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Status of carnitine and circulating amino acids and its association with pre-frailty, sarcopenia and diet in an Uruguayan older population

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Abstract.
BACKGROUND: Frailty is a clinical-biological syndrome in older adults that carries an increased risk for poor health outcomes. Biomarkers of disability are being studied and some acylcarnitines and amino acids are part of the predictive models.
OBJECTIVE: To characterize the status of L-carnitine, some acylcarnitines and amino acids and relate them to frailty, sarcopenia and diet in a community-dwelling Uruguayan older population.
METHODS: Participants were enrolled and assessed through a multi-step process, that included frailty and sarcopenia criteria. L-carnitine, its acyl derivatives and amino acids were determined in blood by LC-MS/MS and dietary intake by a 24-h recall and a food frequency questionnaire.
RESULTS: Sixty-three older adults were enrolled, and 54 completed the initial assessment. Pre-frailty criteria were fulfilled by 41 participants and frailty only by one. No nutritional indicators of undernutrition were found. Probable sarcopenia was found in 20 cases. Males consumed more total meat and red meat than women. Hexanoylcarnitine levels were higher in pre-frail/frail individuals and in weak ones. Analysis by sex showed a distinct pattern between gender, being significant only for weak females.

Methionine also showed some differences between sexes. Weak males presented significantly higher levels of methionine, whereas weak females showed significantly lower ones.
CONCLUSIONS: No associations were found for diet components and L-carnitine, acylcarnitines and amino acids values, except for the percentage of animal protein that was higher in weak males. The clinical impact of these results needs further investigation.

Keywords: Sarcopenia, frailty, diet, carnitine, methionine, elderly

1. Introduction

Frailty is commonly defined as a clinical-biological syndrome in older adults that affects

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multiple body systems and carries an increased risk for poor health outcomes, including falls, incident disability, hospitalization, and mortality [1]. Sarcopenia is the cornerstone of physical frailty, thus, preventing the loss of muscle mass quantity, quality, strength, and function is crucial. Interventions aim to preserve muscle mass include nutrition and physical activity [2–5].

Recommended protein intake, protein source (vegetable and animal) and specific AAs role on muscle health in old age are in debate and being investigated [6–9]. BCAAs, Leu, isoleucine, and valine and some BCAA-related metabolites are positively associated with muscle mass [10]. Leu and leucine-containing supplements have been shown to improve sarcopenia, mainly by improving lean muscle-mass content [11].

Recently, some AAs profiles are proposed as plasma biomarkers of poor muscle quality [12], and protein or specific AAs restriction (i.e. methionine) has been suggested as a strategy to improve longevity in diverse organisms through anti-inflammatory mechanisms [13, 14]. In older persons, there is no evidence of benefit [15], and in fact, this could have negative effects [16].

Supplementation with LCAR is another nutrition intervention studied. LCAR is a non-essential amino acid involved in intermediate metabolism and energy production. LCAR has shown utility to improve muscular resistance in athletes, and it is included in many supplements. However, it is still under investigation for older persons [17–19].

The association among AAs, LCAR, ACs, and frailty is being studied through metabolomic profiles. Omics approach contributes to detecting biomarkers of disability in pre-frail older persons to allow early interventions. Pujos Guinot et al. [20] performed an untargeted metabolomic analysis to identify compounds to be proposed as biomarkers of evolution towards frailty. Rattray et al. [21] identified significant metabolites that differentiate frail and non-frail phenotypes, and Calvani et al. [22] described a distinct pattern of circulating AAs that characterizes older persons with frailty and sarcopenia. Some ACs and AAs are part of these predictive models, but the results are still inconclusive.

The objective of the study was to characterize the status of LCAR, some ACs and AAs and relate them to frailty, sarcopenia and diet in a community-dwelling physically independent or mildly dependent Uruguayan older population.

2. Materials and methods

This study is part of the baseline assessment of the L-Carnitine Intervention Study, a randomized controlled clinical trial performed in a community-dwelling physically independent or mildly dependent Uruguayan older population (65 years or older). The clinical trial was registered in Clinical Trials.gov with the identifier NCT03180424.

Participant recruitment took place from June 2016 through December 2017 from the patients assisted at the Geriatric and Gerontology Department of the University Hospital and the users of the Exercise groups of the National Secretary of Sports. After obtaining written informed consent, data collection was performed in the Geriatric and Gerontology Department of the University Hospital.

The study protocol was approved by the Ethics Committee of the University Hospital Dr. Manuel Quintela, from Montevideo, Uruguay.

2.1. Participant assessment

All participants recruited for the L-Carnitine Intervention Study (selection/exclusion criteria summarized in Supplementary Table 1) were included in this analysis. Participant assessment was done by a trained team and carried out through a multi-step process: (a) the collection of medical history and assessment of all medical criteria (b) anthropometric measures, body composition and dietary assessment (c) gait speed at 4.5 m and 6 m. (d) handgrip strength (e) dried blood spot from a finger prick to determine L-CAR, ACs and AAs.

2.2. Anthropometric and body composition

Weight, height, waist, and calf circumference were measured using the ISAK (International Society for the Advancement of Kinanthropometry) protocol. BMI and waist circumference were analyzed using WHO criteria [23], and calf circumference using Rolland reference cutoff [24]. BIA was performed with a single frequency equipment (ImpediMed DF50). Raw data (resistance and reactance measurements) were recorded. Data of BIA was used to calculate muscle quantity as ASMM using a prediction equation Sergi et al. [25]. ASMM was normalized to height and expressed as ASMMI (kg/m2).

2.3. Dietary assessment

Dietary intake was assessed by a 24-h recall and a food frequency questionnaire, focused on the consumption of meat products in urban cities, red meat (beef and pork), and white meat (fish and poultry). Both were performed by a registered dietitian. Nutritional intakes were evaluated using the CERES+ software version 1.1 (FAO 2003). Total animal protein and total vegetable protein were calculated from the sum of each food source and mixed dishes.

Total energy intake was expressed as total energy per day (Kcal/d), and also as a ratio with actual body weight (Kcal/kg BW).

Dietary protein intake was expressed on a daily basis in grams per day (g/d) and also as a ratio with actual body weight (g/kg BW). Total meat intake, red meat and white meat intake were expressed on a daily basis (g/d).

Animal protein intake was expressed as percentage of total protein intake (% animal protein from total protein intake).

2.4. Frailty and sarcopenia definitions

Frailty criteria were measured according to Fried et al. [26]. The original criteria were used, and some metrics were adapted according to Avila-Funes et al. modifications [27].

Weakness was defined by handgrip strength in dominant hand equal of below the sex and BMI specific cutoffs defined by Fried et al. [26]. Handgrip strength was measured using the Southampton grip-strength measurement protocol and the Jamar hand dynamometer (Lafayette Instrument Company, USA).

Exhaustion was assessed by the Center for Epidemiologic Studies Depression (CES-D) test. Gait speed over 4.5 m was used for the definition of slowness. Slow gait was defined according to Fried et al validated cutoffs [26].

An Spanish validated short version of the Minnesota Leisure Time Activity Questionnaire (VREM) was used to measure physical activity [28].

Participants were considered "frail" if they had three or more frailty components, "prefrail" if they fulfilled one or two frailty criteria, and "non-frail" if none.

Sarcopenia was identified according to the European Working Group on Sarcopenia in Older People 2 (EWGSOP2) (2019) criteria [29]. Sarcopenia was considered probable when low muscle strength was present, defined as hand-grip strength less than 27 for men and less than 16 for women. Sarcopenia was diagnosed when ASMMI was less than 7.0 for men or less than 5.5 for women. Severe sarcopenia was considered when low physical performance is present, defined as gait speed over 6 m below 0.8 m/seg.

2.5. Blood sample collection and analysis of LCAR, ACs, AAs

For LCAR, its acyl derivatives and specific AAs determination, few drops of blood from a finger prick were collected onto filter paper cards and dried overnight. Extraction was performed on 3.2 mm filter paper disks punched out from the dried blood spot specimens using 100% methanol solution containing internal standards. The internal standards used were the Cambridge isotopes internal standards NSK which contained the following stable isotopes for LCAR and the following acylcarnitines: acetylcarnitine (C2), propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5), hexanoylcarnitine (C6), among others, and the AAs, Alanine (Ala), Arginine (Arg), Citrulline (Cit), Glycine (Gly), Leucine (Leu), Methionine (Met), Ornithine (Orn), Phenylalanine (Phe), Creatine (CRE), Guanidinoacetic Acid (GUAC), Creatinine (CRN), Succinylacetone (SUAC), Tyrosine (Tyr), Valine (Val) and Xleu (including the sum of Leu, isoleucine, alloisoleucine and hydroxyproline). The extracted samples were derivatized with 3 N butanolic HCl at 65°C and finally reconstituted with acetonitrile/water (50:50) solution containing 0.02% formic acid (mobile phase). Samples were analyzed with HPLC-MS/MS (Dionex-ABSiex 3200 triple quadrupole) using precursor-ion of 85 m/z following techniques previously reported, 4 level quality control [30, 31]. All results were reported in μmol/L.

2.6. Statistical analysis

A descriptive analysis of the variables involved in the study was carried out using summary measures. The quantitative variables were summarized by means and standard deviation, while the qualitative variables were described by absolute and relative frequencies. For each variable presented, differences were assessed by Mann-Whitney test (for the quantitative variables) and Pearson chi squared test (for the categorical variables).

Statistical significance was determined when the *p*-value was lower than 0.05. All statistical procedures were performed in R software (R 3.5.2. version, R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

Sixty-three older people were enrolled, and 54 completed the initial assessment.

The anthropometric/body composition, dietetic characteristics, blood AAs and ACs results of the population studied were summarized in Table 1.

Some compounds were selected to be reported: LCAR, C3, C5, C6, Met and XLeu. These compounds were prioritized in the presentation of results because they have been either reported to be related to frailty in previous research [10, 20, 22, 40] or because they presented statistically significant differences in the present work. No significant differences were found for any other compound analyzed.

No nutritional indicators of undernutrition were found. Mean BMI shows predominantly overweight, with high waist circumference. Muscular quantity, measured by calf circumference and ASMMI, is preserved. As expected, sex differences were found in muscular quantity.

Mean protein intake/kg BW was 1.00 g/kg, and more than half of the proteins were animal. Males consumed more total meat and red meat than women. No differences in LCAR, ACs and AAs were found between males and females.

Pre-frailty criteria were fulfilled by 41 participants and frailty only by one subject. For this reason, the pre-frail/frail category was presented (77.8%). Weakness was the main criteria to diagnose pre-frailty, followed by exhaustion. Only 3.7% (2 out of 54) presented low physical activity. Using handgrip strength criteria, probable sarcopenia was found in 20 cases (37.1%). Gait speed was not affected in the population. Men were more likely to have probable sarcopenia than women ($p < 0.05$), but no sex differences were found for the pre-frailty/frailty group. Results are shown in Table 1.

Significant differences were found between C6 levels and frailty, being higher in pre-frail/frail individuals and for weak ones (Table 2). Analysis by sex shows a distinct pattern between gender for C6 (Table 3). Regarding weakness and C6, females showed the same significant association that was seen in the general analysis, while men showed no association (Table 3).

Differences were found for C5 levels in the physical activity component of frailty, being higher for good physical activity. However, this result should be taken cautiously because of the difference in the number of subjects between groups (52 vs 2) (Table 2).

Concerning AAs, no differences were found for Xleu and Met in the general population (Table 2). However, Met levels showed some differences between sexes that are depicted in Table 3. The non-difference in Met levels in the general population hides the opposite differences in Met concentration between sexes. Weak males presented significantly higher levels of Met, whereas weak females showed significantly lower ones. For women, significantly lower levels were found in the pre-frail/frail category and for men, in the non sarcopenia group (Table 3).

No associations were found for diet components and LCAR, ACs or AAs values (data not shown). Percentage of animal protein consumption is associated with weakness only for males, being higher in weak ones (Table 3).

This study includes mainly a pre-frail, well-nourished with preserved muscle mass population. Weakness measured by handgrip strength was the principal component to diagnose pre-frailty and probable sarcopenia. From all the ACs analyzed, certain short/medium-chain (C5 and C6) showed association with frailty, with gender differences. Met is the only AA linked with frailty, with an opposite relation between sexes. Source of protein seems to be of interest in relation to frailty, but only for men.

Concerning diet, participants fulfilled the calorie and protein intake recommendations for older persons (mean calorie intake of 26.56 kcal/kg BW and 1.00 g protein/kg BW). Traditionally, protein intake recommendation was 0.8 g/kg BW per day [32, 33]. Nowadays, this recommendation is being reviewed since growing evidence remarks that higher amounts are needed. Several expert groups have suggested daily amounts of 1.0–1.2 g/ kg BW for healthy older persons [34, 35] and the general orientation for calorie intake is about 30 kcal/kg BW, with individual adjustments regarding gender, nutritional status, physical activity, and clinical conditions [36].

In the present study, protein and calorie/kg BW are reported related to actual BW, so calorie and protein/optimal BW are underestimated. More than half of the proteins came from animal sources, showing good availability of these proteins in the population and therefore to essential AAs.

Table 1
Characteristics of the study population

	Mean (SD)			
	Total ($n = 54$)	Male ($n = 23$)	Female ($n = 31$)	p-value
Age (years)	72.56 (4.38)	73.47 (5.38)	71.87 (3.40)	0.217
Anthropometric/body composition characteristics				
BMI	29.36 (4.72)	28.39 (4.12)	30.08 (5.06)	0.182
Waist circumference (cm)	100.65 (11.06)	103.13 (10.82)	98.81 (11.04)	0.157
Calf circumference (cm)	38.82 (3.34)	40.07 (2.95)	37.90 (3.35)	**0.014**
ASMMI (kg/m^2)	7.78 (1.38)	8.75 (0.91)	7.09 (1.24)	**<0.01**
Dietetic characteristics				
Total energy intake per day (Kcal/d)	1980.3(557.9)	2011.1 (464.7)	1891.4 (610.3)	0.159
Energy intake per kg body weight (Kcal/kg BW)	26.56 (8.99)	25.65 (6.83)	27.24 (10.38)	0.501
Total protein intake per day (g/d)	74.12 (22.86)	75.91 (20.90)	72.78 (24.47)	0.616
Total protein intake g/kg body weight (g/kg BW)	1.00 (0.38)	0.95 (0.35)	1.05 (0.41)	0.304
% animal protein from total protein intake	60.88 (18.39)	59.13 (21.71)	62.19 (15.73)	0.570
Total meat intake per day (g/d)	107.62 (60.72)	132.01 (68.07)	88.86 (47.69)	**0.022**
Red meat intake per day (g/d)	41.18 (46.80)	61.55 (61.66)	25.51 (21.58)	**0.020**
White meat per day (g/d)	66.44 (43.06)	70.46 (40.52)	63.34 (45.47)	0.578
L-carnitine, Amino acids and Acylcarnitines levels in blood (micromol/L)				
Met	11.33 (3.46)	10.90 (3.78)	11.66 (3.23)	0.422
Xleu	118.44 (25.89)	123.07 (25.69)	115.00 (25.92)	0.258
LCAR	39.89 (9.31)	38.94 (8.59)	40.59 (10.32)	0.536
C3	2.04 (0.90)	2.12 (0.94)	1.97 (0.86)	0.544
C5	0.20 (0.09)	0.21 (0.10)	0.19 (0.08)	0.392
C6	0.08 (0.04)	0.07 (0.02)	0.09 (0.04)	0.064
	n (%)			
	Total ($n = 54$)	Male ($n = 23$)	Female ($n = 31$)	
Frailty				
Non frail	12 (22.2%)	4 (17.4%)	8 (25.8%)	0.686
Pre frail/frail	42 (77.8%)	19 (82.6%)	23 (74.2%)	
Sarcopenia				
Non	34 (62.9%)	10 (43.5%)	24 (77.4%)	**0.011**
Probable	20 (37.1%)	13 (56.5%)	7 (22.6%)	

Body mass index (BMI); Appendicular skeletal muscle mass index (ASMMI) (kg/m2); Methionine (Met); Leucine + isoleucine + alloisoleucine + hydroxyproline (Xleu); L-carnitine (LCAR); Propionyl carnitine (C3); Isovalerylcarnitine (C5); Hexanoylcarnitine (C6). For each variable presented, differences between male and female were assessed by Mann-Whitney test (for the quantitative variables) and Pearson chi squared test (for the categorical variables).

No gender differences were found for AAs, ACs or LCAR values in our study. During metabolism, LCAR suffers acylations, and ACs are also important in intermediate metabolism. Some studies correlate advancing age and LCAR and/or ACs disbalances [37–39]. Kouchiwa et al. [40] found decreasing values in AAs with age for both sexes and described differences between gender. Serum concentrations of Leu, threonine, Met, histidine, glycine, serine and taurine decreased with age in males, whereas threonine and serine decreased with age in females.

In relation to ACs, findings in C6 levels (higher concentration in weak and pre-frail/frail subjects) have not been reported for this population before. When sexes were compared, this difference corresponds to the one presented by women (only for weakness) since men showed no difference in these ACs. Nevertheless, the clinical impact of these results needs further analysis.

Previous studies included C5 in the predictive model of the evolution toward pre-frailty. For men, dimethyloxazole, glutamine and C5 are included, and dihydroxyphenyl acetic acid, threonine, and mannose for women [20]. Another study found that C5 (and also deoxycarnitine) were positively associated with muscle mass and fat-free mass index [10]. In our study, significant differences were found between C5

Table 2
Mean LCAR, ACs and AAs values (micromol/L) in blood vs frailty criteria

	n	Met	Xleu	LCAR	C3	C5	C6
Frailty							
Non-frail	12	12.84	128.40	43.02	1.96	0.19	0.06
Pre- frail/ frail	42	10.90	115.92	38.99	2.06	0.20	0.09
p-value		0.087	0.131	0.187	0.724	0.527	**0.043**
Frailty components							
Weakness							
Non	16	11.70	126.60	42.45	1.95	0.18	0.07
Weak	38	11.18	115.42	38.81	2.07	0.21	0.09
p-value		0.613	0.187	0.190	0.633	0.501	**0.042**
Exhaustion							
Non	44	11.51	119.70	40.59	2.07	0.20	0.08
Exhausted	10	10.55	112.88	36.77	1.89	0.19	0.09
p-value		0.424	0.452	0.240	0.564	0.603	0.361
Physical activity							
Good	52	11.48	118.23	39.98	2.04	0.21	0.08
Low	2	7.45	123.98	37.49	1.83	0.08	0.06
p-value		0.106	0.758	0.800	0.738	**0.043**	0.298
Sarcopenia							
Non	34	10.86	117.81	39.74	1.96	0.19	0.080
Probable	20	12.13	119.49	40.14	2.17	0.22	0.087
p-value		0.306	0.851	0.817	0.424	0.194	0.505

Methionine (Met); Leucine + isoleucine + alloisoleucine + hydroxyproline (Xleu); L-carnitine (LCAR); Propionyl carnitine (C3); Isovalerylcarnitine (C5); Hexanoylcarnitine (C6). For each blood component, concentrations were compared between levels of frailty, frailty components categories and sarcopenia by Mann-Whitney test.

Table 3
Mean Met, C6 (micromol/L) in blood and % of animal protein from total protein intake vs frailty criteria and sex

	Met		C6		% animal protein	
	Males	Females	Males	Females	Males	Females
Frailty						
Non-frail	10.12	14.21	0.05	0.06	49.53	59.57
Pre frail/frail	11.06	10.77	0.07	0.10	60.57	62.90
p-value	0.625	**0.009**	0.207	0.054	0.450	0.172
Frailty components						
Weakness						
Non-weak	8.45	14.23	0.06	0.07	38.84	61.29
Weak	11.97	10.61	0.07	0.10	64.76	62.50
p-value	**0.041**	**0.005**	0.467	**0.042**	**0.038**	0.299
Exhaustion						
Non-exhausted	11.36	11.65	0.068	0.09	60.87	62.16
Exhausted	7.83	11.71	0.113	0.09	47.53	62.29
p-value	0.131	0.963	0.066	0.973	0.321	0.985
Sarcopenia						
Non	8.56	11.83	0.062	0.09	52.50	62.49
Probable	12.69	11.08	0.075	0.10	64.23	61.16
p-value	**0.009**	0.586	0.269	0.398	0.199	0.844

Methionine (Met); Hexanoylcarnitine (C6). For variables presented, results were compared between levels of frailty, frailty components categories and sarcopenia in males and females by Mann-Whitney test.

levels in the physical activity component of frailty for the whole population, being higher for subjects with good physical activity.

Concerning LCAR and ACs, no other clinically relevant results were found. Nowadays, isolated levels of carnitines are not studied, since they are integrated into metabolomic profiles that include several components. However, the present study remarks that C6 might be of interest in this context.

Analyzing Met and frailty by gender, a distinct pattern between sexes was found. Weak and probable sarcopenia men have higher levels of Met, probably suggesting a detrimental effect of this AA. This is supported by studies of Met dietary reduction, that have demonstrated to extend lifespan in animal models [41–43] through mechanisms that promote metabolic flexibility, improving insulin sensitivity, lipid metabolism, and decreasing systemic inflammation [44–46]. However, the results of Met reduction are still not conclusive [13, 47–50]. Scarce investigation [51–53] has been done in humans, and its extrapolation to the elderly may result at least controversial [14]. For women, in our study, Met seems to act as a protective factor towards frailty, which matches with previous reports. Calvani et al. [22] found that non-frailty non-sarcopenic participants' profile was defined by higher concentrations of Met and α-aminobutyric acid. With our results, it is not possible to identify the mechanism by which Met affects differentially weakness in men and women.

No association was found between protein intake (as grams of total protein/kg BW), protein source or meat intake (total and red) with concentrations of LCAR, ACs or AAs. However, in literature, dietary LCAR intake is related to plasma carnitine concentrations in adults [39]. Concerning dietary sources, meat is the food that contains higher amounts of LCAR [54]. Metabolomic studies have associated some ACs with meat intake [55, 56]. Meat also contains many essential AAs, nutritive factors of high quality and availability, and other compounds that can influence protein metabolism [57, 58]. Previous studies reported an association between dietary protein sources or red meat intake [59] with BCAAs and short-chain ACs or between western dietary patterns and levels of AAs and short-chain ACs [60]. Another study showed that some ACs (acetylcarnitine (C2), propionylcarnitine (C3), and 2- methylbutyrylcarnitine) appeared to be indicators of meat and fish intake [55].

No relationship was found between diet and frailty in the general population, but an association was described for men. Animal protein consumption is associated with weakness, being higher in weak males. Since Leu and Met are ubiquitously found in different protein sources, dissociating from total protein intake is practically impossible and the methodology of our study did not allow us to estimate AAs intake.

4.1. Limitations

Limitations of the study include the small sample size and the marked domain of pre-frail/frail subjects in the population, which limits the comparison between groups. Dietary analysis was focused on protein intake and source, while other dietary components linked with sarcopenia or other AAs associated with muscle mass were not considered. Finally, the methodology of our study did not allow us to determine specific AAs intake in the population, and therefore to associate them with blood concentrations of these AAs.

In the hypothesis tests where the null hypothesis was not rejected, the power of the Mann-Whitney tests ranged from 0.069 to 0.406 and in the test using Chi2 the power was 0.053. This may reflect that future research on the subject should consider larger sample sizes.

5. Conclusions

Our study found a relationship between C6 and weakness in women, which has not been described before. In addition, Met emerges as an AA of interest in relation with frailty. Although there are similar levels between men and women, a distinct pattern between sexes was found.

The results of the analysis of ACs and AAs with frailty, emphasize that it should be done considering gender, as the associations are different between sexes. In addition, only one dietary component, the percentage of animal protein was associated with weakness, and this correlation was found only in men. The clinical impact of these results needs further investigation.

Abbreviations

Acylcarnitines (ACs)
Appendicular skeletal muscle mass (ASMM)
Amino acids (AAs)

ASMM index (ASMMI)
Bioelectrical impedance analysis (BIA)
Body mass index (BMI)
Body weight (BW)
Branched Chain Amino Acids (BCAAs)
Hexanoylcarnitine (C6)
Isovalerylcarnitine (C5)
L-carnitine (LCAR)
Leucine (Leu)
Leucine + isoleucine + alloisoleucine + hydroxyproline (Xleu)
Methionine (Met)
Propionyl carnitine (C3)

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