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From mitochondria to healthy aging: The role of branched-chain amino acids treatment: MATeR a randomized study.

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18 **Title: From Mitochondria To Healthy Aging: The Role Of Branched-chain Amino Acids Treatment: MATeR**
19 **A Randomized Study**

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35

36

37 **Abstract**

38 *Rationale:* Malnutrition often affects elderly patients and significantly contributes to the reduction in
39 healthy life expectancy, causing high morbidity and mortality. In particular, protein malnutrition is one of
40 the determinants of frailty and sarcopenia in elderly people.

41 *Methods:* To investigate the role of amino acid supplementation in senior patients we performed an open-
42 label randomized trial and administered a peculiar branched-chain amino acid enriched mixture (BCAAem)
43 or provided diet advice in 155 elderly malnourished patients. They were followed for 2 months, assessing
44 cognitive performance by Mini Mental State Examination (MMSE), muscle mass measured by
45 anthropometry, strength measure by hand grip and performance measured by the Timed Up and Go (TUG)
46 test, the 30 seconds Chair Sit to Stand (30-s CST) test and the 4 meters gait speed test. Moreover we
47 measured oxidative stress in plasma and mitochondrial production of ATP and electron flux in peripheral
48 blood mononuclear cells.

49 *Results:* Both groups improved in nutritional status, general health and muscle mass, strength and
50 performance; treatment with BCAAem supplementation was more effective than simple diet advice in
51 increasing MMSE (1.2 increase versus 0.2, $p=0.0171$), ATP production (0.43 increase versus -0.1, $p=0.0001$),
52 electron flux (0.50 increase versus 0.01, $p<0.0001$) and in maintaining low oxidative stress. The
53 amelioration of clinical parameters as MMSE, balance, four meter walking test were associated to
54 increased mitochondrial function.

55 *Conclusions:* Overall, our findings show that sustaining nutritional support might be clinically relevant in
56 increasing physical performance in elderly malnourished patients and that the use of specific BCAAem
57 might ameliorate also cognitive performance thanks to an amelioration of mitochondria bioenergetics.

58 **Keywords**

59 Malnutrition, elderly patients, branched-chain amino acids, muscle mass and strength, mitochondrial
60 activity and biogenesis, oxidative stress.

61 **Abbreviations**

62 30-s CST, 30 seconds chair sit to stand test; ADL, activity of daily living; BCAAem, branched chain amino
63 acid enriched mixture; BCAAs, branched chain amino acids; BMI, body mass index; CIRS, cumulative index
64 rating scale; COX-1 and 4, cytochrome C oxidase 1 and 4; FOXO, forkhead box O; GAPDH, glyceraldehyde 3-
65 phosphate dehydrogenase; GDS, geriatric depression scale; GLM, linear regression models; MFN-1 and 2,
66 mitofusin-1 and 2; MMSE, mini-mental state examination; MNA, mini nutritional assessment test; mTOR,
67 mechanistic target of rapamycin; NRF-1, nuclear respiratory factor-1; NO, nitric oxide; OECD, organisation
68 for economic co-operation and development; PBMCs, peripheral blood mononuclear cells; ROS, reactive
69 oxygen species; RT-PCR, real time PCR; TBARs, thiobarbituric acid reactive substances; TFAM, mitochondrial
70 transcription factor A; TUG, timed up and go test.

71

72 **Introduction**

73 Thanks to an increased life expectancy, an improvement in health status and medical services, the older
74 population is constantly increasing. In 2015, 617 million (8.5%) people in the world were aged 65 and over
75 (older adults) and these numbers are estimated to rise to 1.6 billion by 2050 (1). Despite the increase in life
76 expectancy, there is no corresponding increase in healthy life expectancy: recent findings in 2015 show
77 that, despite a life expectancy at the age of 65 of 21.2 years for women and 17.9 years for men, only 9.4
78 years are healthy years (2). Concomitantly, health maintenance in older age will be one of the most
79 relevant societal challenges in the future years. Lifestyle changes appear to be fundamental in increasing
80 healthy life expectancy, and adequate nutrition is enormously important, given that malnutrition (i.e.,
81 undernutrition), particularly as protein-energy deficit is very common amongst the elderly population. This
82 is due to the effects of aging *per se* that causes decreased salivation, difficulty swallowing, and delayed
83 emptying of the stomach and oesophagus, as well as slower gastrointestinal movement (3). Other
84 conditions associated with aging, such as drug use, loneliness, depression, lack of oral health, low quality of
85 life, in addition to chronic non-communicable diseases, markedly increase the undernutrition risk (4). It has
86 been estimated that undernutrition affects between 20 and 50% of hospitalised patients (5) and 5 and 10%
87 of patients living at home in the community (6), the great variations reported in different studies depends

88 not only to differences in the population analysed, but also on the adopted definition. Recently a large
89 study (7), that applies harmonized criteria to define malnutrition in different clinical settings and
90 population, shows a great underestimation of the problem and confirms higher prevalence of malnutrition
91 in residents of nursing homes and hospitalized patients evaluated by Mini Nutritional Assessment test
92 (MNA). Malnutrition is more prevalent in patients affected by acute and chronic disorders with reduced
93 functional status (7) and is associated with poor clinical outcomes and prognosis. Malnutrition is associated
94 with reduced immune function, anaemia, impaired cognitive function, and higher hospitalisation rate and is
95 a strong independent predictor of mortality [for a review, see A. Granic et al, 2018 (8)]. Malnutrition causes
96 body weight loss and muscle atrophy, decreased muscle strength and function, impaired balance, and
97 increased fall and fall-related injuries. The malnutrition-linked muscle atrophy accelerates transition to
98 frailty (9) and has been considered as one of the determinants of sarcopenia (10,11). Importantly,
99 sarcopenia is defined as low muscle mass, with defective muscle strength (also named dynapenia) and
100 decline of physical performance (12) and is, *per se*, associated with increased morbidity and mortality (13).
101 Although several guidelines and consensus documents on nutritional care of malnourished elderly subjects
102 have been proposed (14), and protein needs established in the range from 0.8 g/kg/day (healthy adults)
103 and up to 1.5 g/kg/day (in some cases even higher) according to age, disease and degree of protein
104 depletion (15), the daily protein consumption in older subjects is often inadequate and undernutrition and
105 sarcopenia are underestimated and considered as one of the factors of aging (4, 7).

106 It has been suggested that the aging process significantly affects protein metabolism and enhances the
107 muscle wastage that accompanies undernutrition and sarcopenia. Some studies show lower plasma
108 concentrations of branched-chain amino acids (BCAAs) in elderly subjects (16,17), whereas others do not
109 (18,19). This may be due to the increased first-pass splanchnic extraction of amino acids in older people,
110 with a consequent decrease in delivery to the skeletal muscle tissue and availability for muscle tissue
111 anabolism (20). Most kinetics studies show no difference in the ability of older subjects to retain and
112 metabolise BCAAs (21–24).

113 These observations suggest that the dietary requirement of proteins and essential amino acids is higher in
114 the elderly than in young adults (25) and that an increased intake of a mixture of amino acids or essential
115 amino acids can increase amino acid availability and result in the stimulation of muscle protein anabolism
116 (26).

117 A number of reports, including a recent well conducted meta-analysis concludes that dietary supplement of
118 essential amino acids is more effective than non-essential amino acid or whole protein supplementations in
119 malnourished patients (27).

120 Notably, amino acid mixtures enriched in BCAAs have been shown to promote mitochondrial biogenesis
121 and function, in addition to decrease oxidative stress via nitric oxide (NO) and mechanistic target of
122 rapamycin (mTOR) signalling pathways in middle-aged mice (28). A more recent study has shown the
123 stimulatory effect of leucine on mitochondrial respiration and ATP production in human macrophages (29).

124 These results are important because mitochondrial dysfunction is a hallmark of the aging processes and
125 age-related disorders, including sarcopenia and cognitive decline, are characterized by reduced
126 mitochondrial mass and function [for a comprehensive review, see N. Sun et al, 2016 (30)]. Dietary
127 supplementation of BCAA-enriched mixtures (BCAAem) may contribute to slow-down mitochondrial
128 decline and to ameliorate clinical status of malnourished elderly patients (31).

129 The MATeR study thus aimed to evaluate the efficacy of a specific BCAAem compared to diet advice to
130 promote mitochondrial function and improve clinical outcomes, particularly muscle and cognitive
131 performance, in malnourished elderly community-dwelling subjects.

132

133 **Materials and methods**

134 *Study design.*

135 We conducted a parallel, randomized, controlled, open-label trial to determine the efficacy of dietary
136 BCAAem supplementation, as compared with diet advice, in the slow-down of both muscle and cognitive
137 deficit in malnourished community-dwelling men and women aged 80 years or older. Randomisation was
138 performed by computer generated tables to allocate treatments, with a simple randomization method; the

139 patients received a consecutive number after enrolment and were subsequently allocated to randomization
140 list, according with Kim et al (32). The randomisation was carried out by the principal investigator, scientists
141 performing lab measurement and statistical analyses were blind to treatment.

142 The inclusion criterion was malnutrition defined as MNA lower than 17. The MNA test is composed of 18
143 items that can be completed in less than 10 minutes. It provides a multidimensional assessment of senior
144 patients nutritional status taking into account four domains: anthropometry, general status, dietary habits,
145 and self-perceived health and nutrition states. Anthropometry includes the measurement of calf and arm
146 circumferences, Body Mass Index (BMI), calculated after the measurement of weight and height, and
147 questions about weight loss (4 items); general status comprehends 7 questions related to general health,
148 medication and mobility; the assessment of dietary habits comprehends 5 questions on the number of
149 meals, food and fluid intake and autonomy of feeding; 2 questions evaluate self-perceived health and
150 nutrition states (33,34).

151 Exclusion criteria were known malignancy, life expectancy of less than two months, heart failure (NYHA IV),
152 end stage renal disease, liver cirrhosis (Child B-C), tube/percutaneous endoscopic gastrostomy feeding or
153 parenteral nutrition, Mini-Mental State Examination (MMSE)≤18 and MNA>17. MMSE ≥ 18 identifies
154 patients with mild form of cognitive impairment, those patients generally do not have problems in
155 swallowing and are able to take drugs. We evaluated for inclusion 336 malnourished patients presenting to
156 our out-patients service for a geriatric evaluation, the evaluation was done by expert geriatricians, of the
157 evaluated patients 181 were excluded for presence of exclusion criteria; one hundred and fifty-five
158 malnourished elderly patients living at home in the community and who were admitted to the outpatients'
159 department of our Unit were enrolled. Patients were evaluated at baseline and randomised to receive diet
160 advice, summarised in an easy-to-use brochure for lay persons (77 patients) or to BCAAem supplements (78
161 patients, Aminotrofic[®], kindly supplied by Errekappa Euroterapici S.p.A. and Professional Dietetics S.p.A, 2
162 sachets/day). Aminotrofic[®] is a BCAA enriched mixture, it contains Leucine (1,250 mg), Lysine (650 mg),
163 Isoleucine (625 mg), Valine (625 mg), Threonine (350 mg), Cystine (150 mg), Histidine (150 mg),
164 Phenylalanine (10 mg), Methionine (50 mg), Tyrosine (30 mg), Tryptophan (20 mg), Vitamin B 6 (0.1 mg),

165 Vitamin B1 (0.15 mg). We suggested the patients to take the BCAAem in the mid-morning and afternoon,
166 regardless of food ingestion. In order to check the compliance we asked the patients to bring back at center
167 the empty sachets at follow-up visit.

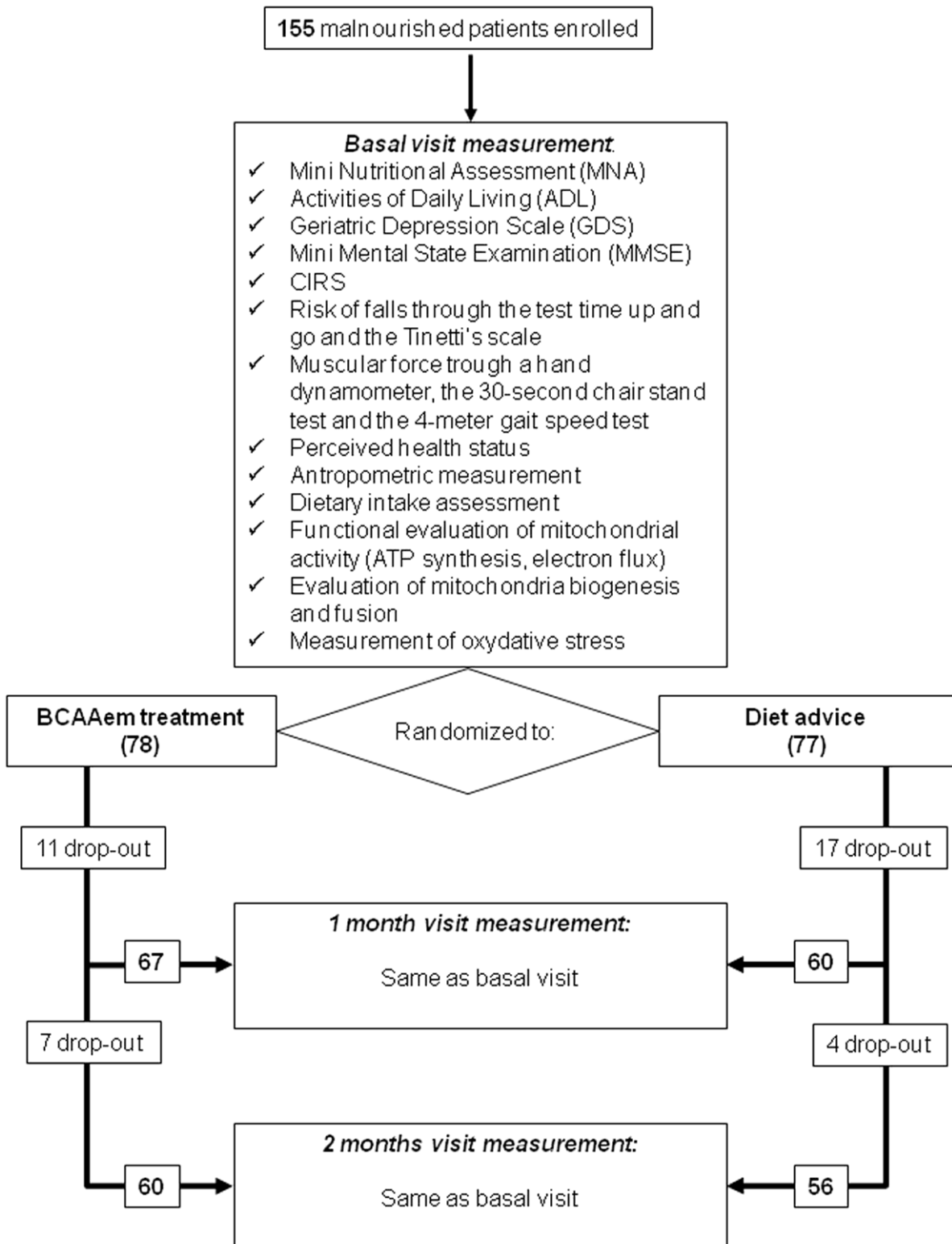
168 Diet advice comprised general advice on the meaning and consequences of malnutrition and dietary
169 recommendation based on the principle of "Food First" to maximize the patient nutritional intake from
170 regular food and drink according with the ESPEN Guidelines on Enteral Nutrition for geriatric patients (35).
171 According to the "Food First" approach we suggested the patients to increase the frequency of eating,
172 maximize the nutrient and energy density of food and drink and fortify food with the addition of fats and
173 sugars, suggested recommendations were given to the patients both orally by the physician and by the use
174 of a brochure; provided dietary recommendations are summarized in the **Supplementary Table 1**.

175 Period of patient enrolment: February 2013-September 2017. Patients were called back to the centre and
176 all the measurements were performed after 1 and 2 months. 116 patients completed the study (60 treated
177 with BCAAem and 56 with diet advice). In the BCAAem group, 2 patients died after the first month, 1 was
178 admitted to hospital within the first month, 10 patients did not return for the month-1 visit and 5 patients
179 did not return for the month-2 visit for personal reasons. In the diet advice group, 1 patient died during the
180 first month, 2 patients did not return for the month-1 visit since they were hospitalised, 14 patients did not
181 return for the month-1 visit and 4 patients for the month-2 visit for personal reasons, there were no
182 collateral effects. Only data from patients with complete follow up were included in the statistical analyses
183 **(Fig. 1)**.

184 **Fig.1. Diagram of the study design.**

185 The diagram shows the study design and the number of patients at each visit in bold. The tests performed
186 at each visit are specified.

187



188

189 The main outcome measures were muscle mass, strength and performance and mitochondrial ATP
 190 production; secondary measurements were cognitive performance, nutritional status, health perceived
 191 status, mitochondrial biogenesis and activity in peripheral blood mononuclear cells (PBMCs). The
 192 measurements were carried out at baseline and after 1 and 2 months of treatment.

193 *Clinical assessment.*

194 *Global clinical assessment:* Self-sufficiency was measured by assessing the Katz Index of Activity of Daily
195 Living (ADL), which evaluates overall performance in six functions: bathing, dressing, going to toilet,
196 transferring, continence and feeding; cognitive performance was evaluated by MMSE that is a brief test
197 used to routinely track cognitive changes in an individual both cognitively intact or with severe cognitive
198 impairment over time. Patients' mood was evaluated by the short form of Geriatric Depression Scale (GDS),
199 the scale consists of 15 questions related to the patient's mood answered "yes" or "no". The cut-off point
200 adopted to define a patient as depressed is a GDS higher than 7 (36). Perceived health status was measured
201 by asking the patients to answer the question "How is your health in general?". The patients' answers:
202 "very good", "good", "fair", "bad" or "very bad" were rated from 5 (Very good) to 1 (Very bad), although
203 there is not yet full standardisation of the measurement of perceived health status across Organisation for
204 Economic Co-operation and Development (OECD) Countries, here we used a standard health interview
205 survey instrument used in the OECD Health Statistics 2007 (37), confirmed in OECD Health Statistics 2018
206 (available on line at <http://www.oecd.org/els/health-systems/health-data.htm>) and accepted in Italy. The
207 Cumulative Index Rating Scale (CIRS) was also recorded, this scale accounts for both the presence and the
208 severity of co-morbidities (38).

209 *Nutritional status assessment:* We assessed patients' dietary intake using the PROGEO software (Progeo
210 S.r.l., Italy), it provides an extensive food database and allows to record and to accurately estimate
211 patients' average nutritional intake. The Photo Intake tool helps patients to recognize the amount of food
212 by the visual weight method, ingested showing pictures of food in 3 portions, food quantities can also be
213 recorded as conventional standard units (spoon, glass, cup, etc.). The software provides a large food
214 database and automatically displays patients' average daily calories and nutrients intake. The interview was
215 based on the recall method on 7 days making reference to the "standard week" as suggested by the
216 manufacturer. The interview was done by geriatricians trained by a nutritionist.
217 BMI was assessed by weighing patients by a precision scale and measuring their height using an altimeter
218 wall, BMI was calculated as weight in kg/height in meters squared. Percentage of fat mass was measured

219 using a plicometer (Mahr GMBH Esslingen), the Pollock, Schmidt and Jackson's formula on three sites
220 (triceps, subscapular and abdomen) was applied (39,40). Skinfold thickness measurements were performed
221 by trained staff according to standard technique: the skinfold thickness was measured by lifting a fold of
222 skin and subcutaneous fat away from the underlying muscle and bone, the skinfold thickness was measured
223 in duplicate with the plicometer. When a difference between the first and the second measurement
224 exceeded 6 mm, a third measurement was taken. The plicometer is applied 1cm from the ridge of skin; take
225 reading 3 seconds after application, to standardise any effects produced by deformation of tissues. The
226 triceps skinfold was measured at the back of the left arm, midway between the acromial process of the
227 scapula and the olecranon process of the ulna. The subscapular skinfold is picked up just under the lower
228 angle of the scapular lifted horizontally below the tip of right scapula. The abdominal skinfold was lifted
229 diagonal midway between umbilicus and right anterior superior iliac spine.

230 *Muscle mass, strength and performance:* Appendicular muscle mass was measured using arm and calf
231 circumference. Arm circumference was measured in duplicate to the nearest 0.001 m at a point midway
232 between the lateral projection of the acromion process of the scapula and the inferior margin of the
233 olecranon process of the ulna. The mean of the two measurements was used in the analyses. The calf
234 circumference was measured to the nearest 0.001 m on the left leg with the participant standing straight,
235 feet 20 cm apart, body weight equally distributed on both feet and at the level of the widest circumference
236 of the calf; measurements were taken according to the Longitudinal Aging Study Amsterdam (LASA -
237 <http://www.lasa-vu.nl/themes/physical/anthropometry.htm>).

238 Muscle strength was measured via the hand grip test, using a hydraulic hand dynamometer (MSD, Europe)
239 (41) to assess muscle performance and mobility the Timed Up and Go (TUG) test, 30 seconds Chair Sit to
240 Stand (30-s CST) test and the 4 meters gait speed test were performed.

241 TUG is a simple test used to assess a person's mobility and requires both static and dynamic balance. TUG is
242 performed by measuring the time that the patient takes to rise from a chair, walk three meters, turn
243 around, walk back to the chair and sit down. TUG performed in ten seconds or less indicate normal
244 mobility; 11–20 seconds are within normal limits for frail, elderly and disabled patients and greater than 20

245 seconds means that the person needs assistance outside and indicates further examination and
246 intervention. A score of 30 seconds or more suggests that the person may be prone to falls (42). The 30-s
247 CST allows the evaluation of lower body strength and to assess the fatigue effect due to the number of sit-
248 to-stand repetitions. It is performed with a chair without arms, the patient seated in the middle of the chair
249 with the arms crossed over his/her chest, then is instructed to stand up as quickly as possible safely without
250 using his/her arms. The number of stands the patients completed in 30 seconds is manually recorded (43).

251 The 4-meter gait speed test was performed using a stopwatch and measures the time, in seconds, the
252 patients take to complete a 4-meter walk.

253 The risk of falls further was evaluated using the Tinetti Gait and Balance Instrument as follows: to test the
254 patient's balance, the patient has to sit in a hard, armless chair and is asked to rise and stay standing, then
255 turn 360° and sit back down. Next, the patient walks a few meters at a normal speed, turns, walks back and
256 sits down. The evaluator observes several features and scores the patient's performance: the higher the
257 score, the better the performance. The maximum score for Gait is 12 points, while the maximum for
258 Balance is 16 points, with a total maximum for the overall Tinetti Instrument of 28 points. Score
259 Interpretation: <19 high risk of falls, 19-28 low risk of falls.

260 *Laboratory tests.*

261 *Functional evaluation of mitochondrial activity:* In order to isolate mitochondrial fractions, blood cells were
262 washed twice in ice-cold PBS, then lysed in 0.5 mL buffer A (50 mM Tris, 100 mM KCl, 5 mM MgCl₂, 1.8 mM
263 ATP, 1 mM EDTA, pH 7.2), supplemented with protease inhibitor cocktail III (Calbiochem), 1 mM PMSF and
264 250 mM NaF. Samples were clarified by centrifuging at 650×g for 3 min at 4°C, and the supernatant was
265 collected and centrifuged at 13000×g for 5 min at 4°C. This supernatant was discarded and the pellet
266 containing mitochondria was washed in 0.5 mL buffer A and suspended in 0.25 mL buffer B (250 mM
267 sucrose, 15 mM K₂HPO₄, 2 mM MgCl₂, 0.5 mM EDTA, 5% w/v BSA). A 50 µL aliquot was sonicated and used
268 for the measurement of protein content, as reported in Campia et al, 2009 (44); the remaining part was
269 diluted to a protein concentration of 10 µg/µL and stored at -80°C until the use. The activity of Complex I-
270 III was measured on 10 µL of non-sonicated mitochondrial samples (44), suspended in 0.59 mL buffer C (5

271 mM KH₂PO₄, 5 mM MgCl₂, 5% w/v BSA). Then 0.38 mL buffer D (25% w/v saponin, 50 mM KH₂PO₄, 5 mM
272 MgCl₂, 5% w/v BSA, 0.12 mM cytochrome c-oxidized form, 0.2 mM NaN₃) was added for 5 min at room
273 temperature. The reaction was started with 0.15 mM NADH and was followed for 5 min. The absorbance
274 was read using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, Winooski, VT). Results
275 were expressed as nmol reduced cytochrome C/min/mg mitochondrial proteins. In each experimental set,
276 the complex I inhibitor rotenone (100 μM) was added as an internal negative control. In the presence of
277 rotenone, the electron flux was reduced to below 5%.

278 The amount of ATP was measured on 20 μg of mitochondrial extracts using the ATP Bioluminescent Assay
279 Kit (FL-AA, Sigma Aldrich Co., St. Louis, MO). Data were converted into nmol/mg mitochondrial proteins,
280 using a previously set calibration curve.

281 Under these experimental conditions, the rate of cytochrome C reduction, expressed as nmol cytochrome C
282 reduced/min/mg cell protein, was dependent on the activity of both Complex I and Complex III.

283 *Real time PCR and assessment of mitochondria biogenesis and fusion:* Real time PCR (RT-PCR) was used to
284 evaluate the mRNA levels of Cytochrome C Oxidase 1 and 4 (COX-1 and COX-4), Mitofusin-1 and 2 (MFN-1
285 and MFN-2), Nuclear Respiratory Factor-1 (NRF-1) and Mitochondrial Transcription Factor A (TFAM) from
286 whole blood nucleated cells.

287 Red cells were lysed in all peripheral blood samples, total nucleated cells were collected and dissolved in
288 TRIZOL reagent (TRISure, Bioline Reagents Ltd, UK) and frozen at -80 °C until RNA extraction. RNA was
289 isolated using chloroform extraction and subsequent isopropanol precipitation according to the
290 manufacturer's protocol. 1 μg of RNA was reverse-transcribed to single-stranded cDNA using the SensiFAST
291 cDNA Synthesis Kit (Bioline Reagents Ltd, UK). RT-PCR was performed using the SensiFAST SYBR Hi-ROX Kit
292 (Bioline Reagents Ltd, UK). The housekeeping control gene was β-actin, and gene expression was quantified
293 using the $2^{-\Delta\Delta Ct}$ method. The primers used (Invitrogen, California, USA) are shown in **Supplementary Table**
294 **2**.

295 All the lab experiments were performed in duplicate, data presented are averages of the duplicates. The
296 coefficient of variation intra-operator ranges between 0.03 and 1.00 for all the measurements.

297 *Oxidative stress:* To determine the oxidative stress level, we measured plasma thiobarbituric acid reactive
298 substances (TBARs), as indicators of lipid peroxidation, using ELISA (TBARS Assay Kit, Cayman Chemical, MI,
299 USA), according to the manufacturer's protocol.

300 *Statistical analyses:* The sample size was calculated on both clinical and lab outcomes; amongst clinical
301 outcome muscle mass was used; in particular sample size provide an 85% power ($p < 0.05$), 50 patients per
302 group have to be enrolled to detect a difference (alpha error = 0.05) of at least 2% variation in muscle mass,
303 based on a study on the effect of BCAAs administration on muscle mass and performance in humans (45).

304 As the patients were old and frail we assumed a possible 35% of drop out at the follow-up. In order to
305 calculate sample size for lab tests we considered as significant an increase of 1.5 fold in ATP production as
306 shown by D'Antona et al with BCAAem in aged mice (28), data on ATP production in humans by PBMCs
307 derives from Avis et al (46) based on this analyses sample size was calculated to provide 95% power
308 ($p < 0.05$) to detect a 1.5-fold difference in ATP production was 13 patients per group .

309 All the analysed variables were tested for normality by the kurtosis test, TUG, 30-s CST, 4 meters walking
310 test, TBARs, electron flux were non-Gaussian.

311 To evaluate possible differences between patients treated with BCAAem or diet advice at baseline the
312 patients were compare by one-way ANOVA for Gaussian variables and by the Mann-Whitney U test for
313 non-Gaussian ones. Gender was compared amongst patients treated with BCAAem or diet advice by χ^2 test.

314 The effect of treatment was evaluated per protocol using the two-way ANOVA for repeated measurements
315 for Gaussian variables, non-Gaussian variables were evaluated after logarithmic transformation. To
316 evaluate possible influences of mitochondrial function on muscle and cognitive performance six linear
317 regression models (GLM) were fitted, between ATP and electron flux and TUG, 30-s CST, Tinetti and 4
318 meters walking test, hand grip and MMSE, non-Gaussian variables were logarithmically transformed.

319 SPSS 24.0 were used for the analyses and $p < 0.05$ was considered statistically significant. Graphs were
320 drawn using GraphPad 7.0 for Windows.

321 *Ethics Committee approval and consent to participate.*

322 The study was approved by the Ethics Committee of our Hospital ("Comitato Etico Interaziendale A.O.U.
323 Città della Salute e della Scienza di Torino - A.O. Ordine Mauriziano - A.S.L. TO1", protocol number
324 0002637), in accordance with the ethical standards of the Declaration of Helsinki and its subsequent
325 amendments. Informed consent was obtained from all individual participants included in the study. The full
326 protocol is available upon request to the corresponding author.

327

328 **Results**

329 Patients treated with BCAAem or diet advice were comparable for all the clinical variables analysed, this
330 excludes possible selection bias that could influence our results (**Supplementary Table 3**). Compliance to
331 BCAAem was good, none of the patients have a compliance lower than 75%, the compliance ranges
332 between 75% and 90%.

333 *Treatment significantly improves general health and cognitive performance.*

334 Patients' general health measured by perceived health status equally improved in both treatment groups
335 and significantly correlated with nutritional status (MNA: $R=0.50$, $p<0.0001$; fat percentage: $R=0.26$,
336 $p=0.005$) at the end of the follow-up period. Also, the mood measured by GDS significantly improved. The
337 level of independence was not significantly influenced by treatment.

338 Patients' overall cognitive performance measured by MMSE significantly improved in patients treated with
339 BCAAem, not in patients treated with diet advice; MMSE significantly correlated with MNA ($R=0.28$,
340 $p=0.002$) as well as GDS ($R=-0.32$, $p<0.0001$) at the end of the follow-up period (**Table 1**).

341 *Treatment significantly improves nutritional status.*

342 Patients adhered to the dietary recommendation as shown by the increased caloric intake. Caloric intake
343 increased in both groups and was particularly consistent in the group treated with diet advice, where
344 dietary recommendations were reinforced by the use of an easy-to-use illustrated brochure, in this group

345 also protein intake was significantly higher. During treatment, we observed a significant improvement in
346 nutritional status measured by MNA, fat mass and BMI in both groups (**Table 2**).

347 *BCAAem treatment increases muscle mass, strength and performance.*

348 Muscle mass measured by calf and arm circumferences significantly increased in both groups as well as
349 muscular strength measured by hand grip strength (**Table 3**).

350 To evaluate whether muscular performance was influenced by treatment, we performed the TUG, the 30-s
351 CST test to evaluate both performance and resistance to fatigue. Muscular performance improved with
352 treatment as did muscle mass. Risk of falls was measured using the Tinetti scale and the TUG, mobility was
353 measured using the 4-meter gait speed test, these tests improved equally in the two groups (**Table 3**).

354 *BCAAem improve bioenergetic capacity of PBMCs.*

355 Here we show that ATP production and electron flux significantly increased over time only in mitochondria
356 from patients treated with BCAAem, and that BCAAem maintain oxidative stress at baseline values,
357 whereas, in patients treated with diet advice, oxidative stress increased over time (**Table 4**).

358 To model the relationship between mitochondria stimulation on PBMCs and effects of BCAAem or diet
359 advice on muscular and cognitive performance we applied a linear regression approach.

360 Our data showed that mitochondrial ATP production significantly predicts balance measured by Tinetti
361 after 2 months of treatment and 4 meters walking test after 1 month of treatment (**Table 5**). MMSE is
362 significantly predicted by ATP after 1 month and by electron flux after 2 months of treatment (**Table 6**).

363 We also evaluated the effect of BCAAem treatment on some of the main mitochondrial biogenesis and
364 fusion markers. Our data showed that treatment increases the expression of COX-1 and COX-4 and TFAM,
365 whereas NRF-1 shows only a non-significant trend towards the increase, significant differences versus
366 baseline levels were measured only in patients treated with BCAAem. The expression of MFN-1 and MFN-2

367 was increased although not significantly by treatment; in the BCAAem treated patients we observed an
368 increased expression of these two molecules after one month of therapy (**Table 7**).

369

370 **Discussion**

371 As life expectancy increases, adequate nutrition is fundamental for successful aging, here, we confirm that
372 amelioration of nutritional status is associated with improvement in general health status, muscle and
373 cognitive performance in old malnourished patients, and show that this may be due to an improvement of
374 their mitochondrial bioenergetics profile and decreasing oxidative stress.

375 Here we show that the diagnosis of malnutrition and its treatment, albeit using different approaches, is
376 fundamental in improving patients' general health and nutritional status. Indeed, in both our treatment
377 groups, there was an improvement in MNA, and weight gain, however the increase in caloric and protein
378 intake was higher in patients treated only with diet advice; the use of this approach instead of the use of an
379 isonitrogenous mix of non-essential aminoacids with a double blind design may be considered as a
380 limitation of the study, however the use of the "food first" approach underlines the role of the physician's
381 counselling in patients' adherence to diet. Another possible limitation of the study is the use of a "non-
382 standard" recall method for diet evaluation, here we use a recall on the 7 days before the interview as our
383 old patients usually follow a standard diet with little variation day by day, the evaluation of 7 days allow us
384 to make a more comprehensive evaluation asking the patients "what is your usual meal (breakfast, lunch,
385 dinner and snacks)? Do you change your meal during one week? If yes when and how during the previous
386 week?". The improvement in cognitive performance, measured by MMSE, was significant in patients taking
387 BCAAem supplements: indeed, patients treated with BCAAem gain, on average, 1.2 points of MMSE. This
388 result is in accordance with a previous study in a cohort of patients with severe chronic obstructive
389 pulmonary disease. However, in this study, the increase in cognitive performance was associated with
390 improved pulmonary gas exchange and there was no mechanistic explanation (47). The effect of the
391 administration of BCAAs in cognitive function was also previously assessed in severely brain damaged

392 patients with hepatic encephalopathy or traumatic brain injuries: in these patients, intravenous BCAAs
393 improve consciousness, assessed using the Glasgow Coma Scale and performance, assessed using the
394 Disability Rating Scale. However, no cognitive tests were performed in these studies and the underlying
395 mechanism was not investigated (48–52). A mechanistic explanation has been provided by the study of
396 animal models of brain injury, demonstrating the efficacy of dietary supplementation with BCAAs in
397 promoting cognitive performance, by restoring hippocampal function, given that BCAAs act as glutamate
398 and GABA precursors (53). In humans, a recent, very large retrospective study showed an association
399 between serum level of isoleucine, leucine and valine with a lower risk of dementia. This study does not
400 report any causal mechanism (54).

401 Poor nutrition and, in particular, protein-energy deficit further accelerate the loss of muscle mass and
402 function associated with age: sarcopenia (55,56). Sarcopenia increases the risk of falls, disability, frailty, loss
403 of independence and death increasing healthcare costs (57,58). Here, we show that intervention on
404 malnutrition can improve muscle mass and performance. In particular, we show an improvement in
405 balance by the Tinetti balance test and the TUG test, with a consequent reduction of the risk of falls and
406 fall-related injuries in a population with increased risk of falls (average TUG higher than 14 seconds).
407 Treatment increases muscle performance, increasing mobility, and resistance to fatigue; the improved
408 performance in the 4-meter gait speed test observed during treatment demonstrates an overall increase in
409 health and the performance status of the patients according to previous study (62). Also, muscle strength
410 measured using hand dynamometer increased in both treatment groups.

411 Taken together, these data demonstrate that dietary intervention can counteract the decrease in physical
412 and cognitive performance in old malnourished patients and that loss of muscle mass and function can be
413 countered using an appropriate treatment strategy in a rapidly aging population.

414 Several studies suggest a major role of mitochondrial dysfunction as a major contributor to aging and age-
415 related diseases [for a review, see Cedikova et al, 2016 (63)]. Mitochondrial biogenesis, ATP production and
416 oxidative phosphorylation capacity decrease with aging and production of reactive oxygen species,

417 damaged mitochondrial DNA and protein increase [for a review, see Cedikova et al, 2016 (63)]. In this
418 study, we further investigate the mechanisms underlying the better clinical effects obtained with the
419 administration of BCAAem, besides diet advice, by evaluating the treatment effects on mitochondrial
420 function, biogenesis and fusion, as well as oxidative stress. In animal models, the administration of BCAAs
421 has been shown to be effective in increasing the number and the function of mitochondria (28). In a small
422 cohort of young healthy obese and non-obese subjects, the infusion of a mixture of aminoacids increased
423 ATP production rates of muscular mitochondria in lean, but not in obese subjects (64). The increase in size,
424 number and energy production of mitochondria that follows the administration of BCAAem was associated
425 with better muscle performance, on both at skeletal and cardiac muscle level in mice (28). Here we
426 evaluated mitochondria from PBMCs, it has been previously shown that mitochondria isolated from
427 skeletal muscle and from PBMCs have a similar bioenergetics profile and are associated with gait speed in
428 older adults (65), as regards neurological tissues other authors measured mitochondrial function in PBMCs
429 and correlates its reduction with neurodegeneration (66). According with these observations we show that
430 mitochondrial activity measured using ATP production and electron flux increase is associated with both
431 cognitive and muscular performance amelioration in elderly patients; these data are particularly interesting
432 as they suggest a possible non-invasive and reliable measure to follow up treatment outcomes. We show
433 that the increase of TFAM, mitochondrial respiratory chain (COX-1 and COX-4) and mitofusin gene
434 expressions correlate with an increase in ATP production and electron flux, and suggest that BCAAem
435 treatment induces biogenesis, activity and fusion of mitochondria, stimulating at the end the bioenergetic
436 capacity of PBMCs.

437 Mitochondrial biogenesis and fusion are particularly increased after the first month of treatment.
438 Afterwards, there is a plateau with no further increase, whereas mitochondrial activity increases during the
439 entire follow-up period. This suggests that, after an increase in the number and fusion of mitochondria, the
440 organelles maintain an increased function without any further increase in their number.

441 Other than the effect on muscle mass and function, treatment of malnutrition is associated with improved
442 general health. This may be due to better energy production associated with an increase in respiratory

443 chain activity and a decrease in oxidative stress, in an experimental model of progeroid aging characterised
444 by increased DNA damage, boosting mitochondrial preserves mammalian health and increases longevity
445 (67).

446 It is well known that oxidative damage is one of the components of aging: the increase in free radical
447 production and the decrease in the defence against oxidative stress cause molecular alteration and
448 functional decay; the free radical theory of aging suggests that oxidative stress is an important factor in
449 age-associated diseases (68). Here, we show that BCAAem supplementation lowers the levels of oxidative
450 stress in elderly patients.

451 In conclusion, this study, for the first time, suggests that BCAAem treatment in old malnourished patients
452 could be a good strategy able to ameliorate the bioenergetic capacity of PBMCs, this effect may partially
453 explain the positive trend on muscle and cognitive performance in these patients.

454

455 **Conflict of interest statement and funding**

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460

461 **Statement of authorship**

462 FS and IB performed the lab experiments, acquired and analysed the lab data, and participated in
463 drafting and critically revising the manuscript. CRavetta, GC, FD, CF, FGP and PP performed the
464 clinical evaluation of patients and managed the dataset. MM, EN, CRiganti, CRuocco and GCI
465 participated in the study design and were major contributors in writing the manuscript. PD designed
466 the study, performed the statistical analyses and wrote the paper. All authors read and approved the
467 final manuscript.

468

469 **Availability of data and materials**

470 The datasets generated and/or analysed during the current study are not publicly available but are
471 available from the corresponding author upon reasonable request.

472

473

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656

657 **Table 1. Global clinical assessment at baseline and follow-up according to treatment.**

658 The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as
 659 mean \pm SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

660 * Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2
 661 months; \$ Denotes differences between 1 and 2 months; Significant values are in bold.

662

Perceived health status						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	3 \pm 0.1	-0.56 to -0.02*	2.9 \pm 0.1	-0.56 to -0.02*	Time	< 0.0001
1 month	3.3 \pm 0.1	-0.5 to 0.02\$	3.2 \pm 0.1	-0.6 to -0.04\$	Treatment	0.8437
2 months	3.5 \pm 0.1	-0.8 to -0.3**	3.5 \pm 0.2	-0.8 to -0.3**	Interaction	0.9300
MMSE ²						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	25.1 \pm 0.4	-1.1 to -0.04*	25.6 \pm 0.4	-0.2 to 0.8*	Time	0.0139
1 month	25.7 \pm 0.4	-0.8 to 0.2\$	25.3 \pm 0.5	-1.0 to 0.06\$	Treatment	0.9422
2 months	26 \pm 0.4	-1.4 to -0.3**	25.8 \pm 0.4	-0.7 to 0.4**	Interaction	0.0171

GDS ³						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	6.1±0.3	-0.4 to 0.8*	6.0±0.4	-0.2 to 0.9*	Time	0.0084
1 month	5.9±0.4	-0.3 to 0.9\$	5.7±0.4	-0.3 to 0.8\$	Treatment	0.7448
2 months	5.6±0.4	-0.1 to 1.1**	5.4±0.3	0.01 to 1.2**	Interaction	0.9184

ADL ⁴						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	10.2±0.3	-0.2 to 0.4*	9.7±0.3	-0.2 to 0.5*	Time	0.3569
1 month	10.1±0.3	-0.2 to 0.5\$	9.5±0.3	-0.5 to 0.1\$	Treatment	0.1130
2 months	10.0±0.3	-0.1 to 0.6**	9.7±0.3	-0.3 to 0.30**	Interaction	0.2294

663 ¹Branched Chain Amino Acid Enriched Mixture; ²Mini-Mental State Examination; ³Geriatric Depression Scale;

664 ⁴Activity of Daily Living.

665

666

667 **Table 2. Patients' nutritional status at baseline and follow-up according to treatment.**

668 The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as

669 mean \pm SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

670 * Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2

671 months; \$ Denotes differences between 1 and 2 months; Significant values are in bold.

672

Caloric Intake (Kcal/day)						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	Mean \pm SE	95% CI of difference	Mean \pm SE	95% CI of difference	Effect of	p
baseline	1095 \pm 36	-137 to -15.8*	1042 \pm 29	-169 to -48*	Time	<0.0001
1 month	1172 \pm 36	-80 to 42.4\$	1151 \pm 31	-168 to -47\$	Treatment	0.9740
2 months	1189 \pm 36	-156 to -34.1**	1259 \pm 38	-277 to -156**	Interaction	0.0031
Daily protein Intake (g/Kg weight)						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	Mean \pm SE	95% CI of difference	Mean \pm SE	95% CI of difference	Effect of	p
baseline	0.85 \pm 0.02	-0.03 to 0.09*	0.87 \pm 0.02	-0.20 to -0.04*	Time	0.0874
1 month	0.83 \pm 0.02	-0.06 to 0.06\$	0.97 \pm 0.02	-0.04 to 0.08\$	Treatment	0.0001

2 months	0.83±0.02	-0.03 to 0.09**	0.96±0.02	-0.14 to -0.02**	Interaction	0.0007
MNA²						
	BCAAem¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	14.8±0.26	-0.03 to 0.09*	14.9±0.28	-0.16 to -0.04*	Time	<0.0001
1 month	18.0±0.43	-0.06 to 0.06\$	17.8±0.38	-0.04 to 0.08\$	Treatment	0.9057
2 months	18.9±0.5	-0.03 to 0.09**	18.8±0.47	-0.14 to -0.02**	Interaction	0.8366
Fat mass (%)						
	BCAAem¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	18.8±0.94	-1.68 to 0.61*	20.2±0.88	-1.7 to 0.6*	Time	0.0009
1 month	19.3±0.92	-2.16 to 0.13\$	20.8±0.88	-1.6 to 0.7\$	Treatment	0.1708
2 months	20.3±0.94	-2.70 to -0.41**	21.2±0.88	-2.2 to 0.1**	Interaction	0.6438
BMI³						
	BCAAem¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>

baseline	20.5±0.42	-1.42 to 0.10*	20.8±0.41	-1.38 to 0.14*	Time	0.0010
1 month	21.2±0.68	-0.92 to 0.60\$	21.5±0.42	-0.81 to 0.71\$	Treatment	0.7239
2 months	21.3±0.66	-1.57 to -0.05**	21.5±0.41	-1.43 to 0.09**	Interaction	0.9482

673 ¹Branched Chain Amino Acid Enriched Mixture; ²Mini Nutritional Assesment test; ³Body Mass Index.

674

675

676 **Table 3. Muscle mass, strength and performance at baseline and follow-up according to treatment.**

677 The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as

678 mean \pm SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

679 * Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2

680 months; \$ Denotes differences between 1 and 2 months; Significant values are in bold.

Muscle mass						
Calf circumference (cm)						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	30.4 \pm 0.35	-1.14 to -0.04*	30.7 \pm 0.43	-1.08 to 0.02*	Time	0.0004
1 month	31.0 \pm 0.38	-0.87 to 0.23\$	31.2 \pm 0.40	-0.54 to 0.56\$	Treatment	0.8560
2 months	31.3 \pm 0.39	-1.45 to -0.36**	31.19 \pm 0.39	-1.07 to 0.03**	Interaction	0.4521
Arm circumference (cm)						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	22.7 \pm 0.36	-0.75 to 0.23*	23.0 \pm 0.40	-0.83 to 0.15*	Time	0.0045
1 month	23.0 \pm 0.37	-0.77 to 0.21\$	23.3 \pm 0.40	-0.54 to 0.44\$	Treatment	0.6754

2 months	23.3±0.38	-1.03 to -0.04**	23.4±0.44	-0.88 to 0.10**	Interaction	0.7351
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Muscle strength

Hand grip (Kg)

	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	17.9±1.0	-2.01 to 0.47*	17.9±1.0	-1.84 to 0.65*	Time	0.0474
1 month	18.5±1.0	-1.6 to 0.87\$	18.7±0.9	-1.0 to 1.50\$	Treatment	0.7796
2 months	18.3±1.0	-2.40 to 0.10**	19.1±1.0	-1.59 to 0.90**	Interaction	0.5231

TUG (sec)²

	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	19.8±2.14	1.5 to 7.6*	20.5±1.5	-1.2 to 4.9*	Time	0.0001
1 month	15.2±1.0	-2.9 to 3.2\$	18.7±1.6	-2.1 to 4.0\$	Treatment	0.2780
2 months	15.1±1.1	1.6 to 7.8**	17.7±1.7	-0.3 to 5.9**	Interaction	0.3215

30-s CST³

	BCAAem ¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>

	<i>difference</i>		<i>difference</i>			
baseline	6.8±0.5	-2.6 to -0.7*	6.0±0.5	-2.4 to -0.5*	Time	<0.0001
1 month	8.4±0.6	-1.0 to 0.88\$	7.4±0.5	-1.6 to 0.3\$	Treatment	0.3328
2 months	8.5±0.7	-2.7 to -0.7**	8.1±0.6	-3.0 to -1.1**	Interaction	0.5810
Tinetti						
	BCAAem¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	20.4±0.8	-2.1 to -0.1*	18.3±0.8	-3.2 to -1.1*	Time	< 0.0001
1 month	21.5±0.7	-1.7 to 0.3\$	20.4±0.8	-1.3 to 0.7\$	Treatment	0.1503
2 months	22.2±0.7	-2.8 to -0.8**	20.7±0.9	-3.4 to -1.4**	Interaction	0.2076
4 meters walking test (sec)						
	BCAAem¹		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	8.2±0.6	-0.3 to 1.7*	9.8±0.7	0.4 to 2.3*	Time	<0.0001
1 month	7.5±0.6	-0.7 to 1.3\$	8.4±0.7	-0.6 to 1.4\$	Treatment	0.1684
2 months	7.2±0.6	0.04 to 2.0**	8.0±0.7	0.8 to 2.8**	Interaction	0.3955

681 ¹Branched Chain Amino Acid Enriched Mixture; ²Timed Up and Go test; ³30 seconds Chair Sit to Stand test.

682

683 **Table 4. Mitochondrial activity and oxidative stress at baseline and follow-up according to treatment.**

684 The table shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as
 685 mean ± SE and 95% CI of the difference between basal, 1 and 2 months of treatment.

686 * Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2
 687 months; \$ Denotes differences between 1 and 2 months; Significant values are in bold.

688

ATP (changes vs baseline)						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	Mean±SE	95% CI of difference	Mean±SE	95% CI of difference	Effect of	p
baseline	1.00±0.00	-0.45 to -0.15*	1.00±0.00	-0.13 to 0.17*	Time	0.0001
1 month	1.30±0.09	-0.28 to 0.016\$	0.98±0.01	-0.16 to 0.14\$	Treatment	0.0005
2 months	1.43±0.10	-0.58 to -0.28**	0.99±0.02	-0.14 to 0.16**	Interaction	0.0001
Electron flux (changes vs baseline)						
	BCAAem ¹		Diet advice		Two-way ANOVA	
	Mean±SE	95% CI of difference	Mean±SE	95% CI of difference	Effect of	p
baseline	1.00±0.00	-0.38 to -0.13*	1.00±0.00	-0.13 to 0.13*	Time	< 0.0001
1 month	1.26±0.05	-0.37 to -0.12\$	1.00±0.01	-0.14 to 0.12\$	Treatment	< 0.0001

	TBARs ² (µg/M)				Two-way ANOVA	
	BCAAem ¹		Diet advice		Effect of	p
	Mean±SE	95% CI of difference	Mean±SE	95% CI of difference		
2 months	1.50±0.09	-0.62 to -0.38**	1.01±0.04	-0.14 to 0.12**	Interaction	< 0.0001
baseline	2.3±0.4	-2.8 to 1.2*	4.1±0.7	-3.02 to 0.97*	Time	0.0007
1 month	3.0±0.57	-2.4 to 1.6\$	4.5±0.9	-4.61 to -0.61\$	Treatment	0.0289
2 months	3.2±0.70	-3.1 to 0.85**	6.7±1.3	-5.64 to -1.64**	Interaction	0.0332

689 ¹Branched Chain Amino Acid Enriched Mixture; ²Thiobarbituric Acid Reactive Substances.

690

691

692 **Table 5. Muscle performance and balance.**

693 The table shows the results for linear regression models (GLM), non-Gaussian variables indicated by * were

694 logarithmically transformed. Significant values are in bold.

695

Dependent variable	Co-variate	$\beta \pm SE$	t	p	95% CI
Tinetti baseline	<i>Slope</i>	18.9 \pm 2.7	7.0	0.000	13.5 to 24.4
	<i>Electronflux baseline</i>	-0.02 \pm 0.01	-1.4	0.179	-0.05 to 0.009
	<i>ATP baseline</i>	0.05 \pm 0.03	1.5	0.134	-0.016 to 0.119
Tinetti 1 month	<i>Slope</i>	20.0 \pm 2.9	6.8	0.000	14.1 to 25.8
	<i>Electronflux 1 month*</i>	0.000 \pm 0.013	0.02	0.984	-0.03 to 0.26
	<i>ATP 1 month</i>	0.011 \pm 0.04	0.32	0.752	-0.06 to 0.08
Tinetti 2 months	<i>Slope</i>	48.9 \pm 13.5	3.6	0.001	21.8 to 75.9
	<i>Electronflux 2 months*</i>	0.05 \pm 0.04	1.5	0.131	-0.02 to 0.122
	<i>ATP 2 months</i>	-13.5 \pm 6.2	-2.2	0.033	-25.9 to -1.1
Dependent variable	Co-variate	$\beta \pm SE$	t	p	95% CI
4 meters walking test baseline*	<i>Slope</i>	0.89 \pm 0.08	11.2	0.000	0.7 to 1.0
	<i>Electronflux baseline</i>	0.000 \pm 0.000	0.8	0.394	0.000 to 0.001
	<i>ATP baseline</i>	-0.001 \pm 0.001	-1.4	0.175	-0.003 to 0.001

4 meters walking test 1 month*	<i>Slope</i>	0.96±0.10	9.6	0.000	0.76 to 1.16
	<i>Electronflux 1 month*</i>	0.000±0.000	0.40	0.691	-0.001 to 0.001
	<i>ATP 1 month</i>	-0.002±0.001	-2.0	0.045	-0.005 to -6.1E⁻⁵
4 meters walking test 2 months*	<i>Slope</i>	0.42±0.46	0.92	0.361	-0.49 to 1.33
	<i>Electronflux 2 months*</i>	-0.002±0.001	-1.73	0.089	-0.004 to 0.000
	<i>ATP 2 months</i>	0.23±0.21	1.08	0.283	-0.19 to 0.644

696

697 **Table 6. Cognitive performance.**

698 The table shows the results for linear regression models (GLM), non-Gaussian variables indicated by * were

699 logarithmically transformed. Significant values are in bold.

Dependent variable	Co-variate	$\beta \pm SE$	t	p	95% CI
MMSE ¹ baseline	<i>Slope</i>	24.3±1.4	17.2	0.000	21.5 to 27.2
	<i>Electronflux baseline</i>	0.002±0.007	0.22	0.829	-0.01 to 0.02
	<i>ATP baseline</i>	0.02±0.018	0.99	0.327	-0.02 to 0.05
MMSE ¹ 1 month	<i>Slope</i>	22.7±0.17	12.9	0.000	19.2 to 26.20
	<i>Electronflux 1 month*</i>	-0.002±0.08	-0.27	0.790	-0.02 to 0.013
	<i>ATP 1 month</i>	0.048±0.02	2.29	0.026	0.006 to 0.09
MMSE ¹ 2 months	<i>Slope</i>	29.9±7.9	3.8	0.000	14.01 to 45.7
	<i>Electronflux 2 months*</i>	0.05±0.02	2.5	0.014	0.01 to 0.09

	<i>ATP 2 months</i>	-3.31±3.6	-0.9	0.366	-10.6 to 3.9
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700 ¹Mini-Mental State Examination.

701

702

703 **Table 7. Mitochondria biogenesis and fusion at baseline and follow-up according to treatment.** The table704 shows multiple T-test and Two-way ANOVA for multiple measure results. Values are shown as mean \pm SE

705 and 95% CI of the difference between basal, 1 and 2 months of treatment.

706 * Denotes differences between baseline and 1 month; ** Denotes differences between baseline and 2

707 months; § Denotes differences between 1 and 2 months; Significant values are in bold.

708

COX-1 ¹ (changes vs baseline)						
	BCAAem ²		Diet advice		Two-way ANOVA	
	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	1.0 \pm 0.0	-23.8 to -1.9*	1.0 \pm 0.0	-11.4 to 10.6*	Time	0.1155
1 month	13.9 \pm 8.5	-4.4 to 17.5§	1.4 \pm 0.3	-13.2 to 8.7§	Treatment	0.1967
2 months	7.3 \pm 3.6	-17.3 to 4.7**	3.7 \pm 1.2	-13.6 to 8.3**	Interaction	0.1409
COX-4 ³ (changes vs baseline)						
	BCAAem ²		Diet advice		Two-way ANOVA	
	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Mean\pmSE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	1.0 \pm 0.0	-2.3 to -0.10*	1.0 \pm 0.0	-1.39 to 0.76*	Time	0.0459
1 month	2.2 \pm 0.7	-0.7 to 1.47§	1.3 \pm 0.09	-1.1 to 1.04§	Treatment	0.2373

2 months	1.8±0.5	-1.9 to 0.3**	1.3±0.19	-1.4 to 0.73**	Interaction	0.3786
TFAM⁴ (changes vs baseline)						
	BCAAem²		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	1.0±0.0	-6.9 to -0.6*	1.0±0.0	-3.8 to 2.5*	Time	0.0178
1 month	4.8±1.0	-2.4 to 3.9\$	1.7±0.5	-4.5 to 1.8\$	Treatment	0.0932
2 months	4.2±1.15	-6.2 to 0.1**	3.0±1.5	-5.1 to 1.2**	Interaction	0.2235
NRF-1⁵ (changes vs baseline)						
	BCAAem²		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	1.0±0.0	-20.2 to 3.5*	1.0±0.0	-13.5 to 10.2*	Time	0.6599
1 month	9.4±6.4	-14.1 to 9.6\$	2.6±0.7	-12.8 to 10.9\$	Treatment	0.3507
2 months	11.6±9.5	-22.4 to 1.3**	3.6±1.2	-14.4 to 9.3**	Interaction	0.2055

MFN-1 ⁶ (changes vs baseline)						
	BCAAem ²		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	1.0±0.0	-22.4 to -2.1*	1.0±0.0	-10.8 to 9.4*	Time	0.0746
1 month	13.2±7.7	-7.0 to 13.3\$	1.7±0.3	-10.3 to 10.0\$	Treatment	0.1648
2 months	10.1±6.1	-19.3 to 1.0**	1.8±0.3	-11.0 to 9.3**	Interaction	0.1320

MFN-2 ⁷ (changes vs baseline)						
	BCAAem ²		Diet advice		Two-way ANOVA	
	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Mean±SE</i>	<i>95% CI of difference</i>	<i>Effect of</i>	<i>p</i>
baseline	1.0±0.0	-11.6 to -1.1*	1.0±0.0	-6.0 to 4.5*	Time	0.0772
1 month	7.3±4.1	-1.9 to 8.6\$	1.7±0.3	-5.9 to 4.6\$	Treatment	0.2046
2 months	3.9±1.6	-8.2 to 2.3**	2.4±0.5	-6.6 to 3.9**	Interaction	0.1810

¹Cytochrome C Oxidase-1; ²Branched Chain Amino Acid Enriched Mixture; ³Cytochrome C Oxidase- 4;

⁴Mitochondrial Transcription Factor A; ⁵Nuclear Respiratory Factor-1; ⁶Mitofusin-1; ⁷Mitofusin-2.