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ORIGINAL ARTICLE

Leucine and citrulline modulate muscle function in malnourished aged rats

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Abstract Protein energy malnutrition in the elderly causes preferential loss of muscle mass which is associated with poor functional states. Leucine and citrulline are able to stimulate muscle protein synthesis in aged rats but no study has been undertaken to evaluate their effect on muscle function. Sprague–Dawley male rats aged 23 months were used in the experiment. Part of them were subjected to a dietary restriction for 12 weeks and then assigned to four groups: a group was euthanized (restricted group), and the others were refed for 1 week with either a leucine-, a citrulline-supplemented diet, or a standard diet. The other rats were fed ad libitum. Muscle mass and motor activity significantly increased during the refeeding with either leucine or citrulline (respectively, +51 and +37% for muscle mass, $P < 0.05$). The improvement of muscle mass and of motor activity induced by leucine and citrulline was highly associated with that of maximal tetanic isometric force ($r = 0.769$, $P < 0.0001$; $r = 0.389$, $P < 0.05$, respectively) but only leucine improved maximal tetanic isometric force (+101%, $P < 0.05$). In conclusion, this is the first study to demonstrate the ability of two amino acids (leucine and citrulline) to modulate muscle function.

Keywords Amino acids · Muscle mass · Maximal tetanic isometric force · Motor activity · Ageing

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Abbreviations

AA	Amino acids
Cit	Citrulline
Leu	Leucine
AL	Ad libitum
R	Restricted
NEAA	Non-essential amino acids
isoN	Isonitrogenous
TCA	Trichloroacetic acid

Introduction

Malnutrition in the elderly is a major health and societal concern as it contributes to increased morbidity and mortality (Bouillanne et al. 2005; Kagansky et al. 2005). Indeed, 4–10% of elderly people who live in community and up to 70% of those who are hospitalized are concerned

by protein energy malnutrition (HAS 2007; Sullivan et al. 1995). Malnutrition contributes to enhanced risk of falls, fractures, infections and blunted response to aggression, which leads to disability and poor quality of life in the elderly (Capodaglio et al. 2007; Liu and Latham 2009). Considering these alarming data and the rising number of elderly people in western countries, there is an urgent need to find a way to limit or avoid malnutrition among them.

The ability to recover from a malnourished state is decreased in elderly patients and in rodents (Hebuterne et al. 1995, 1997; Seiler 2001; Walrand et al. 2000). The resistance to renutrition is multifactorial but seems to be mainly due to the significant increase in amino acid (AA) catabolism within the splanchnic area which occurs in the elderly (Boirie et al. 1997; Volpi et al. 2001) and in aged rats (Jourdan et al. 2007). Moreover, it has been shown that post-prandial hyperaminoacidemia is lower in aged rats than in young rats (Mosoni et al. 1993). This results in inadequate systemic plasma levels of AA (Mosoni et al. 1995), low aminoacidemia and blunted protein synthesis rates at the postprandial state (Dardevet et al. 2002). In addition, there is a resistance to anabolic stimuli at muscle level: anabolic response of muscle to orally ingested AA is impaired in the elderly compared with adults, in whom similar AA load stimulates muscle protein synthesis (Guillet et al. 2004).

However, recent research suggests that nutritional manipulations may be an efficient way to preserve muscle mass and to improve muscle function. Indeed, leucine (Leu) does not escape the splanchnic area extraction in aged subjects, but it has been shown to be the most efficient amino acid among other essential AA to stimulate muscle protein synthesis in the elderly (Dardevet et al. 2002; Katsanos et al. 2006; Rieu et al. 2003, 2007) in post-prandial state. Conversely, citrulline (Cit), a non-protein AA, that has been for long under-estimated (Curis et al. 2005), escapes the splanchnic extraction. Two recent studies have shown that Cit is an activator of muscle protein synthesis in malnourished aged rats (Osowska et al. 2006) and in healthy subjects fed a hypoprotein diet (Jourdan et al. 2008), respectively (for a complete review, see Moinard and Cynober 2007).

An improvement of muscle protein synthesis per se has limited interest (Clark and Manini 2008) if it does not lead to an improvement of muscle function, which enables aged people to preserve their mobility and independence. To the best of our knowledge, so far, no nutritional intervention alone has been shown to improve muscle function and the link between increased muscle protein synthesis and improvement of muscle function is not established. As it is already established that Leu and Cit both restore muscle protein synthesis in the late ages of life, we intended to determine whether this anabolic action is associated with an improvement of muscle function. If so, these AA could represent an interesting nutritional strategy to prevent and treat protein-energy malnutrition that occurs in elderly subjects.

The aim of this study was to evaluate the effect of diets supplemented with Cit or Leu on parameters reflecting muscle function and associated behaviours in a validated model of protein-energy malnutrition in aged rats (Chambon-Savanovitch et al. 1999; Felgines et al. 1999; Walrand et al. 2000), at the post-absorptive state.

Materials and methods

Animal care complied with the French regulations for the protection of animals used for experimental and other scientific purposes (D 2001-486) and with European Community regulations (Official Journal of the European Community, L538 12:18:1986). The experimental protocol was approved by the local ethics committee for animal research (registered number P2.CM.035.07).

All the chemicals and AA used in this experiment were purchased from Sigma (Saint Quentin, Fallavier, France) except for Cit which was a gift from Kyowa Hakko (Tokyo, Japan).

Animals

Thirty-seven male Sprague–Dawley rats, 23 months of age (Charles-River, L'Arbresle, France) were housed individually at 20–23°C with an alternate 12-h light–dark cycle. They had free access to water. During the 3-week acclimatization period all rats were fed ad libitum (AL) with a standard diet containing 17% (w/w) proteins, 3% (w/w) lipids, 59% (w/w) carbohydrates, 21% (w/w) water, fibres, vitamins and minerals (Safe, Villemoisson-sur-Orge, France). Daily spontaneous intakes were recorded.

Study design

After the acclimatization period, the rats were randomized into the following five groups (Fig. 1):

- AL group: the rats were fed AL during 13 weeks and then killed.
- Restricted group (R): the rats underwent a dietary restriction for 12 weeks (50% of their spontaneous intakes recorded during the acclimatization period) (Osowska et al. 2006; Walrand et al. 2000) and were killed at the end of this period of restriction.
- CIT group: after 12 weeks of dietary restriction, the rats were refed for 1 week with a standard diet supplemented with 5 g Cit/kg/day, which represents 1.2 g N/kg/day,

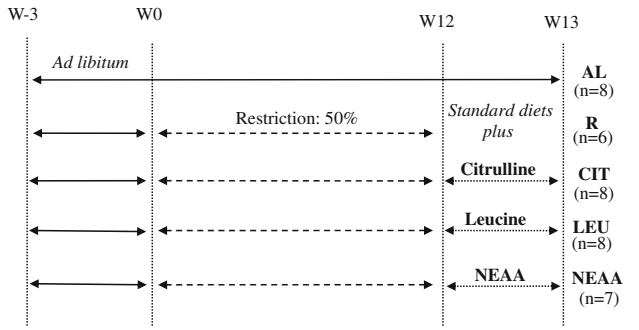

Fig. 1 Study design. *AL* rats fed ad libitum for 12 weeks, *R* rats diet-restricted (50% of spontaneous intake) for 12 weeks, *NEAA*, *LEU* and *CIT*, rats restricted for 12 weeks and then refed for 1 week with an NEAA-, a Leu- or a Cit-enriched diet, respectively. Diets were isonitrogenous and isoenergetic

and then killed. This dose of Cit is the extrapolation of doses used in humans taking into consideration that the metabolic rate and nitrogen requirements of rats are 10 times those of humans (Osowska et al. 2006). Food was limited to 90% of spontaneous intake to ensure the rats ate their entire ration and hence received the same amounts of food.

- LEU group: after 12 weeks of dietary restriction, the rats were refed for 1 week with a standard diet supplemented with Leu (11.2 g/kg/day) in isonitrogenous quantity to the Cit diet, i.e. 1.2 g N/kg/day.
- Non-essential amino acids (NEAA) group: after 12 weeks of dietary restriction, the rats were refed for 1 week with a standard diet supplemented with NEAA (alanine, glycine, proline, histidine, aspartate and serine, in equimolar ratio) isonitrogenous to the Cit and Leu diets, i.e. 1.2 g N/kg/day.

On the last day of the experiment, the rats at the post-absorptive state were submitted to a behavioural test aimed to determine motor activity and spontaneous alternation. Maximal tetanic isometric force was measured by electrophysiological methods (see below for details); blood was sampled and the *tibialis* anterior muscle was rapidly removed and weighed just after the rat was killed. *Tibialis* muscle was chosen because of its richness in type 2 fibres, mainly affected by age.

Motor activity and spontaneous alternation behaviour

The rats were tested for spontaneous alternation and motor activity behaviour in a *Y*-maze. The apparatus consisted of a symmetrical *Y*-maze with 40-cm long, 15-cm wide and 35-cm high arms as described previously (Moinard et al. 2004). The apparatus was illuminated from above (35 lux) and the floor was covered with a small amount of sawdust. Behaviour was observed by a trained experimenter. The rats were put in one arm of the maze and the sequence and number of arm entries were recorded over a period of 10 min. An arm visit was recorded when a rat moved all four paws into the arm. Scoring of alternations consisted in the evaluation of response sequences, in which entering the arm least recently visited was considered an alternation response. The proportion of alternations was computed by dividing the number of alternations by the total number of arm visits. To obtain an accurate evaluation of the qualitative aspects of the behaviour, a minimum of nine visits was retained to calculate the percentage of spontaneous alternation behaviour. This test allowed us to determine two behavioural parameters as the proportion of alternations is a good indicator of attention and/or related functions, and the total number of visited arms is a good indicator of motor activity.

Maximal tetanic isometric force

At the end of the experiment, the rats were anaesthetized with isoflurane and the *tibialis* anterior muscle function was evaluated by measuring in situ muscle contraction in response to nerve stimulation (Vignaud et al. 2003). Their knees and feet were fixed using clamps and pins. The distal tendons of the *tibialis* muscles were attached to an isometric transducer (Harvard Bioscience, Les Ulis, France) using a silk ligature. All data provided by the isometric transducer were recorded and analysed on a microcomputer (PowerLab system: ADInstruments, Paris, France). Great care was taken to ensure that blood and nerve supply remained intact during surgery. The sciatic nerves were proximally crushed and distally stimulated by bipolar silver electrodes using supramaximal square wave pulses of 0.1 ms duration. All isometric measurements were then made after a 5-min rest period and at an initial muscle length of L_0 (length at which maximal tension was obtained during the twitch). Several tetani responses (75–145 Hz, 500 ms) were recorded in succession, with at least a 1-min interval between tetani contractions. Maximal tetanic isometric force was determined. In this study, the aforementioned muscle function was limited to maximal tetanic isometric force and motor activity. The rats were then killed by decapitation.

Muscle mass

The rats were killed and *tibialis* muscles were rapidly removed, weighed, frozen in liquid nitrogen and stored at −80°C until analysis. The other leg muscles were then removed and weighed to assess muscle:body weight ratios in order to evaluate how supplements affected muscle mass relative to whole body mass.

Table 1 Animal characteristics

	AL	R	NEAA	LEU	CIT
Dietary intakes (g/day)	32 ± 1	35 ± 1	34 ± 2	32 ± 2	34 ± 1
Animal weights (g) W0	661 ± 18	657 ± 12	695 ± 27	663 ± 18	687 ± 21
Animal weights (g) W12	599 ± 30	406 ± 10*	433 ± 12*	443 ± 13*	436 ± 8*
Animal weights (g) W13	577 ± 33		444 ± 14*	459 ± 11*	456 ± 7*

Weights of the animals are given at WO, W12 after the restriction period, and W13 after renutrition. Dietary intakes measured during the acclimatization period are expressed in g/day. ANOVA + PLSD Fischer test: * $P < 0.05$ versus AL

Muscle protein content

The frozen *tibialis muscles* were pulverized and homogenized in ice-cold 10% trichloroacetic acid (TCA) (1 ml TCA/100 mg tissue) using an Ultra-Turrax T25 tissue disrupter (Ika Labortechnik, Staufen, Germany). After delipidation with ethanol–ether (1:1, vol/vol), the precipitate was dissolved in 1 N NaOH (4 ml/100 mg tissue) for 12 h at 40°C. The total protein content was then determined according to the method described by Fleury and Eberhard (1951) on a Genesys spectrophotometer (ThermoSpectronic, New York, NY).

Aminoacidemia

Just after decapitation, blood was sampled in heparinized tubes, which were rapidly centrifuged. Plasma free amino acid (AAs) concentrations were analyzed by ion-exchange chromatography, as described in Osowska et al. 2006.

Statistical analysis

Data are expressed as means ± SEM. Comparisons between sets of data were made using one-way analysis of variance (ANOVA) followed by the PLSD Fisher test. Correlations were made by using the *Z*-correlation test. The Statview software was used. Differences at $P < 0.05$ were considered significant.

Results

Animal characteristics

Animal weights at the beginning of the experimental procedure (i.e. W0), at the end of the restriction period (i.e. W12) and after renutrition (i.e. W13) are given in Table 1, together with dietary intakes measured during the acclimatization period.

Muscle mass

Tibialis anterior muscles of the Leu-refed ($n = 8$) rats were significantly heavier (0.62 ± 0.03 g) than those of the AL-fed rats ($n = 8$), R rats ($n = 6$) and NEAA-refed rats ($n = 7$) (0.41 ± 0.05, 0.44 ± 0.03 and 0.47 ± 0.03 g, respectively; $P < 0.05$). The rats refed with Cit ($n = 8$) had a significantly increased *tibialis* mass (0.56 ± 0.04 g) compared with the AL-fed rats ($P < 0.05$) (Fig. 2). Weights of the other leg muscles are given in Table 2. There were no significant between-group differences. Muscle:body weight ratios were higher in the Cit- and Leu-refed rats than AL-fed rats (1.13 ± 0.06 and 1.11 ± 0.05 vs. 0.68 ± 0.04, respectively; $P < 0.05$). Cit also improved muscle:body weights ratio compared with NEAA (i.e. 0.95 ± 0.03; $P < 0.05$).

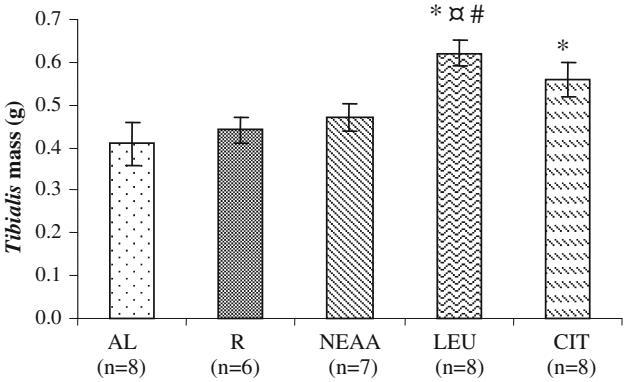

Fig. 2 Effects of the different diets on *tibialis* mass. *AL* rats fed ad libitum for 12 weeks, *R* rats diet-restricted (50% of spontaneous intake) for 12 weeks, *NEAA*, *LEU* and *CIT*, rats restricted for 12 weeks and then refed for 1 week with an NEAA-, a Leu- or a Cit-enriched diet, respectively. Diets were isonitrogenous and isoenergetic. ANOVA + PLSD Fischer test: $^{*}P < 0.05$ versus AL, $^{¤}P < 0.05$ versus NEAA, $^{\#}P < 0.05$ versus R

Muscle protein content

Muscle protein content of the Cit- and Leu-refed rats was strongly higher (i.e. +66 and 71%, respectively) compared with the R rats (116 ± 9 and 113 ± 10 vs. 68 ± 8 mg, respectively) and around 15% greater than that of the AL-fed rats (100 ± 18 mg) (Fig. 3). However, the differences did not reach significance. Of note, the muscle protein content and muscle mass were correlated ($r = 0.854$, $P < 0.0001$) (Fig. 4).

Table 2 Weights of the rate leg muscles, expressed in g

	AL	R	NEAA	LEU	CIT
Soleus	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.19 ± 0.01
Plantaris	0.23 ± 0.02	0.26 ± 0.02	0.24 ± 0.02	0.29 ± 0.02	0.30 ± 0.01
Gastrocnemius	1.07 ± 0.09	1.10 ± 0.06	1.10 ± 0.05	1.28 ± 0.04	1.37 ± 0.09
EDL	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.17 ± 0.01

AL rats fed ad libitum for 12 weeks, *R* rats diet-restricted (50% of spontaneous intake) for 12 weeks, *NEAA*, *LEU* and *CIT*, rats restricted for 12 weeks and then refed for 1 week with an NEAA-, a Leu- or a Cit-enriched diet, respectively. Diets were isonitrogenous and isoenergetic. ANOVA

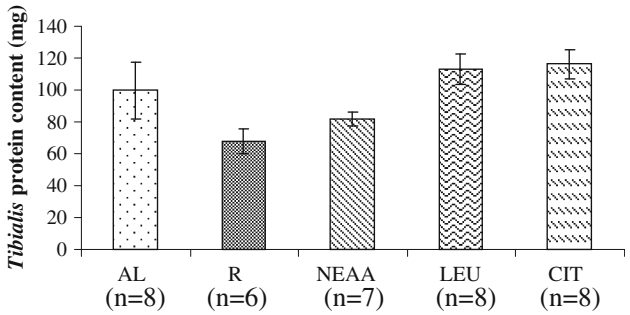

Fig. 3 Effects of the different diets on *tibialis* protein content. *AL* rats fed ad libitum for 12 weeks, *R* rats diet-restricted (50% of spontaneous intake) for 12 weeks, *NEAA*, *LEU* and *CIT*, rats restricted for 12 weeks and then refed for 1 week with an NEAA-, a Leu- or a Cit-enriched diet, respectively. Diets were isonitrogenous and isoenergetic. ANOVA

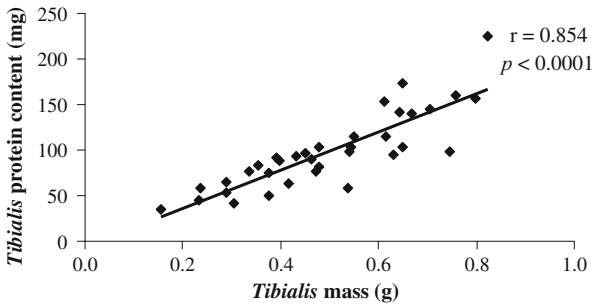

Fig. 4 Correlation between *tibialis* mass and *tibialis* protein content. *Z*-correlation test: $r = 0.854$, $P < 0.0001$

Maximal tetanic isometric force

Maximal tetanic isometric force was significantly increased ($P < 0.05$) in the Leu-refed (459 ± 42 g) rats compared with the AL-fed rats and the NEAA-refed rats (228 ± 51 and 260 ± 38 g, respectively) (Fig. 5). For Cit (360 ± 31 g), the increase did not reach significance ($P = 0.068$).

Y-maze test

The rats refed with either Leu or Cit visited significantly more arms of the *Y*-maze than the AL-fed rats (21 ± 2 and 19 ± 2 vs. 13 ± 1, respectively; $P < 0.05$) (Fig. 6), and this latter parameter was correlated with *tibialis* mass ($r = 0.417$, $P < 0.01$) (Fig. 7). The spontaneous alternation, ranging from 60 to 70%, was not affected by the different diets (data not shown).

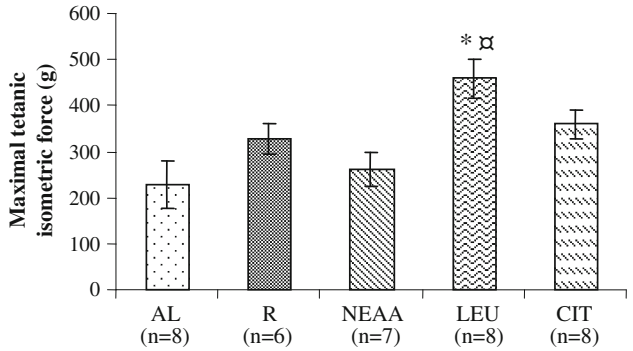

Fig. 5 Effects of the different diets on the maximal tetanic isometric force. *AL* rats fed ad libitum for 12 weeks, *R* rats diet-restricted (50% of spontaneous intake) for 12 weeks, *NEAA*, *LEU* and *CIT*, rats restricted for 12 weeks and then refed for 1 week with an NEAA-, a Leu- or a Cit-enriched diet, respectively. Diets were isonitrogenous and isoenergetic. ANOVA + PLSD Fischer test: $*P < 0.05$ versus AL, $¤P < 0.05$ versus NEAA

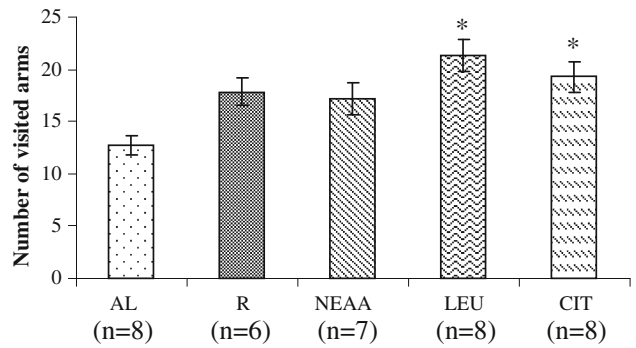

Fig. 6 Effects of the different diets on the number of visited arms in the *Y* maze. *AL* rats fed ad libitum for 12 weeks, *R* rats diet-restricted (50% of spontaneous intake) for 12 weeks, *NEAA*, *LEU* and *CIT*, rats restricted for 12 weeks and then refed for 1 week with an NEAA-, a Leu- or a Cit-enriched diet, respectively. Diets were isonitrogenous and isoenergetic. ANOVA + PLSD Fischer test: $*P < 0.05$ versus AL

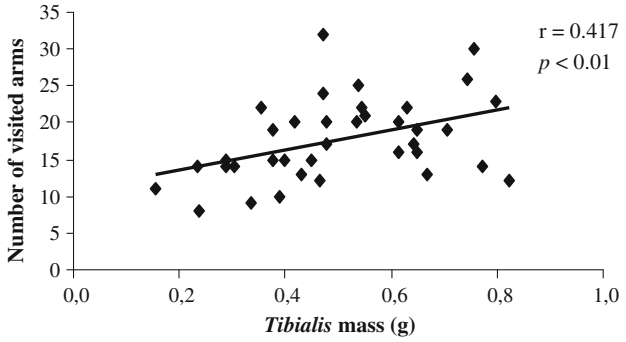

Fig. 7 Correlation between *tibialis* mass and number of visited arms. *Z*-correlation test: $r = 0.417$, $P < 0.01$

Other correlations between parameters

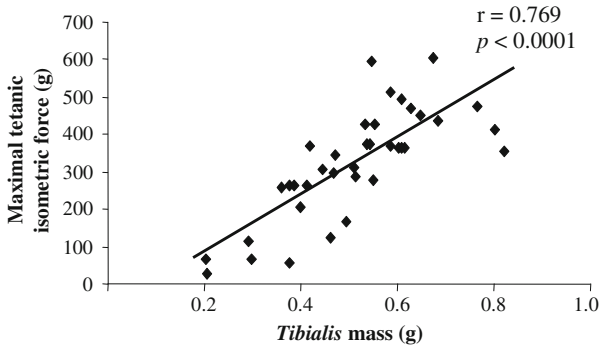

Fig. 8 Correlation between *tibialis* mass and maximal tetanic isometric force. *Z*-correlation test: $r = 0.769$, $P < 0.0001$

Maximal tetanic isometric force was strongly correlated with *tibialis* mass ($r = 0.769$, $P < 0.0001$) (Fig. 8). A relationship was also shown between maximal tetanic isometric force and the number of visited arms, which reflects motor activity and exploration ($r = 0.389$, $P < 0.05$, not shown).

Plasma free amino-acid concentrations

Table 3 reports the plasma amino acid concentrations that were significantly modified by the diets. Plasma Cit, ornithine and arginine concentrations were significantly enhanced in the Cit-supplemented-rats. Plasma Leu concentrations were increased in Leu-refed rats.

Discussion

Decreased muscle mass (i.e. sarcopenia, Rolland and Vellas 2009) and function (i.e. dynapenia, Clark and Manini 2008) may lead to frailty and disability in the elderly. To our knowledge, there is in this population no evidence of benefits of nutritional supplements per se to improve maximal tetanic isometric force and motor activity represented here by the mention muscle function.

A recent Cochrane review showed protein-energy supplementation induces weight gain, reduces mortality and complications in malnourished elderly patients, but there is no evidence for functional benefit (Milne et al. 2009). The effect of dietary counselling and multivitamin supplementation during 4 months was studied in discharged geriatric patients at risk of malnutrition. This nutritional intervention allowed them to maintain weight and improve activities of daily living, but muscle strength (grip strength) recovering was not improved (Persson et al. 2007). In a 12-week-resistance training program in aged men, muscle

Table 3 Plasma amino acid concentrations (μmol/L)

AA plasma	AL	R	NEAA	LEU	CIT
Asp	14 ± 1	9 ± 0*	9 ± 1*	9 ± 1*	8 ± 1*
Thr	340 ± 10	453 ± 31*	294 ± 22$^{\#}$	390 ± 33$^{¤}$	214 ± 20$^{*,\#,\circ}$
Ser	346 ± 12	392 ± 10	338 ± 31	424 ± 17$^{*,¤}$	234 ± 11$^{*,\#,¤,\circ}$
Asn	59 ± 1	70 ± 3	60 ± 5	69 ± 4	44 ± 2$^{*,\#,¤,\circ}$
Glu	91 ± 5	50 ± 4*	58 ± 6*	57 ± 4*	60 ± 4*
Gln	701 ± 28$^{\#}$	891 ± 44	669 ± 53$^{\#}$	723 ± 35$^{\#}$	653 ± 31$^{\#}$
Gly	293 ± 38$^{\#}$	489 ± 61	304 ± 22$^{\#}$	258 ± 13$^{\#}$	228 ± 18$^{\#}$
Cit	115 ± 11	137 ± 12	113 ± 8	94 ± 7	1044 ± 148$^{*,\#,¤,\circ}$
Met	39 ± 2	36 ± 2	32 ± 2*	45 ± 2$^{\#,¤}$	29 ± 1$^{*,\circ}$
Leu	140 ± 5	144 ± 10	155 ± 12	280 ± 37$^{*,\#,¤}$	153 ± 12°
Tyr	55 ± 4	40 ± 2*	47 ± 4	44 ± 2*	39 ± 2*
Orn	39 ± 2	48 ± 4	36 ± 2	35 ± 2	135 ± 10$^{*,\#,¤,\circ}$
His	59 ± 3	80 ± 3*	60 ± 2$^{\#}$	80 ± 5$^{*,¤}$	68 ± 3
Lys	325 ± 11	359 ± 13	323 ± 18	368 ± 19	236 ± 20$^{*,\#,¤,\circ}$
Arg	132 ± 7	125 ± 6	109 ± 8	116 ± 4	404 ± 31$^{*,\#,¤,\circ}$

AL rats fed ad libitum for 12 weeks, *R* rats diet-restricted (50% of spontaneous intake) for 12 weeks, *NEAA*, *LEU* and *CIT*, rats restricted for 12 weeks and then refed for 1 week with an NEAA-, a Leu- or a Cit-enriched diet, respectively. Diets were isonitrogenous and isoenergetic. ANOVA + PLSD Fischer test: * $P < 0.05$ versus AL, $^{¤}$ $P < 0.05$ versus NEAA, $^{\#}$ $P < 0.05$ versus R, $^{\circ}$ $P < 0.05$ versus LEU

strength was not further increased when supplemented with a mix of AA containing Leu (Godard et al. 2002). Resistance training does improve muscle strength in healthy or frail elderly (Capodaglio et al. 2007; Liu and Latham 2009) but is sometimes difficult to achieve due to poor compliance, and maybe even more so in malnourished elderly patients. Nutritional manipulations may represent an easy complementary strategy in this population.

Since Leu and Cit benefit muscle protein synthesis in old rats (Dardevet et al. 2002; Rieu et al. 2003; Osowska et al. 2006), we investigated whether these two AA could also lead to an improvement of the parameters reflecting muscle function in malnourished old rats. Of note, the effect of short-term Leu and Cit supplementation on muscle function during renutrition has never been explored before. In our study, we showed for the first time that nutritional manipulations per se are able to improve maximal tetanic isometric force and motor activity in malnourished aged rats.

Leu improved maximal tetanic isometric force (i.e. +101 and 76% vs. AL and NEAA, respectively) in our model of malnourished old rats. In the elderly, the improvement in maximal tetanic isometric force is important because muscle strength predicts mortality, whatever its causes (Gale et al. 2007; Newman et al. 2006; Ruiz et al. 2008). A recent work (Verhoeven et al. 2009) including 30 healthy elderly men showed no improvement in muscle mass and maximal strength after a 3-month-Leu-supplementation. Therefore, the effect of Leu may differ whether short- or long-term supplementation and whether malnourished or healthy individuals are studied. So, further studies are needed to clarify the effects of Leu supplementation in malnourished elderly patients

Leu and Cit had a similar action on motor activity. For both Leu- and Cit-refed old rats, the total number of visited arms increased to the level observed in younger adult rats (Moinard et al. 2004), leading to the conclusion that these two AA restore motor activity to that observed in the young. The rats' spontaneous alternation behaviour tested in the *Y*-maze test, which appraises attention and related functions, was not different between groups. Therefore, the increase in motor activity in Leu- and Cit- supplemented rats was not due to changes in cognitive functions (i.e. attention, memory, fear or anxiety) (Moinard et al. 2004). Values obtained here for spontaneous alternation are very close to the references we got for younger rats (Moinard et al. 2004), which means that exploratory behaviour is preserved in aged rats. Increase in motor activity obtained by Leu- or Cit-treatment may thus ensue from improvement of muscle function.

Although differences do not reach significance, muscle protein content of the Cit- and Leu-refed rats was 66 and 71% greater than the R rats, respectively, and around 15% greater than that of the AL-fed rats, which is in line with previous observations (Moinard and Cynober 2007; Osowska et al. 2006). Although *tibialis* mass was not reduced in R-rats compared with AL-fed rats, protein content was. This hardly suggests a preferential loss of protein in the R rat muscles. These data underline the importance of preventing protein loss in protein energy malnutrition. Relative quantity of proteins in muscle (expressed in mg of protein/g of muscle) was decreased by 30% in R-rats compared with AL-fed rats (data not shown), showing the effectiveness of our model of malnutrition on muscle protein store. Moreover, this decreased muscle protein content observed in R-rats was not followed by a decreased muscle force and underlines the complexity about the relationship between muscle protein content and muscle function (Clark and Manini 2008). Indeed, our model of malnutrition had no effect on muscle mass or muscle function. The protein-energy malnutrition detected by clinicians is due to anorexia, which is tied to depression, polymedication, socioeconomic factors, and so on, making it impossible to reproduce in animals. The rats in this study were forced to undergo dietary restriction. We personally observed that the restricted rats demonstrated an increase in physical activity (linked to searching for food), which could explain why muscle mass and function were not impaired. Similar results have been observed in similar models of food restriction (Zangarelli et al. 2006). However, this model is of major interest since it efficiently reproduces the impairment of protein metabolism that occurs in the elderly, while aged rats exhibited the same resistance to renutrition as humans (Osowska et al. 2006; Chambon-Savanovitch et al. 2001; Walrand et al. 2000). In other studies, the decrease in muscle protein content was significant in diet-restricted rats (Osowska et al. 2006; Walrand et al. 2000). Unfortunately, in our study this decrease did not reach significance, largely due to the large heterogeneity found in aged rat populations. An alternative would be to express protein:DNA ratio as a measure of muscle mass, which would be stable.

Leu and Cit both increased muscle mass and this latter parameter was strongly correlated with the increased muscle protein content, as previously observed for Cit (Osowska et al. 2006). Muscle mass was also positively correlated with maximal tetanic isometric force and motor activity (number of visited arms in the *Y* maze), suggesting that Leu and Cit have beneficial effects on muscle that enable the restoration of muscle function. In the literature (Clark and Manini 2008; Harris 1997), the association between muscle mass and strength is controversial. Initial investigations concluded that muscle mass is a major determinant of the changes of maximal muscle strength in old individuals, with a linear relationship between the two (Frontera et al. 1991; Hughes et al. 2001; Newman et al. 2003). However, in further studies, no association was

found: either an improvement of muscle mass without any improvement of maximal muscle strength was shown or the reverse (Beliaeff et al. 2008; Goodpaster et al. 2006; Rolland and Vellas 2009). In fact, other parameters, such as neurological and other skeletal muscle factors, may contribute to a decreased muscle function independently of changes in muscle mass (Clark and Manini 2008; Rolland and Vellas 2009). Interestingly, an isolated improvement of maximal muscle strength (with no increase in mass) allows for an improvement of muscle functional performance, whereas an increase in muscle mass only does not improve it (Rolland and Vellas 2009). In our study, increased muscle mass was associated with both improvement of maximal tetanic isometric force and motor activity.

Cit and Leu are both known to modulate muscle protein synthesis but an alternative mechanism may be proposed: many works (Meneilly et al. 1995; Rasmussen et al. 2006) have clearly established that the physiological insulin-stimulated increase in endothelial-dependant vasodilatation is blunted in healthy elders. A recent study (Timmerman et al. 2010) has shown that increasing blood flow and muscle perfusion during hyperinsulinemia in older adults enhances muscle protein synthesis. It is also known that decline in muscle function is associated with blood flow restriction (Corcondilas et al. 1964; Laughlin and Armstrong, 1982; Radegran and Saltin 1998). Based on these overall data, we may suggest both Leu and Cit would be able to improve muscle performance: Leu by stimulating insulin secretion (Anthony et al. 2002), and Cit by increasing arginine production (Deutz 2008; Moinard and Cynober 2007).

In conclusion, this work is the first to establish that a specific nutritional manipulation (i.e. Leu supplementation) improves muscle mass and function (i.e. motor activity and maximal tetanic isometric force), at the post-absorptive state, when administered for a short time-period. We also show a significant relationship between muscle mass and relevant functional end-points, both for Leu- or Cit-treatment. Thus, Leu or Cit may be efficient and promising strategy to prevent and treat dynapenia that occurs in the elderly and therefore might limit functional impairment and protein energy malnutrition-related morbi-mortality in the elderly.

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Conflict of interest CF, LC and CM are share holders of Citrage company. Other authors do not declare any conflict of interest.

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