.59	0.53 (0.05; 1.01)	<0.05
ALM/H² (kg/m²)	-0.06	ns	-0.22; 0.09	0.12	<0.001	0.02; 0.22	0.19 (0.00; 0.37)	<0.05
FM (g)	240.70	ns	-665.29; 1146.69	-77.05	ns	-647.47; 493.36	-317.75 (-1408.20; 772.69)	ns
Gynoid FM (%)	1.35	ns	0.03; 2.66	0.17	ns	-0.65; 1.00	-1.17 (-2.76; 0.41)	ns
Android FM (%)	0.35	ns	-1.92; 2.62	-0.65	ns	-2.09; 0.77	-1.01 (-3.74; 1.73)	ns
VAT (g)	-39.83	ns	-130.23; 50.56	-76.25	<0.001	-136.84; -15.67	-34.11 (-139.49; 71.27)	ns
VAT/FM	0.00	ns	-0.00; 0.00	-0.00	ns	-0.00; 0.00	-0.001 (-0.003; +0.002)	ns
Indirect calorimetry								
RMR (kcal)	-97.17	ns	-227.90; 33.56	12.89	ns	-69.41; 95.20	110.07 (-47.28; 267.42)	ns
R	0.00	ns	-0.04; 0.05	-0.03	ns	-0.06; 0.00	-0.04 (0.95; 0.23)	ns
FAT (%)	0.06	ns	-16.60; 16.74	12.00	<0.001	1.51; 22.50	11.94 (-8.13; 32.01)	ns
CHO (%)	-3.05	ns	-20.46; 14.36	-6.27	ns	-17.49; 4.94	-3.22 (-25.30; 18.85)	ns
Strength and Power assessment								
MVC (kg)	-0.86	<0.001	-1.51; -0.20	0.52	<0.001	0.08; 0.96	1.38 (0.57; 2.19)	<0.05
PP (W)	-5.04	ns	-10.13; 0.04	4.82	<0.001	1.41; 8.23	9.87 (3.58; 16.15)	<0.05
sEMG fatigue assessment								
FD iv	0.04	ns	-0.02; 0.10	-0.03	ns	-0.06; 0.00	-0.07 (-0.14; 0.00)	ns
FD slopes (%/s)	-0.03	ns	-0.08; 0.02	-0.01	ns	-0.04; 0.02	0.02 (-0.05; 0.08)	ns
CV iv (m/s)	-0.98	ns	-2.01; 0.05	0.04	ns	-0.48; 0.56	1.02 (-0.06; 2.21)	ns
CV slopes (%/s)	0.04	ns	-0.23; 0.31	0.01	ns	-0.13; 0.16	-0.26 (-0.34; 0.28)	ns
TtT (s)	-12.53	ns	-30.58; 5.51	0.58	ns	-8.90; 10.07	13.11 (-7.48; 33.71)	ns

Notes: BMI, Body Mass Index; DXA, Dual Energy X-Ray Absorptiometry; FFM, Fat Free Mass; ALM, Appendicular Lean Mass; FM, Fat Mass; VAT, Visceral Adipose Tissue; RMR, Resting Metabolic Rate; R, Respiratory quotient; MVC, Maximal Voluntary Contraction; PP, Peak Power; sEMG, surface electromyography; FD iv, Fractal Dimension initial value; CV iv, Conduction Velocity initial value; TtT, Time to perform the Task. ns, not significant. In bold the statistically significant evidences.

Primary outcomes (FFM; ALM, ALM/H²; MVC; PP and myoelectric manifestation of fatigue): 1) no statistical difference was found in total FFM in either group; 2) a statistically significant increase in all index of sarcopenia (ALM: +0.34 kg and ALM/H²: +0.12 kg/m²; p<0.001) were found in SUPP, with no changes in PLA; 3) increases of MVC (+0.52 kg; p<0.001) and PP (+4.82 W; p<0.001) were significantly observed in the SUPP, whereas the same variables showed a negative change in PLA, with a significant decrease registered for MVC: -0.86 kg; p<0.001; 4) no statistical changes were found for all sEMG descriptors of fatigue (FD iv, CV iv, FD and CV slopes) and TtT.

Secondary outcomes (vitamin D blood levels; RMR, R, CHO%, FAT%; FM and VAT): 1) we measured a significant increase of vitamin D levels in SUPP (+8.73 ng/ml; p<0.001) with a negative change in PLA (-2.98 ng/ml; p<0.001) (Figure 2); 2) a statistically significant increase was observed for fasting FAT oxidation rate (+12%; p<0.001), whereas no other changes of fasting metabolic markers (RMR, R, CHO%) were measured; 3) no changes were found for FM absolute values or FM gynoid or android distribution (FM%) in either group, but we highlighted a statistical decrease of VAT (-76.25 g;

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p<0.001) in SUPP.

Treatment effect between groups

The results of intergroup analysis (PLA vs. SUPP) are shown in Table 3. The variables that showed significant changes between the two groups over the course of the study are indicated as follows. Primary outcomes: 1) a positive legs FFM response, with a mean difference of 443.70 g (p<0.05); 2) an increase of ALM (+0.53 kg; p<0.05) and ALM/H^2 (+0.19 kg/m^2; p<0.05); 3) an increase of MVC (+1.38 kg; p<0.05) and PP (+9.87 W; p<0.05). Secondary outcomes: we registered an increase of vitamin D level of 11.72 ng/ml (p<0.001).

Figure 1

Flow diagram of the study: multi-ingredient supplementation (SUPP) compared to placebo (PLA) in elderly subjects. The diagram indicates the total number of subjects assessed from the identification phase to the group allocation and the number of subjects included in the final statistical analysis after 12 weeks of treatment
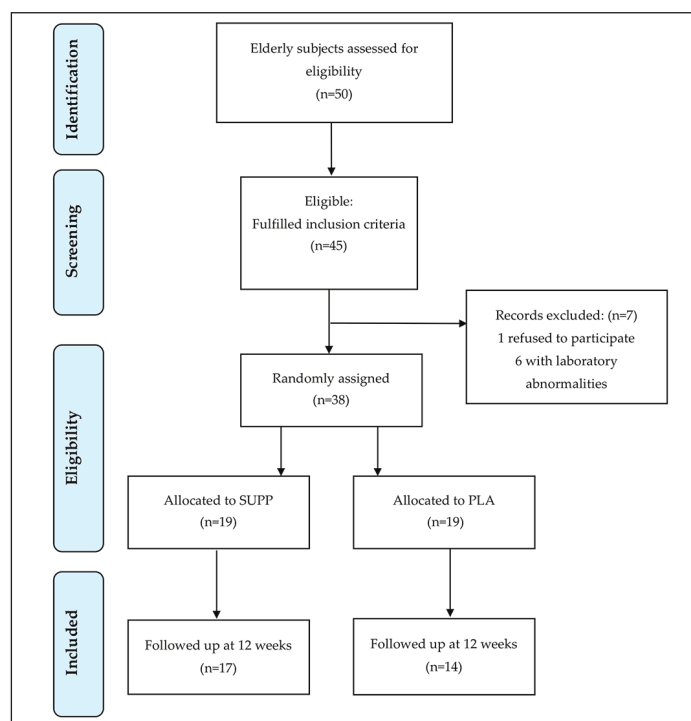
Pearson's correlations between mean differences in treatment group

A significant correlation was found between vitamin D and ALM/H^2 (r=0.706; p<0.001) (Figure 3A), between VAT/FM and MVC (r=-0.572; p<0.001) (Figure 3B) and between Legs FFM and ALM/H^2 (r=0.857; p<0.001) (Figure 3C). R is computed as the partial correlation, adjusted for age, sex and BMI.

Figure 2

Mean variation of vitamin D blood levels in PLA (-2.98 ng/ml) and SUPP (+ 8.73 ng/ml) after 12 weeks of treatment. Error bars indicate standard error of the mean
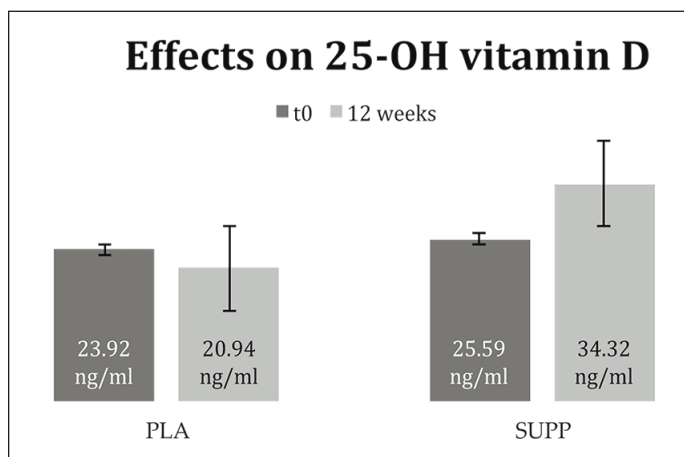

Discussion

Effects on muscle mass, muscle strength and muscle power

The experimental results agree with comparable previously published works in which the use of a similar mixture of compounds has shown enhancements of muscle mass (ALM) with no exercise intervention (11, 22). In particular, Bauer et al. (22) after 13 weeks of supplementation using a formula containing leucine-enriched whey protein (40 g) with 6 g of leucine, vitamin D (1600 IU) and carbohydrates, showed an absolute increase of 0.25 kg and a relative increase of 0.17 kg compared to control. More recently, Bell et al. (11) after 6 weeks of supplementation using a multi-ingredient mix including whey protein (60 g), vitamin D (1000 IU), creatine (5 g) and n-3 PUFA, observed an improvement of ALM of 0.40 kg in treated group, whereas the difference with matched controls was not described. In our experimental conditions (ALM: + 0.34 kg from baseline; + 0.53 kg between groups), compared to the study by Bauer et al. (22) which shows lower ALM improvement after treatment and compared to placebo, the higher ALM gain may be attributed to the coexistence of EAA and creatine in the mixture, which is probably more effective than a higher dose of amino acids and leucine alone. In fact, although the leucine-enriched whey protein blend seems to be an appropriate approach to preserving muscle mass and function in older sarcopenic adults (44), a recent systematic review (45) found this effect only in 3 out of 12 Randomized Control Studies (RCTs) whereas an additional anabolic action of creatine was found in 4 out of the 5 RCTs considered. In the study by Bell et al. (11) comparable results were obtained in half the time (6 weeks). However, the authors used a very high dose of protein/amino acids and creatine compared to our formula and the subjects involved were male. Although it is not known, at present, whether elderly males and females

respond differently to a multi-ingredient supplementation, we should consider a "gender effect" of based on recent findings (46) underlying that aged females' muscle displays higher heterogeneity in myofibers size and phenotype composition compared to males' (about 5-fold).

Figure 3

Pearson's correlations between mean differences in SUPP group (n=17): Vitamin D Vs. ALM/H² (A); VAT/FM Vs. MVC (B); Legs FFM Vs. ALM/H² (C)
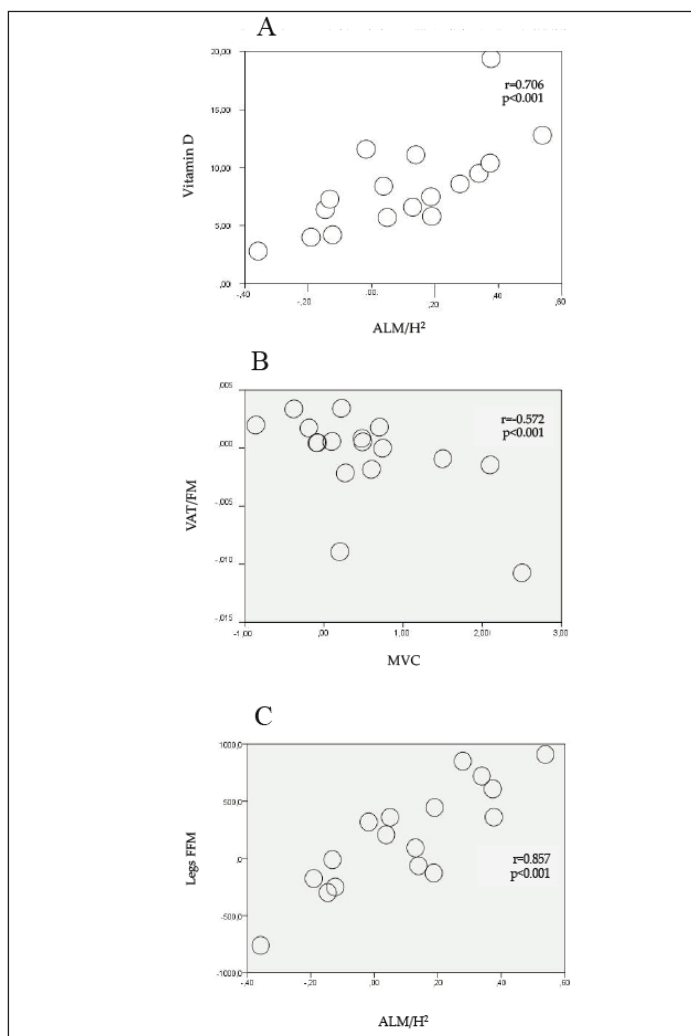
Notes: ALM/H², Appendicular Lean Mass index; VAT/FM, Visceral Adipose Tissue to Fat Mass ratio; FFM, Fat Free Mass; MVC, Maximal Voluntary Contraction

Although it is not possible to isolate which compounds in the supplement were responsible for the outcomes assessed, we believe that the observed reversing of vitamin D inadequacies (Figure 2) may have contributed to the overall favorable effect on muscle strength (MVC). This hypothesis is based on previous meta-analysis revealing a small but significant positive effect of vitamin D supplementation on global muscle force expression (47). Furthermore, published data indicated that serum 25-hydroxyvitamin D concentrations between 60 and 75 nmol/L (24.04 and 30.05 ng/ml, respectively) correlate with lower-extremity strength (48) and, possibly, with the amelioration of Legs FFM. This correlation could explain the increase of Legs FFM we observed after supplementation (Table 3) and the positive correlation between Vitamin D and ALM/H² (Figure 3A), Legs FFM and ALM/H² (Figure 3C) we observed by the treatment effect.

In the present study a gain in muscle power (PP) was found as an important functional outcome of nutrient supplementation. Considering that an additional effect of EAA and vitamin D on muscle power tests is improbable, as outlined by recent meta-analysis (47) and systematic review (45), we suppose that creatine in the mixture could be highly effective in increasing power-based functional tests (49) and may have contributed to the observed effect.

Potential muscle and metabolic role of Muscle Restore Complex® (ALA, CoQ10 and resveratrol)

With the aim to obtain more insight for the design of "the most effective formula", capable of maximally preventing muscle wasting due to ageing, and considering a likely role of free radicals production and inflammation in its development and progression, compounds with antioxidants and anti-inflammatory properties (ALA, CoQ10 and resveratrol) (32-36, 50-54) were added to the mix. This is the first time that a similar blend was added to an EAA-based formula for the prevention of aged-related loss of muscle mass and function. However, considering that the bioavailability of each single component was not measured, and the sub-group analysis is also missing, at this stage we can only speculate that their presence in the formula may have played a potential synergic role leading to the obtained results. In particular, compared to the study of Bauer et al. (22), the grater ALM improvement compared to placebo may be due at least in part to antioxidant and/or anti-inflammatory mechanisms.

The anti-inflammatory properties of ALA, although rarely investigated in humans, have shown a 15% significant decrease in serum interleukin-6 levels following 4 weeks of supplementation (55). CoQ10 blood levels were recently correlated with muscle strength in two independent humans cohorts studies (52), and modulating effects of CoQ10 supplementation on inflammatory (53) and chronic oxidative stress response (54) were found after 4 weeks of treatment in the elderly. Other interesting data suggest that cellular energy delivery may be positively conditioned by a combination of creatine, ALA and CoQ10 use in subjects carrying mitochondrial dysfunctions (56). The resveratrol, a plant-derived polyphenol, is probably the most promising of such compounds as it showed: 1) to protect skeletal muscle from the aging-induced oxidative stress (57); 2) to enhance, at least in rodents, skeletal muscle fibers size (type IIA and IIB fibers) and myonuclear number thus leading to hypertrophy (35); 3) to reverse the atrophy, on isolated myotubes, caused by TNF-α, through the regulation of the Akt/mTOR/FoxO1 signaling

pathways and inhibition of the atrophy-related ubiquitin ligase (58); 4) to improve mitochondrial capacity by activating the AMPK/SIRT1/PGC-1α pathway (59-61). On this latter point, Most et al. (62) showed that a 12-week supplementation with 80 mg/day of resveratrol (a dosage similar to our experimental condition) can improve skeletal muscle oxidative capacity leading to amelioration of fasting substrate oxidation. The authors (62), according to our results, underlined that, although body weight, total FM and resting R were not affected by treatment, a significant decrease of VAT (~11%) was found. Considering that VAT is detrimental to metabolic health (63), the reduction we observed may be of clinical importance in the long term. Furthermore, the negative association between VAT and MVC, we found (Figure 3B), is in agreement with previously published data (64).

Effects on Time to perform the Task (TtT) and myoelectric manifestations of fatigue

We measured the effect of supplementation on TtT failure, as a measure of endurance, and on myoelectric descriptors as reliable indicators of peripheral (CV initial values and slopes) and central fatigue (FD initial values and slopes), during submaximal isometric contractions at 60% MVC to exhaustion. No statistical changes were found for all the measured outcomes, either compared to baseline or between groups. These data demonstrate, for the first time, that 12 weeks of EAA-based multi-ingredient nutritional supplementation failed to positively affect muscle endurance capacity (TtT) and myoelectric manifestations of central and peripheral fatigue in older adults. We consider this finding to be indirect confirmation of a preferential effect of the mixture on the structure and function of type II fibers. In fact, changes in muscle mass, strength and power are mainly attributable to relative expression of fast fibers within the muscle without amelioration in endurance and relative proportion of slow type I fibers.

This is the first study to have assessed the effects of a multi-ingredient supplementation on myoelectric manifestation of fatigue in the elderly and this precludes further considerations on the subject. So far, only two middle-term studies have investigated the effects of amino acids-based supplementations on fatigue in healthy elderly people but different procedures to induce and detect fatigue and the combination with a specific training program was used. In particular, Reule et al. (65) documented a reduction of fatigue after 12 weeks of leucine-rich (3.2 g/day) amino acid supplementation by measuring the capacity of this treatment to counteract the loss of strength measured as MVC, and not during submaximal contractions, in the acute phase recovery (0-3 hours) after an eccentric stress test (downhill walking). Gryson et al. (66) described the effect of 16 weeks of a leucine-fortified milk protein supplementation on TtT failure during a sustained isometric contraction (dominant leg until exhaustion at 75% MVC) performed after a fatiguing protocol (3 isometric MVCs). Authors referred that

the TtT failure improved in the trained participants receiving a 10 g/day of the protein compared to controls. However, it is important to highlight that in this study TtT was measured at % MVC higher than those generally described in literature to assess the arising of peripheral fatigue (67) and the findings from Gryson et al. probably describe the overall contribution of type II fibers to peripheral fatigue rather than giving information on the role of type I fibers within the muscle. In fact, since fiber type composition has been proposed as a major determinant of CV rate of change during submaximal isometric contractions (68), it is well known that 70% of MVC, with a higher decrease of CV, may indicate a major recruitment of type II fibers (69) compared to 60% MVC, more suitable to detecting the contribution of type I fibers to TtT (67).

Limitations

This study has limitations. As other authors have outlined (11), it is very difficult to create an experimental design finalized to statistically discriminate the effects of each single compound included in a multi-ingredient supplement. To obtain reliable results, this would require a very large sample size and several subgroups to be analyzed. Therefore, we consider the results obtained to be suitable for further investigations towards the effectiveness of each compound and their bioavailability.

Furthermore, compared to the two studies we mainly analyzed in the discussion (11, 22), we used different procedures for evaluating upper limb muscle strength and power. While on the one hand this represent an unconventional method to measure the muscle function, increasing the possibilities of studying muscular strength and power compared to classical methods used on the elderly (handgrip test, gait speed test, sit-to-stand chair test or other lower extremity function tests), on the other hand it limits the comparison to results obtained with more validated tests available in literature.

Conclusions and future directions

The study demonstrate that a mixture with EAA, creatine, vitamin D and MRC® (ALA, CoQ10, resveratrol) may improve muscle aging-related outcomes, such as muscle mass, muscle strength and muscle power in a medium-short period and without physical activity programs. In particular, given that from the fifth decade of life muscle mass and strength decline at rates of ~0.5-1% and ~1-3% annually, respectively (70), the changes in ALM (+ 1.68%) and MVC (+5.22%) observed in SUPP after 12 weeks of treatment are to be considered clinically relevant. In fact, in absolute values the increase of these variables is equivalent to an offsetting of more than one year of age-related decline, suggesting that this formula, similarly to previous studies (11, 22), can effectively counterbalance progression of muscle mass and strength loss.

As a preliminary study, in this frame we first aimed to compare our original formula with others available in literature and we now hope for future studies that imply much more effort

in terms of bioavailability assessment, biochemical responses and number of enrolled subjects.

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