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Supplementation with essential amino acids in the early stage of carbohydrate reintroduction after a very-low energy ketogenic therapy (VLEKT) improves body cell mass, muscle strength and inflammation

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Abstract

Background Although little is yet known about the long-term maintenance of very low-energy ketogenic therapy (VLEKT) effects on body composition, muscle strength and inflammation, it is plausible to assume that changes may occur, particularly during the steps following the ketogenic step, due to the loss of the protective effects of ketones and the concomitant reintroduction of carbohydrates. For this reason, the present study aimed to evaluate the effects of supplementation with 8 g *per* day of essential amino acids (EAAs) on these parameters.

Methods A total of 68 women of reproductive age and with grade I obesity who had completed 45 days of the ketogenic phase with VLEKT (KeNuT protocol) and 40 days of non-ketogenic phase of KeNuT protocol with VLEKT (phase 3, fruit reintroduction) were included in the study. Half of them ($n=34$) followed this first step of the re-educational phase of KeNuT protocol with VLEKT with supplementation with EAAs (Aminotrofic®: 4 g twice daily). Anthropometric parameters, body composition *via* bioelectrical impedance analysis and high-sensitivity C-reactive protein (hs-CRP) levels were assessed at baseline, pre- and post both dietary interventions.

Results At the end of 45 days of ketogenic phase with VLEKT (before fruit reintroduction and EAA supplementation), the two groups did not differ in any of the parameters assessed. At the end of the fruit reintroduction phase, the supplemented group showed greater magnitudes of reduction in weight, waist circumference, fat mass (FM) and

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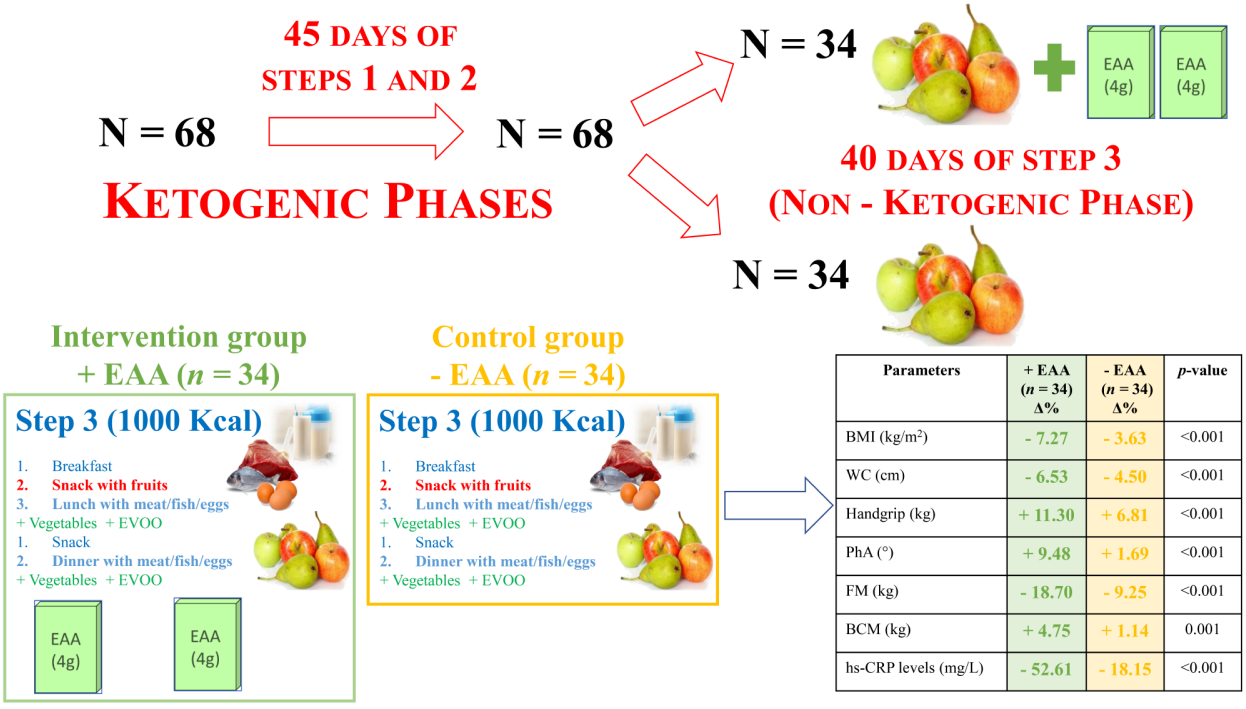
hs-CRP ($p < 0.001$ for all) and of increase in muscle strength ($p < 0.001$), phase angle ($p < 0.001$), body cell mass (BCM) ($p = 0.001$), and muscle mass (%) ($p < 0.001$).

Conclusion These results underline the usefulness of supplementation with EAAs during the first transitional phase post VLEKT to improve body composition (specifically reduction in FM and increase in BCM), muscle strength, and inflammatory status.

Graphical Abstract



Parameters	+ EAA ($n = 34$) Δ%	- EAA ($n = 34$) Δ%	*p*-value
BMI (kg/m^2)	- 7.27	- 3.63	<0.001
WC (cm)	- 6.53	- 4.50	<0.001
Handgrip (kg)	+ 11.30	+ 6.81	<0.001
PhA (°)	+ 9.48	+ 1.69	<0.001
FM (kg)	- 18.70	- 9.25	<0.001
BCM (kg)	+ 4.75	+ 1.14	0.001
hs-CRP levels (mg/L)	- 52.61	- 18.15	<0.001

Keywords Very-low energy ketogenic therapy, Obesity, Essential amino acids, Body cell mass, Muscle strength, Inflammation, Body composition, Bioelectrical impedance analysis

Background

The very-low calorie ketogenic diet (VLCKD), recently renamed as very-low energy ketogenic therapy (VLEKT), emphasising both its low-calorie characteristics (in addition to low-carbohydrate intake) and the need to be considered as a medical nutrition therapy (MNT) [1], is recognised as a valid and useful therapeutic tool to manage obesity and related comorbidities [2–4].

The advantage of using VLEKT as an MNT in such clinical settings lies essentially in its ability to induce a rapid and constant weight reduction represented by an almost exclusive, or at least, selective reduction in fat mass (FM), particularly visceral fat, preserving fat-free mass (FFM), hence skeletal muscle mass (SMM). This mainly results from its peculiar hormonal and metabolic effects, especially on regulation of glycaemic, insulinemic and lipidemic levels [2–4]. According to Ketogenic Nutritional Therapy (KeNuT) protocol [5], however, the above mentioned results can be achieved only if VLEKT involves the use of replacement meals. In addition to a low-calorie intake (650–800 kcal), and a strictly controlled amount of macronutrients (particularly carbohydrates and fats), replacement meals have other several advantages: (i) they contain high biological value proteins (mostly whey-derived) or vegetable proteins, (ii) they contribute to the maintenance of intestinal well-being, with particular interest in the composition of the microbiota, and (iii) they increase dietary compliance, due to the fact that they are, for the most part, ready-to-use and available in single portions [5].

KeNuT is a multistep dietary protocol, based on two main phases: a ketogenic phase (divided into phases 1 and 2) which may last between 8 and 12 weeks, and a non-ketogenic phases (split up into phases 3 to 5, of variable duration). During phases 3–5 carbohydrate-containing food groups are progressively reintroduced, with the aim of not abruptly interrupting the state of ketosis, gradually re-sensitising the organism to carbohydrate,

and promoting a nutritional rieducation towards a Mediterranean-style diet [5]. The non-ketogenic phases, however, is particularly critical for several aspects. Firstly, interrupting ketosis determines the absence of protective effects of ketone bodies on skeletal muscle, such as the anticatabolic effect [6]. Moreover, during the first stage of the non-ketogenic phase (phase 3) fruit is the first carbohydrate food group reintroduced in replacement of one or two proteic ready-to-use meals [5], determining a net change in the intake and distribution of macronutrients, mostly, proteins. Therefore, the main concern at this stage lies in a potential negative effect at the SMM level, which could be subject to a net reduction, given the still low caloric intake (about 1000 kcal in phase 3) and the potential reduction in protein intake. To our knowledge, there are currently no studies that have monitored changes in body composition (BC) during the stages following ketosis within a multistep protocol (KeNuT), for this reason inferable conclusions remain purely speculative. However, these dietary steps must be finely tuned and it appears necessary to delineate main strategies in order to counteract any, even hypothetical, negative effect, particularly on SMM.

In this regard, a widely used nutritional strategy to promote skeletal muscle health involves the supplementation with essential amino acids (EAAs), as key elements to support muscle protein synthesis (MPS), which in turn is essential for muscle growth and repair [7, 8]. EAAs, particularly leucine, support MPS both directly, by acting as substrates, and through activation of the mammalian Target Of Rapamycin (mTOR) signalling pathway [7, 8] and its downstream targets involved in mRNA translation initiation, such as ribosomal S6 kinase 1 and eukaryotic translation initiation factor 4E-binding protein 1 [9]. mTOR is a key nutrient sensor for cell growth and proliferation that, when activated, triggers a series of processes that stimulate translation and protein synthesis in skeletal muscle. These mechanisms display a dual benefit: not only EAAs promote trophism, but they also prevent loss of SMM during calorie restriction or under conditions of increased muscle breakdown, by stimulating MPS and reducing muscle protein breakdown [8]. As previously reviewed, encouraging results on the efficacy of EAAs come mainly from studies in elderly subjects in which stimulation of MPS and improvement of muscle strength were observed following oral administration of EAAs [7], which, therefore, represents a practical advantage over other administration strategies, determining the same pharmacological effects. Overall, this evidence suggests the importance of the oral administration of EAAs during nutritional intervention for the management of patients with obesity, particularly in circumstances where a caloric restriction is carried out (as in the case of the VLEKT protocol) in order to maintain patient compliance and, simultanously, promote MPS or, at least, stem the reduction in SMM and muscle strength.

The aim of the present study is to evaluate the effect of a supplementation with a free-form EAAs formula (8 g EAAs *per* day in two administrations) on anthropometric parameters, BC, muscle strength and inflammation in a group of young women (intervention group) with grade I obesity compared with age- and BMI-metched women not supplemented with EAAs (control group) during the 40-day phase 3 of the KeNuT protocol after the ketogenic phase with VLEKT.

Materials and methods

Design and setting

This study was conducted on young women with grade I obesity undergoing a VLEKT protocol for weight loss at the *Centro Italiano per la cura e il Benessere del paziente con Obesità* (C.I.B.O.) of Federico II University Hospital (Naples, Italy). Data were retrospectively collected between January 2023 and May 2024. After being informed about the study protocol, all women provided written consent. The Local Ethical Committee approved the study design (reference n. 50/20).

Population study

In order to reduce sample variability and avoid assessment bias, the study included two groups of young women (a total of 68 participants, 34 for each group) who had completed the ketogenic phase of the KeNuT protocol (i) aged between 25 and 36 years, (ii) with a body mass index (BMI) ≥ 30 kg/m^2, (iii) pre-menopausal, (iv) not following pharmacological teraphies including hormonal contraceptives, (v) non-smokers, (vi) who had not regularly taken antioxidant or EAAs supplements in the three months prior to the start of the VLEKT programme, and (vii) who refuses to undergo full laboratory instrumental examination were matched for age, SMM, and degree of inflammation. The initial eligibility of the participants was determined by an endocrinologist following a medical examination in order to collect anamnestic data and to assess whether the peresciption of EAAs supplementation was appropriate. The practice of regular exercise (at least 30 min *per* day of aerobic exercise) was assessed in all participants, as previously reported in other studies [10–12]. Figure 1 shows a flow chart of women included and excluded.

Study protocol

The study protocol included three sets of evaluations: baseline, after 45 days of ketogenic phase with VLEKT, and after 40 days of non-ketogenic phase with reintroduction of fruit (phase 3) as the first carbohydrate food group. Overall, the protocol consisted of three visits (T0 - T2) corresponding to: baseline (T0, before starting

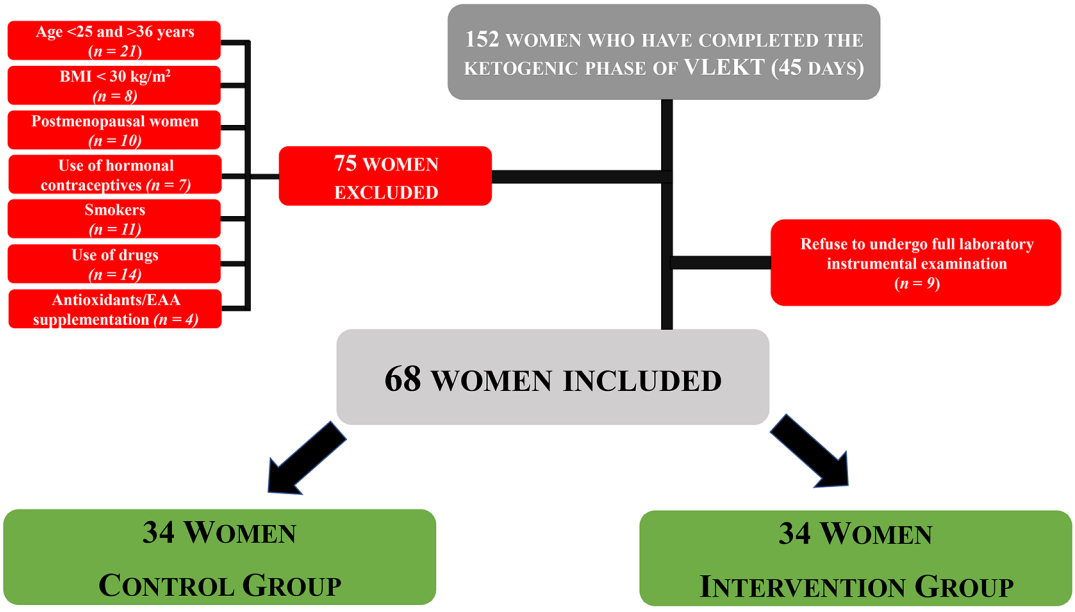


Fig. 1 Flow chart of study design

the VLEKT programme), end of the ketogenic phase of KeNuT protocol (T1, 45 days after VLEKT programme beginning and before fruit reintroduction), and end of the study (T2, 40 days after fruit reintroduction phase of KeNuT protocol). Each visit consisted in a clinical assessment by the same endocrinologist and a nutritional assessment by the same nutritionist. Specifically, at T0 the endocrinologist assessed the clinical condition of the participants in order to ascertain their eligibility and those who met the inclusion/exclusion criteria were asked to sign a written informed consent. Following the endocrinological examination, still at T0, the nutritionist collected lifestyle data, carried out the anthropometric and BC assessment by BIA, and gave the patients an individualised weekly diet plan containing both the indications for adhering to the VLEKT programme and how to prepare and consume the replacement meals. Patients were asked to adhere to the instructions given, with particular attention to the lists of permitted and non-permitted foods, as well as not to vary physical activity levels throughout the entire observational period. At time point T1 the endocrinologist again assessed patients' clinical condition and the persistence of the criteria necessary to continue the protocol. Similarly, the nutritionist again carried out the anthropometric and BC assessment and provided the dietary indications in order to beginthe non-ketogenic phase, based on the reintroduction of fruit. This phase (lasting 40 days) could include the supplementation with 8 g free-form EAAs daily or not (supplemented and not-supplemented groups, respectively). For both groups the dietary indications, and the daily calorie intake, were the same. During the endocrinological and nutritional visits at the last time point (T2) the same data were recorded as in the previous ones. At all-time points (T0 - T2) a nurse collected a blood sample for monitoring high-sensitivity C-reactive protein (hs-CRP) levels. The three visits were interspersed with weekly interviews by a nutritionist aimed at assessing adherence to dietary prescriptions and indications given. In particular, the interview consisted in collecting information on (i) intake of foods/drinks allowed and not allowed during VLEKT, both in the ketogenic and non-ketogenic phases, according to the KeNuT protocol [5], (ii) ketonemia values independently collected by the patients by capillary blood sampling in order to ascertain the actual presence of ketosis during the ketogenic phase, (iii) intake of the EAAs supplement prescribed to the supplemented group during the fruit reintroduction phase, (iv) any changes in physical activity levels. A summary flow-chart of the study protocol is reported in Fig. 2.

Anthropometric assessment

As reported in previous studies [13–15], anthropometric parameters were assessed by the same skilled Nutritionist in the morning (between 8:00 am and 10:00 am) on overnight fasting subjects. The subjects were asked to wear light clothing and remove their shoes. Body weight was measured using a calibrated beam scale (Seca 711; Seca, Hamburg, Germany) to the nearest 0.1 kg. Height was measured using a wall stadiometer (Seca 711; Seca, Hamburg, Germany) to the nearest 0.5 cm. To calculate BMI, body weight was divided by the square of height in metres, according to the formula [weight (kg)/height^2(m^2)] = BMI (kg/m^2). Based on the BMI value,

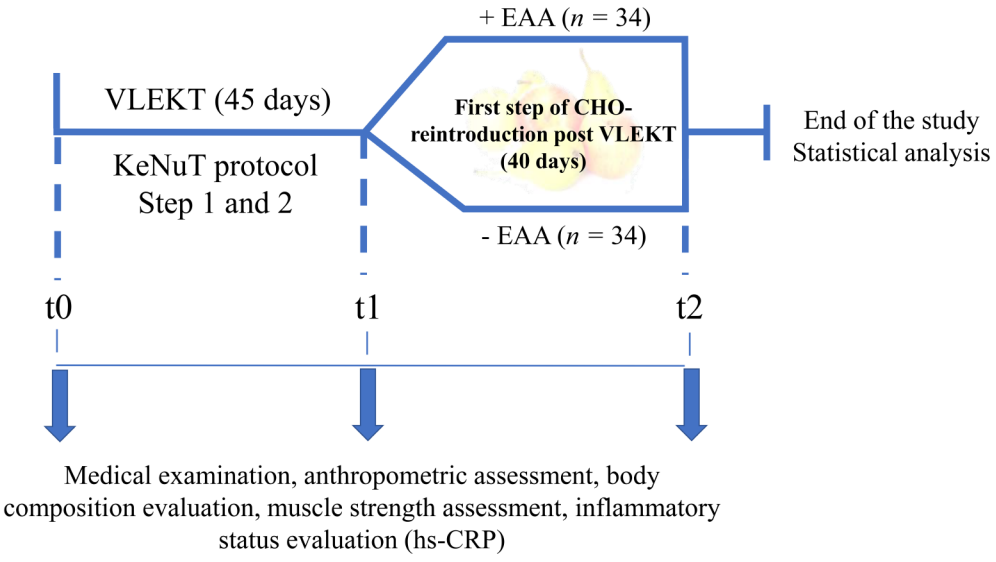


Fig. 2 Study protocol

women were classified as overweight (25.0–29.9 kg/m^2), grade I obesity (30.0–34.9 kg/m^2) and grade II obesity (35.0–39.9 kg/m^2) according to the World Health Organisation classification [16]. Waist circumference (WC) was measured using a non-elastic tape with an accuracy of 0.1 cm. Subjects were standing with their feet together, arms at their sides and were asked to breathe normally [17]. The women were asked to uncover the abdomen and the measurement of WC was taken at the midpoint between the iliac crest and the last rib. In patients with a high degree of obesity in whom it was difficult to identify the point of repere, the measurement was taken with 0.1 cm at the umbilical level [17].

Bioelectrical impedance analysis (BIA)

BC was assessed using a bioelectrical impedance analysis (BIA 101 RJL with a current of 800 A and a frequency of 50 kHz, Akern Bioresearch, Florence, Italy), conducted by the same skilled nutritionist and with the same device to minimise inter- and intra-device and observer variabilities [17]. The exam was conducted in accordance with the guidelines of the European Society of Parenteral and Enteral Nutrition (ESPEN) [18]. Specifically, during the BIA test, participants were supine with limbs slightly separated from the body, had removed shoes and socks and emptied their bladder 30 min earlier. They had not ingested any food or drink or engaged in any physical activity in the six hours prior to the examination and had not consumed any alcohol in the previous 24 h. The skin contact surfaces were cleaned with alcohol immediately before the electrodes were placed. Electrodes (BIATRODES Akern Srl; Florence, Italy) were applied on the right hand (proximal to the phalangeal-metacarpal joint) and on the right foot (distal to the transverse arch). Sensory electrodes were placed on the right wrist (midway between the distal projection of the radius and ulna) and on the right ankle (between the medial and lateral malleoli). The Nutritionist checked the function of the BIA device daily using resistors and capacitors of known value. The test directly measured the bioelectric parameters resistance (R) and reactance (Xc). The phase angle (PhA) at 50 kHz was expressed in degrees (°) and calculated following the formula PhA = arctangent Xc/R*(180/π). The BC parameters measured were FM, FFM and SMM expressed in kg and as percentage on body weight; body cell mass (BCM) expressed in kg; BCM index (BCMI) calculated by dividing the BCM for the height squared, thus expressed as kg/m^2. Hydration status parameters measured were total body water (TBW), intracellular water (ICW) and extracellular water (ECW), expressed in litres and relative percentage. All parameters were measured by the manufacture's proprietary algorithms using Bodygram Plus software (Akern, Florence, Italy). In all participants the BIA test was performed (at baseline) in the early follicular phase.

Handgrip strength test

Muscle strength was assessed using the HGS test, measured using a hydraulic dynamometer (model Lafayette J00105), which records muscle strength in kg. All measurements were carried out under strictly standardised conditions, by the same skilled nutritionist and using the same device, in order to avoid variability between different observers and instruments, after explaining the procedure to each participant, as reported above [19]. Subjects were given guidance to achieve correct positioning, which included a sitting position, with shoulders hunched and in neutral rotation, elbows bent at 90° and

forearms and wrists slightly extended (up to 30°), according to the recommendations of the American Society of Hand Therapists [20]. The participants had to squeeze the dynamometer with their non-dominant hand three times, applying maximum isometric force (for 5 s). To prevent fatigue, a rest interval of 1 min was provided between each repetition [21]. When participants were unable to perform the HGS with the non-dominant hand, the dominant hand was used. In this study, the average of the three attempts was taken for analysis. The dynamometer was regularly calibrated with resistors and capacitors of known value. The threshold value used to define a low HGS in women was < 16 kg [22].

Nutritional intervention and supplement

As reported in our previous studies [10, 23–33], in accordance with the EASO guidelines, VLEKT was divided into the three phases: active or ketogenic, re-educational and maintenance [3]. The ketogenic phase of VLEKT was jointly developed by a nutritionist and approved by an endocrinologist. The diet was formulated in accordance with specific parameters, with a total energy intake < 800 kcal/day coming from the following macronutrient distribution: 13% carbohydrate (< 30 g/day), 43% protein (1.3 g/kg ideal body weight) and 44% fat. The ideal body weight (kg) was determined using the Lorentz equation: ideal body weight = height (cm) – 100-[(height-150)/2] [34]. According to the KeNuT protocol, the ketogenic phase of the VLEKT was based on the consumption of commercially available replacement meals of high biological value, whose protein content was derived from sources such as whey, soy, eggs and peas (New Penta Srl, Cuneo, Italy). In order to ensure nutritional adequacy during the ketogenic phase with VLEKT, supplementation with B-complex vitamins, vitamins C and E, essential minerals such as potassium, sodium, magnesium and calcium, and omega-3 fatty acids (Penta-Cal, New Penta Srl, Cuneo, Italy) was provided [3]. The first step of non-ketogenic phase (re-educational phase or phase 3), as required by the KeNuT protocol, consisted in indicating the reintroduction of a fresh fruit as a snack, leaving the composition of the other meals (breakfast, lunch, dinner) unchanged compared to the preceding phase [5]. The portion of fresh fruit allowed daily was 150 g, the consumption of cooked, syrupy, dried, dehydrated or juiced fruit was not permitted. In this phase, all types of vegetables (both cooked and raw) were reintroduced and supplementation with minerals and vitamins was gradually discontinued [5]. Although all study participants followed the same dietary instructions and caloric intake for the fruit reintroduction phase, one group of women (supplemented group, $n = 34$) was prescribed EAAs supplementation (Aminotrofic®, Errekappa, Milan, Italy). The daily EAAs dose supplemented was 8 g (2 sachets). The supplemented group was instructed to take one sachet of the supplement (containing 4 g EAAs) twice daily with water (a glass), mid-morning and mid-afternoon, at least 2 h after the previous main meal and at least 30–40 min before the next. The complete composition of the daily dose supplemented was: 2,500 mg L-leucine, 1,300 mg L-lysine, 1,250 mg L-isoleucine, 1,250 mg L-valine, 700 mg L-threonine, 300 mg L-cystine, 300 mg L-histidine, 200 mg L-phenylalanine, 100 mg L-methionine, 60 mg L-tyrosine, 40 mg L-tryptophan, 300 mg vitamin B6, and 300 mg vitamin B1. Caloric intake was 41,2 kcal (172,4 kj), 300 mg fat, 500 mg carbohydrate and 0 protein, according to the nutritional table declared by the manufacturer.

Compliance to VLEKT

Adherence to VLEKT and exercise was monitored by individual telephone interviews conducted once a week by an endocrinologist and a nutritionist. To monitor actual ketosis status (YES/NO), β-hydroxybutyrate levels in capillary blood were monitored using test strips (Optium Xceed Blood Glucose and Ketone Monitoring System; Abbott Laboratories, Chicago, IL, USA). Specifically, measurement at the beginning and end of the diet programme (45 days) was carried out on an outpatient basis by the healthcare personnel involved in the weight-loss program. During the 45 days of VLEKT, on the other hand, the women were asked to independently monitor their ketone levels at home in the same way as in the outpatient clinic; this monitoring was to be carried out once a week, in the morning after an overnight fasting and, if possible, at the same time, reporting the results to the nutritionist during the weekly telephone consultation.

Measurement of high sensitivity C reactive protein (hs-CRP) levels

For the monitoring of high-sensitive C reactive protein (hs-CRP) levels, venous blood sampling was performed between 8:00 and 10:00 am, after an at least 8-hour fasting. Hs-CRP levels were measured using a high-sensitivity nephelometric test (CardioPhase hsCRP kit, Siemens Healthcare Diagnostics, Marburg, Germany), with a minimum detection limit of 0.01 mg/L and intra- and inter-test variability of less than 7%. In accordance with Centers for Disease Control and Prevention and American Heart Association guidelines, based on hs-CRP levels measured at baseline, and after 45 days of ketogenic phase with VLEKT, and after 40 days of non-ketogenic phase with reintroduction of fruit (phase 3), all women were classified into three cardiovascular risk categories: low (< 1.0 mg/L), intermediate (1.0–3.0 mg/L) and high (≥ 3.0 mg/L) [35].

Statistical analysis

The overall statistical analysis was conducted using the MedCalc® package (Version 12.3.0, 1993–2012 MedCalc Software bvba—MedCalc Software, Mariakerke, Belgium) and IBM SPSS Statistics Software (PASW Version 21.0, SPSS Inc., Chicago, IL, USA). Data distribution was evaluated using the Kolmogrov-Smirnov test. When normally distributed, continuous variables were expressed as mean ± standard deviation (SD); on the other hands, categorical variables were expressed as numbers and percentages (%). A Student's paired *t*-test was used to compare outcomes between pre- and post-VLEKT ketogenic phase and pre- and post-fruit reintroduction phase for normally distributed variables. A chi-square (χ^2) test was used to test for differences in the frequency distributions among BMI categories, WC and HGS cut-off points, and cardiovascular risk categories according to the hs-CRP levels. A bivariate proportional odds ratio (OR) model, *p*-value, 95% interval confidence (IC), and R^2 were performed to assess the associations between EAAs supplementation and Δ% of all the parameters evaluated. Differences were considered statistically significant when *p* value was < 0.05. Since this was a pilot study, no power calculations were performed.

Results

Effects of ketogenic phase with VLEKT and fruit-reintroduction re-educational phase in the entire study population

A total of 68 young women aged 25–36 years, with obesity, met the inclusion/exclusion criteria, were included in these statistical analyses. All subjects were evaluated at baseline, after 45 days of ketogenic phase with VLEKT, and after 40 days of non-ketogenic phase with reintroduction of fruit (phase 3). Adherence to VLEKT was assessed and the ketosis state was confirmed in all women (only in ketogenic phase). In addition, no women changed their physical activity levels as required.

In Table 1 anthropometric parameters, BC, and hs-CRP levels of the overall study population at baseline, after 45 days of ketogenic phase with VLEKT, and after 40 days of non-ketogenic phase with fruit reintroduction (re-educational phase) were reported.

In particular, significant reductions of body weight (Δ% = -7.30 ± 2.87, $p < 0.001$) and WC (Δ% = -7.60 ± 4.11, $p < 0.001$), and significant increase in HGS value (Δ% = 15.77 ± 12.78, $p < 0.001$), were observed in all study participants after the 45-day of ketogenic phase with VLEKT dietary protocol. Similar results were observed after the 40-day of non-ketogenic phase with fruit reintroduction period for body weight (Δ% = -5.45 ± 2.18, $p < 0.001$), WC (Δ% = -5.52 ± 1.88, $p < 0.001$), and HGS (Δ% = 9.06 ± 4.44, $p < 0.001$). Therefore, the distribution of participants across BMI categories, as well as, WC cut-off points significantly changed. Specifically, after 45-day of ketogenic phase with VLEKT the initial prevalence of women with grade II obesity decreased and all patients fell into the category of grade I obesity; on the other hands, after fruit reintroduction, the prevalence of overweight increased by 23.5%. Similarly, the prevalence of women with WC values above the cut-off of 88 cm fell from 100 to 80.9% after the ketogenic phase with VLEKT, and to 66.2% after the 40-day of non-ketogenic phase with fruit reintroduction. With regard to muscular strength, on the other hand, the prevalence of women with HGS values above the cut-off of 16 kg remained almost similar (from 98.5 to 100% after the ketogenic phase with VLEKT and unchanged after the reintroduction phase of fruit).

BIA reveals significant changes in bioelectric parameters, hydration status and BC in the study participants during the two dietary interventions (Table 1). In particular, R-values remained unchanged at the end of the ketogenic phase with VLEKT, whereas they decreased significantly at the end of the fruit reintroduction phase (Δ% = -0.97 ± 2.62, $p = 0.004$). The values of Xc and PhA, conversely, increased significantly both after the ketogenic phase with VLEKT (Xc Δ% = 7.46 ± 10.40, $p < 0.001$; PhA Δ% = 6.37 ± 10.02, $p < 0.001$) and after the fruit reintroduction phase (Xc Δ% = 4.55 ± 5.09, $p < 0.001$; PhA Δ% = 5.58 ± 5.94, $p < 0.001$).

In addition to significant changes in total hydration and intra- and extracellular distribution of fluids (Table 1), important changes in BC were observed at the end of the two nutritional interventions. More precisely, significant reductions in FM were observed at the end of the ketogenic phase with VLEKT and at the end of the fruit reintroduction phase both in absolute value (kg) (Δ% = -15.93 ± 9.14, $p < 0.$ 001 and − 13.98 ± 6.00, $p < 0.001$, post-ketogenic phase with VLEKT and post-fruit reintroduction, respectively) and in percentage (Δ% = -9.47 ± 7.67, $p < 0.001$ and − 9.10 ± 4.69, $p < 0.001$, post-ketogenic phase with VLEKT and post-fruit reintroduction, respectively). In contrast, BCM increased significantly (Δ% = 2.71 ± 8.33, $p = 0.012$ and 2.94 ± 4.76, $p < 0.001$, post-ketogenic phase with VLEKT and post-fruit reintroduction, respectively). Similarly, BCMI significantly increased (Δ% = 2.68 ± 8.37, $p = 0.017$ and 10.96 ± 1.17, $p < 0.001$, post-ketogenic phase with VLEKT and post-fruit reintroduction). Concerning the SMM expressed in absolute value (kg), a minimal but significant increase (Δ% = 0.93 ± 2.86, $p = 0.009$) was observed only after the 40-day of non-ketogenic phase with fruit reintroduction. When expressed as a percentage of body weight, on the other hand, significant increases were observed at the end of both nutritional interventions (Δ% = 7.21 ± 7.27, $p < 0.001$ and 6.79 ± 3.79, $p < 0.001$, post-ketogenic phase with VLEKT and post-fruit reintroduction, respectively).

Table 1 Anthropometric parameters, body composition, and inflammation at baseline, after the 45-day of ketogenic phase with VLEKT, and after the 40-day fruit-reintroduction in all study participants

Parameters (*N* = 68)	Baseline	Post 45-day VLEKT	*p-value	Δ%	Post 40-day fruit reintroduction	*p-value	Δ%
Anthropometric							
Weight (kg)	96.04 ± 10.15	89.02 ± 9.73	**< 0.001**	-7.30 ± 2.87	84.19 ± 9.55	**< 0.001**	-5.45 ± 2.18
BMI (kg/m^2)	35.46 ± 1.65	32.85 ± 1.43	**< 0.001**	-7.30 ± 2.87	31.07 ± 1.60	**< 0.001**	-5.45 ± 2.18
25.0–29.9 kg/m^2	0	0	-	-	16 (23.5%)	$\chi^2 = 15.94$,	-
30.0–34.9 kg/m^2	33 (48.5%)	68 (100%)	$\chi^2 = 44.47$,	-	52 (76.5%)	***p* = 0.001**	-
35.0–39.9 kg/m^2	35 (51.5%)	0	***p* < 0.001**		0	-	-
Waist circumference (cm)	106.48 ± 10.28	98.46 ± 11.05	**< 0.001**	-7.60 ± 4.11	93.02 ± 10.60	**< 0.001**	-5.52 ± 1.88
< 88 cm	0	13 (19.1%)	$\chi^2 = 12.25$,	-	23 (33.8%)	$\chi^2 = 3.06$;	-
> 88 cm	68 (100%)	55 (80.9%)	***p* = 0.001**		45 (66.2%)	$p = 0.080$	-
Handgrip (kg)	23.00 ± 3.20	26.54 ± 4.23	**< 0.001**	15.77 ± 12.78	28.90 ± 4.40	**< 0.001**	9.06 ± 4.44
< 16 kg	1 (1.5%)	0	$\chi^2 = 0.01$,	-	0	-	0
> 16 kg	67 (98.5%)	68 (100%)	$p = 0.999$		68 (100%)		100
Body composition							
R (Ω)	461.78 ± 67.11	466.59 ± 66.82	0.196	1.25 ± 6.67	461.88 ± 66.33	**0.004**	-0.97 ± 2.62
Xc (Ω)	44.50 ± 8.85	47.32 ± 7.68	**< 0.001**	7.46 ± 10.40	49.35 ± 7.50	**< 0.001**	4.55 ± 5.09
PhA (°)	5.50 ± 0.72	5.82 ± 0.67	**< 0.001**	6.37 ± 10.02	6.12 ± 0.64	**< 0.001**	5.58 ± 5.94
TBW (lt)	41.75 ± 5.45	40.84 ± 5.46	**< 0.001**	-2.14 ± 3.33	40.58 ± 5.49	**0.005**	-0.65 ± 1.81
TBW (%)	43.51 ± 3.43	45.94 ± 3.90	**< 0.001**	5.65 ± 4.85	48.28 ± 4.12	**< 0.001**	5.10 ± 2.50
ECW (lt)	20.19 ± 2.97	19.07 ± 2.62	**< 0.001**	-5.27 ± 5.16	18.36 ± 2.51	**< 0.001**	-3.67 ± 3.08
ECW (%)	48.37 ± 3.72	46.78 ± 3.35	**< 0.001**	-3.13 ± 4.88	45.30 ± 2.90	**< 0.001**	-3.06 ± 2.99
ICW (lt)	21.55 ± 3.29	21.77 ± 3.47	0.191	1.12 ± 6.54	22.22 ± 3.48	**< 0.001**	2.19 ± 4.21
ICW (%)	51.63 ± 3.72	53.22 ± 3.36	**< 0.001**	3.27 ± 5.21	54.70 ± 2.90	**< 0.001**	2.88 ± 3.11
FM (kg)	40.77 ± 6.43	34.25 ± 6.41	**< 0.001**	-15.93 ± 9.14	29.55 ± 6.31	**< 0.001**	-13.98 ± 6.00
FM (%)	42.31 ± 3.69	38.30 ± 4.63	**< 0.001**	-9.47 ± 7.67	34.90 ± 5.12	**< 0.001**	-9.10 ± 4.69
FFM (kg)	55.26 ± 5.74	54.77 ± 6.19	0.050	-0.91 ± 3.70	54.64 ± 6.36	0.269	-0.26 ± 1.83
FFM (%)	57.69 ± 3.69	61.70 ± 4.63	**< 0.001**	7.01 ± 5.58	65.10 ± 5.12	**< 0.001**	5.53 ± 2.73
BCM (kg)	28.17 ± 4.04	28.87 ± 4.32	**0.012**	2.71 ± 8.33	29.68 ± 4.40	**< 0.001**	2.94 ± 4.76
BCMI (kg/m^2)	10.42 ± 1.18	10.67 ± 1.23	**0.017**	2.68 ± 8.37	10.96 ± 1.17	**< 0.001**	2.92 ± 4.87
SMM (kg)	27.11 ± 4.88	26.89 ± 5.01	0.212	-0.74 ± 5.37	27.14 ± 5.09	**0.009**	0.93 ± 2.86
SMM (%)	28.20 ± 3.57	30.19 ± 4.10	**< 0.001**	7.21 ± 7.27	32.23 ± 4.38	**< 0.001**	6.79 ± 3.79
Inflammatory parameter							
hs-CRP levels (mg/L)	3.72 ± 2.54	2.04 ± 1.29	**< 0.001**	-33.91 ± 28.10	1.26 ± 0.99	**< 0.001**	-35.38 ± 24.20
< 1.0 mg/L	2 (2.9%)	8 (11.8%)	$\chi^2 = 2.70$; $p = 0.100$	-	37 (54.4%)	$\chi^2 = 26.04$; ***p* < 0.001**	-
1.0–3.0 mg/L	33 (48.5%)	51 (75%)	$\chi^2 = 9.00$; ***p* = 0.003**	-	26 (38.2%)	$\chi^2 = 17.24$; ***p* < 0.001**	-
> 3.0 mg/L	33 (48.5)	9 (13.2%)	$\chi^2 = 18.00$; ***p* < 0.001**	-	5 (7.4%)	$\chi^2 = 0.72$; $p = 0.397$	-

Data are expressed as number and percentage (n, %) or mean ± standard deviation (mean ± SD). *A *p* value in blod type denotes a significant difference ($p < 0.05$)

SD standard deviation, *VLEKT* very low-energy ketogenic therapy, *BMI* body mass index, *R* resistance, *Xc* reactance, *PhA* phase angle, *TBW* total body water, *ECW* extracellular water, *ICW* intracellular water, *FM* fat mass, *FFM* fat-free mass, *BCM* body cell mass, *BCMI* BCM index, *SMM* skeletal muscle mass, *hs-CRP* high-sensitivity C reactive protein

Finally, a significant reduction in hs-CRP levels was observed at the end of the two nutritional interventions (Δ% = -33.91 ± 28.10, $p < 0.001$ and – 35.38 ± 24.20, $p < 0.001$, post-ketogenic phase with VLEKT and post-fruit reintroduction, respectively). Therefore, the distribution of participants across the three cardiovascular risk categories significantly changed with a progressive and significant reduction of the prevalence of women at high risk (from 48.5 to 13.2% after the 45-day of ketogenic phase with VLEKT, and to 7.4% after the 40-day fruit reintroduction) and relative increase of those at low risk (from 2.9 to 11.8% after the 45-day of ketogenic phase with VLEKT, and to 54.4% after the 40-day fruit reintroduction).

Effects of EEAs supplementation during the fruit-reintroduction re-educational phase

During the first step of the re-educational phase (reintroduction of fruit as the first carbohydrate food group), one group of women (supplemented group, $n = 34$) was prescribed a supplementation with 8 g daily free-form EAAs to be taken in two administrations (4 g EAAs *per* administration), one mid-morning and one mid-afternoon. The remaining women (not supplemented group, $n = 34$), on the other hand, followed the same relative nutritional indications provided for this phase by the KeNuT protocol [5], without taking the EAAs supplement.

Table 2 shows the changes in anthropometric parameters, BC and inflammation observed in the two groups at the end of the 40-day fruit reintroduction phase. Before the start of the reintroduction phase (i.e. at the end of the 45-day of ketogenic phase with VLEKT), no statistically significant difference was observed between the two groups for all parameters assessed. After the fruit reintroduction phase, the magnitude of weight reduction (Δ% = -3.63 ± 1.09 vs. -7.27 ± 1.26, $p < 0.001$, not supplemented group vs. supplemented group) and WC (Δ% = -4.50 ± 1.80 vs. -6.53 ± 1.33, $p < 0.001$, not supplemented group vs. supplemented group) was significantly greater in the supplemented group than in the not supplemented group. Similarly, the magnitude of increase in the HGS value was significantly higher in the supplemented group compared to not supplemented group (Δ% = 6.81 ± 3.39 vs. 11.30 ± 4.25, $p < 0.001$, not supplemented group vs. supplemented group), also resulting in a significantly higher final absolute value in this group (HGS (kg) = 27.79 ± 4.84 vs. -30.00 ± 3.66, $p = 0.038$, not supplemented group vs. supplemented group).

At the bioimpedance level, we observed, firstly, a significantly greater extent of increase in Xc (Δ% = 0.68 ± 2.91 vs. 8.41 ± 3.64, $p < 0.001$, not supplemented group vs. supplemented group) and PhA (Δ% = 1.69 ± 5.08 vs. 9.48 ± 3.82, $p < 0.001$, not supplemented group vs. supplemented group) in the supplemented group than in not supplemented one which, for PhA, also resulted in significantly higher final absolute values (PhA (°) = 5.92 ± 0.56 vs. 6.32 ± 0.65, $p = 0.009$, not supplemented group vs. supplemented group).

In addition to significant changes in total hydration and intra- and extracellular fluids distribution (Table 2), important changes in BC were observed between the two study groups after the fruit-reintroduction phase. In particular, significant greater reductions in FM were observed in the supplemented group compared to the not supplemented one both in absolute value (kg) (Δ% = -9.25 ± 3.67 vs. -18.70 ± 3.69, $p < 0.001$, not supplemented group vs. supplemented group) and in percentage (Δ% = -5.82 ± 3.09 vs. -12.38 ± 3.60, $p < 0.001$ not supplemented group vs. supplemented group). In contrast, BCM increased significantly (Δ% = 1.14 ± 5.48 vs. 4.75 ± 3.04, $p = 0.001$, not supplemented group vs. supplemented group). Similarly, BCMI significantly increased (Δ% = 1.04 ± 5.61 vs. 4.80 ± 3.07, $p = 0.001$, not supplemented group vs. supplemented group). Concerning the SMM, although no significant differences were observed when it was expressed in absolute value (kg), a significant higher increase was observed in supplemented group compared to the not supplemented one (Δ% = 4.86 ± 4.38 vs. 8.72 ± 1.51, $p < 0.001$, not supplemented group vs. supplemented group) when expressed as a percentage of body weight.

Finally, a significant greater reduction in hs-CRP levels was observed in supplemented group than not supplemented one (Δ% = -18.15 ± 19.32 vs. 52.61 ± 14.29, $p < 0.001$, not supplemented group vs. supplemented group), also resulting in significant lower absolute valued (hs-CRP = 1.64 ± 1.24 vs. 0.88 ± 0.29 mg/L, $p = 0.001$, not supplemented group vs. supplemented group).

As expected, the data shown in Table 2 with regard to BMI, WC, HGS and hs-CRP values are reflected in changes in the distribution of women in the two study groups between BMI categories, WC and HGS cut-offs, and cardiovascular risk categories at the end of the fruit reintroduction phase (Table 3). However, significant variations were only observed with regard to the BMI categories, which showed a higher prevalence of women with grade I obesity in the not supplemented group than in the supplemented one (94.1% vs. 58.8%, $p < 0.002$, not supplemented group vs. supplemented group).

The bivariate proportional OR model was used to assess the association of the EAAs supplementation with the Δ variations of the anthropometric, BC and inflammation-related variables evaluated. As reported in Table 4, the EAAs supplementation was significantly associated with the higher values of Δ% for all the variables evaluated, except for Δ% ECW expressed in litres ($p = 0.118$), Δ% FFM expressed in kg ($p = 0.401$), and Δ% SMM expressed in kg ($p = 0.664$).

Discussion

The present retrospective study shows how in young women with grade I obesity who completed 45 days of ketogenic phase with VLEKT, supplementation with EAAs (Aminotrofic® 4 g twice a day) during the fruit reintroduction phase leads to greater improvements in anthropometry, BC, muscular strength and inflammation than in those who followed this re-educational phase without supplementation, suggesting EAAs as a nutritional strategy to optimise this phase of the KeNuT protocol.

In this view, the decision to use this supplementation exclusively in the fruit reintroduction phase has two main reasons. Firstly, since in this phase it is indicated to

Table 2 Anthropometric parameters, body composition, and inflammation before and after the 40-day fruit-reintroduction re-educational phase in supplemented and not supplement groups

Parameters (*n* = 68)	Post 45-day VLEKT	*p*-value	Post 40-days fruit reintroduction	*p*-value	Δ%	*p*-value
Age (years)						
Not supplemented	29.82 ± 3.40	0.473	-	-	-	-
Supplemented	29.26 ± 2.97		-		-	
Anthropometric						
Weight (kg)						
Not supplemented	88.56 ± 10.11	0.698	85.38 ± 10.05	0.308	-3.63 ± 1.09	**< 0.001**
Supplemented	89.48 ± 9.46		83.00 ± 9.01		-7.27 ± 1.26	
BMI (kg/m^2)						
Not supplemented	33.02 ± 0.28	0.315	31.83 ± 1.40		-3.63 ± 1.09	**< 0.001**
Supplemented	32.68 ± 1.50		30.30 ± 1.43	**< 0.001**	-7.27 ± 1.26	
Waist circumference (cm)						
Not supplemented	98.74 ± 11.58	0.833	94.31 ± 11.21	0.323	-4.50 ± 1.80	**< 0.001**
Supplemented	98.17 ± 10.67		91.75 ± 9.96		-6.53 ± 1.33	
Handgrip (kg)						
Not supplemented	26.07 ± 4.78	0.356	27.79 ± 4.84	**0.038**	6.81 ± 3.39	**< 0.001**
Supplemented	27.02 ± 3.63		30.00 ± 3.66		11.30 ± 4.25	
Body composition						
R (Ω)						
Not supplemented	468.79 ± 70.76	0.788	463.41 ± 70.22	0.851	-1.06 ± 3.67	0.762
Supplemented	464.38 ± 63.62		460.35 ± 63.22		-0.87 ± 0.72	
Xc (Ω)						
Not supplemented	47.85 ± 8.57	0.574	48.11 ± 8.32	0.176	0.68 ± 2.91	**< 0.001**
Supplemented	46.79 ± 6.75		50.59 ± 6.46		8.41 ± 3.64	
PhA (°)						
Not supplemented	5.84 ± 0.65	0.747	5.92 ± 0.56	**0.009**	1.69 ± 5.08	**< 0.001**
Supplemented	5.79 ± 0.70		6.32 ± 0.65		9.48 ± 3.82	
TBW (lt)						
Not supplemented	40.48 ± 5.72	0.585	40.42 ± 5.81	0.815	-0.15 ± 2.42	**0.021**
Supplemented	41.21 ± 5.24		40.74 ± 5.22		-1.15 ± 0.53	
TBW (%)						
Not supplemented	45.76 ± 4.13	0.710	47.40 ± 4.20	0.077	3.61 ± 2.60	**< 0.001**
Supplemented	46.12 ± 3.71		49.16 ± 3.89		6.60 ± 1.12	
ECW (lt)						
Not supplemented	18.86 ± 2.87	0.515	18.65 ± 2.88	0.343	-1.4 ± 1.81	**< 0.001**
Supplemented	19.28 ± 2.36		18.07 ± 2.08		-6.19 ± 1.70	
ECW (%)						
Not supplemented	46.65 ± 3.34	0.756	46.15 ± 2.71	**0.015**	-0.97 ± 2.43	**< 0.001**
Supplemented	46.90 ± 3.41		44.46 ± 2.85		-5.15 ± 1.80	
ICW (lt)						
Not supplemented	21.61 ± 3.44	0.715	21.77 ± 3.32	0.291	0.93 ± 5.32	**0.013**
Supplemented	21.93 ± 3.56		22.67 ± 3.62		3.45 ± 2.12	
ICW (%)						
Not supplemented	53.35 ± 3.34	0.756	53.85 ± 2.71	**0.015**	1.05 ± 2.87	**< 0.001**
Supplemented	53.09 ± 3.41		55.54 ± 2.85		4.71 ± 2.14	
FM (kg)						
Not supplemented	34.46 ± 6.90	0.794	31.32 ± 6.54	**0.019**	-9.25 ± 3.67	**< 0.001**
Supplemented	34.05 ± 5.98		27.78 ± 5.61		-18.70 ± 3.69	
FM (%)						
Not supplemented	38.69 ± 4.86	0.491	36.47 ± 4.89	**0.010**	-5.82 ± 3.09	**< 0.001**
Supplemented	37.91 ± 4.44		33.33 ± 4.93		-12.38 ± 3.60	
FFM (kg)						

Table 2 (continued)

Parameters (*n* = 68)	Post 45-day VLEKT	*p*-value	Post 40-days fruit reintroduction	*p*-value	Δ%	*p*-value
Not supplemented	54.10 ± 6.13	0.377	54.06 ± 6.12	0.456	-0.06 ± 2.24	0.377
Supplemented	55.44 ± 6.27		55.22 ± 6.63		-0.46 ± 1.31	
FFM (%)						
Not supplemented	61.30 ± 4.86	0.491	63.53 ± 4.89	**0.010**	3.68 ± 2.47	**< 0.001**
Supplemented	62.09 ± 4.44		66.67 ± 4.93		7.37 ± 1.41	
BCM (kg)						
Not supplemented	28.58 ± 4.12	0.588	28.83 ± 3.84	0.111	1.14 ± 5.48	**0.001**
Supplemented	29.16 ± 4.56		30.53 ± 4.80		4.75 ± 3.04	
BCMI (kg/m^2)						
Not supplemented	10.69 ± 1.19	0.899	10.76 ± 1.02	0.175	1.04 ± 5.61	**0.001**
Supplemented	10.65 ± 1.28		11.15 ± 1.30		4.80 ± 3.07	
SMM (kg)						
Not supplemented	26.58 ± 5.28	0.607	26.86 ± 5.37	0.651	1.08 ± 4.01	0.659
Supplemented	27.21 ± 4.79		27.42 ± 4.86		0.77 ± 0.68	
SMM (%)						
Not supplemented	29.99 ± 4.37	0.689	31.42 ± 1.39	0.128	4.86 ± 4.38	**< 0.001**
Supplemented	30.39 ± 3.88		33.04 ± 4.18		8.72 ± 1.51	
Inflammatory parameter						
hs-CRP levels (mg/L)						
Not supplemented	2.14 ± 1.70	0.526	1.64 ± 1.27	**0.001**	-18.15 ± 19.32	**< 0.001**
Supplemented	1.94 ± 0.68		0.88 ± 0.29		-52.61 ± 14.29	

Data are expressed as number and percentage (n, %) or mean ± standard deviation (mean ± SD). *A *p* value in blod type denotes a significant difference ($p < 0.05$)

SD standard deviation, *VLEKT* very low-energy ketogenic therapy, *BMI* body mass index, *R* resistance, *Xc* reactance, *PhA* phase angle, *TBW* total body water, *ECW* extracellular water, *ICW* intracellular water, *FM* fat mass, *FFM* fat-free mass, *BCM* body cell mass, *BCMI* BCM index, *SMM* skeletal muscle mass, *hs-CRP* high-sensitivity C reactive protein

consume a portion of fruit (approximately 150 g) in place of a replacement meal, there is a net reduction in daily protein intake or, at least, if this is maintained by balancing the portions of protein foods in the remaining meals, a change in its distribution over the day. Furthermore, we considered unnecessary to supplement with EAAs in the later phases in which milk and dairy products are reintroduced, foods rich in proteins of high biological value and sources of leucine [36]. In addition, in the context of optimising the re-educational phase, it should also be considered that supplementation with EAAs in the mid-morning and mid-afternoon can also represent a nutritional strategy because it allows these nutrients to be supplied to the patient without altering the caloric intake envisaged by the KeNuT protocol for this post-keto stage (approximately 1000 kcal *per* day) [5]. Since it is in fact a supplement containing only EAAs and vitamins B6 and B1, as stated on the nutrition label by the manufacturer, the complete daily dose (2 sachets, 8 g of EEAs) only provides 41 kcal, a totally negligible amount. The additional advantage of using this supplement lies in the presence of EEAs in free form, thus not as protein components, which have greater bioaccessibility and bioavailability. Although the plasma levels of EAAs in supplemented women were not monitored in our study, a previous study carried out on the same formula we used, traced its pharmacokinetic profile by comparing it with that of EAAs not administered in free form, but as components of dietary protein (meal test) [37]. The study showed that when EAAs were taken in free form, blood peaks were significantly higher, and no post-peak plateau was observed. According to the authors, these results were due not only to the method of consumption (directly as a bolus), but also to the different nutritional composition of the two formulae, in particular the presence of macronutrients (carbohydrates and fats) in the test meal, which would lead to a slowing of gastric emptying. In contrast, when EAAs are taken in free form, the gastric transit rate is more rapid, hence, intestinal absorption results faster [37]. Also, the rapid increase in plasma levels and the lack of the post-peak plateau of EAAs in free form, indicates an almost complete plasma clearance of EAAs which, in turn, suggests their rapid absorption at extra-intestinal organs and tissues levels, particularly in skeletal muscle [37]. Overall, these results indicate the high bioavailability of the EAAs administered in our study, providing a basis for understanding our observations since, in our study, supplementation was carried out twice a day away from the main meals (breakfast, lunch, and dinner), thus in the absence of co-administration of macronutrients (including complex carbohydrates, fat or high amounts of fibre) that could have had an effect on slowing down gastric emptying.

Table 3 Distribution of women into both supplemented and not supplemented groups across BMI categories, WC cut-off points, HG cut-off points, and cardiovascular risk according to hs-CRP levels after the 40-day fruit-reintroduction re-educational phase

Parameters ($n=68$)	Not supplemented ($n=34$)	Supplemented ($n=34$)	*p*-value
Anthropometric			
BMI (kg/m^2)			
25.0–29.9 kg/m^2	2 (5.9%)	14 (41.2%)	$\chi^2=9.89$; **$p<0.002$**
30.0–34.9 kg/m^2	32 (94.1%)	20 (58.8%)	
35.0–39.9 kg/m^2	0	0	
Waist circumference (cm)			
< 88 cm	9 (26.5%)	14 (41.2%)	$\chi^2=1.05$; $p=0.305$
> 88 cm	25 (73.5%)	20 (58.8%)	
Handgrip (kg)			
< 16 kg	0	0	-
> 16 kg	34 (100%)	34 (100%)	
Inflammatory parameter			
hs-CRP levels (mg/L)			
< 1.0 mg/L	15 (44.1%)	22 (64.7%)	$\chi^2=2.13$; $p=0.144$
1.0–3.0 mg/L	14 (41.2%)	12 (35.3%)	$\chi^2=0.06$; $p=0.803$
> 3.0 mg/L	5 (14.7%)	0	

Data are expressed as number and percentage (n, %) or mean ± standard deviation (mean ± SD). *A *p* value in blod type denotes a significant difference ($p<0.05$)

BMI body mass index, *hs-CRP* high-sensitivity C-reactive protein

Table 4 Bivariate OR model to assess the association of 8 g free-form EAAs daily supplementation with the Δ variations of variables

Variables	OR	*p-value	95% IC	R^2
Δ% weight	0.97	**< 0.001**	0.029–0.328	0.650
Δ% BMI	0.98	**< 0.001**	0.030–0.328	0.649
Δ% WC	0.43	**< 0.001**	0.283–0.665	0.296
Δ% HG	1.38	**< 0.001**	1.164–1.632	0.263
Δ% PhA	1.63	**< 0.001**	1.287–2.062	0.461
Δ% TBW (lt)	0.14	**< 0.001**	0.045–0.406	0.245
Δ% TBW (%)	2.69	**< 0.001**	1.752–4.131	0.421
Δ% ECW (lt)	0.01	0.118	0.001–5.483	0.719
Δ% ECW (%)	0.35	**< 0.001**	0.212–0.580	0.499
Δ% ICW (lt)	1.34	**0.009**	1.076–1.676	0.130
Δ% ICW (%)	2.18	**< 0.001**	1.488–3.190	0.390
Δ% FM (kg)	0.45	**< 0.001**	0.294–0.688	0.613
Δ% FM (%)	0.49	**< 0.001**	0.354–0.691	0.511
Δ% FFM (kg)	0.87	0.401	0.630–1.203	0.013
Δ% FFM (%)	2.95	**< 0.001**	1.843–4.704	0.493
Δ% BCM (kg)	1.37	**0.002**	1.119–1.678	0.193
Δ% BCMI	1.37	**0.002**	1.124–1.677	0.200
Δ% SMM (kg)	0.96	0.664	0.799–1.153	0.003
Δ% SMM (%)	1.83	**< 0.001**	1.384–2.407	0.351
Δ% hs-CRP	0.91	**< 0.001**	0.880–0.948	0.462

*A *p*-value in bold type denotes a significant difference ($p<0.05$)

OR odds ratio, *BMI* body mass index, *WC* waist circumference, *HG* handgrip, *PhA* phase angle, *TBW* total body water, *ECW* extracellular water, *ICW* intracellular water, *FM* fat mass, *FFM* fat-free mass, *BCM* body cell mass, *BCMI* BCM index, *SSM* skeletal muscle mass, *hs-CRP* high-sensitivity C-reactive protein

EAAs are historically known for their effect on muscular anabolism [38], which has consolidated their use in various clinical contexts, first and foremost that of geriatric and critical patients [8]. In particular, the specific composition of EAAs formulae appears to contribute to the anabolic action, with leucine recognised as the main stimulator of MPS, with proven effects on improving muscle mass and physical function [39–41]. The EAAs formula used in our study had a high proportion of leucine (2.5 g *per* day), which is therefore in line with the indications in the scientific literature for promoting an anabolic effect [7]. In our study, however, we did not observe any significant changes in SMM (expressed in absolute value, kg) in either group. This result was expected, since both groups were prescribed the same diet with equal caloric and protein intake and, above all, were advised not to exercise. Thus, in young subjects, in the absence of pathological processes favouring muscle catabolism but, on the contrary, following a nutritional program that resulted in an important and positive metabolic input with weight and FM reductions, no major changes in SMM were expected even with amino acid supplementation. On the other hand, however, a greater increase in SMM expressed as a percentage was observed in the supplemented group. This finding is interesting as it suggests the role of EAAs supplementation in contributing to improve body composition, since SMM% expresses the percentage of muscle tissue in the body. Furthermore, SMM% was identified as an independent predictor of metabolic syndrome in a recent large cross-sectional study [42]. Also, it is also important to note that significantly higher improvements in BCM and BCMI were observed in the supplemented group. This finding is of interest as the BCM expresses the SMM and better describes its changes [43, 44].

The stimulation of MPS is also believed to play a key role in tissue hydration. At muscle cell level, in fact, there are high amounts of actin and myosin within the myofibrils, the interaction of which determines muscle shortening and contraction. For this interaction, the participation of water is particularly relevant, given the hydrophilic nature of proteins, as well as the chemical properties of the aqueous solution [45]. This implies that small changes in protein concentrations result in significant changes in osmotic pressure [45], with a consequent pull of water into the cell (increased ICW). This fluctuation of fluid towards the inside of the cell is due to the particular sensitivity of muscle fibres to osmotic changes, in turn given by the high presence of aquaporine-4 transporters [46]. It is interesting to note that the relationship between muscle protein concentration and intracellular

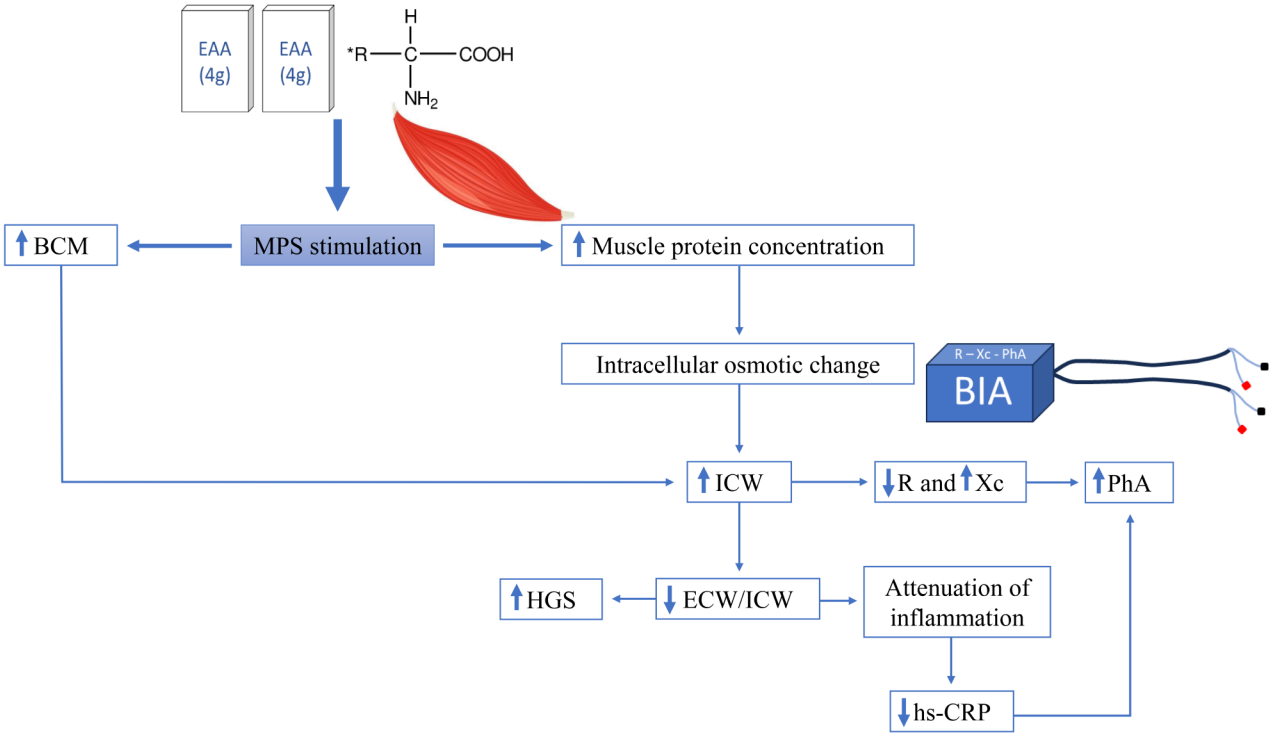


Fig. 3 Main putative mechanisms for the effects of EAA in improving body cell mass, muscle strength and inflammation. The supplementation with essential amino acid stimulate the muscle protein synthesis, resulting in both increasing body cell mass and muscle protein concentration. This latter causes an intracellular osmotic change resulting in increased intracellular water (ICW) levels, which is revealed via bioelectrical impedance analysis with reduction of resistance and increase of reactance, with consequent increased phase angle (PhA). Also, the increased ICW results in a mathematical reduction of the extracellular water/ICW ratio, which is associated with two main outcomes: (i) increased muscle strength (observed as increased handgrip strength values) and (ii) attenuation of pro-inflammatory stimuli (observed as reduced levels of high-sensitivity C-reactive protein) that, in turn, correlates with increase in PhA values. Abbreviations: essential amino acid, EAA; body cell mass, BCM; muscle protein synthesis, MPS; bioelectrical impedance analysis, BIA; intracellular water, ICW; resistance, R; reactance, Xc; phase angle, PhA; handgrip strength, HGS; extracellular water, ECW; high-sensitivity C-reactive protein, hs-CRP

fluids is, in some respects, bidirectional, triggering a virtuous circle in which the increase in ICW itself (i) promotes the pathways of synthesis and (ii) slows down those of protein degradation [47, 48], (iii) stimulates the proliferation of satellite cells, favouring their fusion with myofibrils in the processes of muscle hypertrophy [49]. These changes in hydration status are clearly described at the BIA level, not only by changes in the (absolute and percentage) values of TBW, ECW and ICW, but also by changes in the raw BIA parameters. In this case, since muscle mass is an excellent conductor [50], and given the increase in ICW, reductions in R-values and increases in Xc-values are observed, resulting in an increase in PhA [51, 52]. Our results are in line with what has been described so far. With regard to hydration status, in fact, during the fruit reintroduction phase, we observed a significantly greater increase in ICW in the supplemented group than in the not supplemented one, both in absolute values (litres) and in percentage. With regard to the raw BIA parameters, although there were no significant differences in the R-values between the two groups, significant increases in Xc and PhA values were observed in the supplemented group. Overall, therefore, these results allow us to speculate on the hypothesis that supplementation with EAAs effectively promoted MPS, resulting in an increase in protein concentrations in muscle cells, with a consequent change in osmotic pressure that promoted fluid entry into the cell, thus, improving tissue hydration (Fig. 3).

The assessment of hydration status is particularly relevant in obesity, in which an expansion of ECW has been described, which is reflected in an increased ECW/ICW ratio [53], as a possible consequence of (i) an elevated ECW/ICW ratio in adipose tissue [54, 55], (ii) presence of obesity-associated oedema or (iii) adipose tissue-related hormonal response [56]. This peculiar fluid distribution seems to be a characteristic of obesity itself, as documented by pioneering studies on the subject that reported a higher ECW/ICW ratio in women with obesity than in their counterparts with normal weight [56]. In this context, it is interesting to note that studies have shown an increase in this ratio in women with obesity as a consequence of long-term weight loss achieved through dietary intervention [53] or bariatric surgery [57].

According to some authors, this increase in the ECW/ICW ratio post weight loss could be due to alterations in haemodynamics and fluid regulation [53, 58], as well as a reduction in ICW which, in turn, would reflect a reduction in BCM and cell shrinkage [53]. In contrast to what has been described so far, in our study we observed, overall, a reduction in ECW and an increase in ICW after 45 days of ketogenic phase with VLEKT, which is reflected in a mathematical (although not calculated) reduction of the ECW/ICW ratio. This change in intra- and extracellular fluid distribution followed the same trend after 40 days of fruit reintroduction, being more pronounced in the supplemented group than in the not supplemented one. This discrepancy with the literature data can be explained as the result of a lack of SMM loss. In fact, as previously described, in women with obesity, weight loss accompanied by a reduction in SMM was reflected in a concomitant reduction in ICW [59]. Considering that, as reported by the authors themselves, this reduction in SMM would be the result of increased gluconeogenesis (presumably from amino acid substrates [60]) induced by persistent calorie restriction, it is possible to speculate the hypothesis that the supplementation with EAAs carried out in our study (as well as the optimal protein intake during the VLEKT) counteracts this mechanism by directly supplying the necessary substrates, thus avoiding protein breakdown and sparing the muscle. Consequently, the ICW does not decrease but, on the contrary, increases due to the mechanisms described above.

It is worth mentioning that, as its compartment, measurements of ICW are often used to obtain an accurate estimate of BCM [61], a particularly important component of lean body mass, as it represents the total mass of metabolically active cells, thus, responsible for the basal metabolic rate [62]. In this sense, BCM is considered to be the potassium-rich tissue capable of exchanging oxygen, oxidising glucose and performing work [62], thus representing a fundamental parameter for assessing nutritional status [61]. In our study, we observed that BCM values (kg) increased overall both after 45 days of ketogenic phase with VLEKT and after 40 days of fruit reintroduction. However, during the re-educational phase, a greater increase was observed in the supplemented group than in the not supplemented one. Since increases in BCM values, describing an increased mass of metabolically active cells [50], are reflected in increases in oxygen consumption, carbon dioxide production and energy expenditure [63, 64], it is possible to speculate the hypothesis that this represents the mechanism by which significantly greater reductions in body weight were observed in the supplemented group as well as in FM both in absolute values and as a percentage of body weight.

Hydration status also plays a crucial role in muscle strength. In particular, the ECW/ICW ratio has been defined as a strong predictor of muscle strength, independent of age, gender, BMI and SMM, with ICW playing a key role [65], as also confirmed by studies on athletes demonstrating a linear relationship between muscle strength and ICW levels [66–68]. Conditions of cellular dehydration, in fact, appear to result in inhibitory stimuli on mTOR [69] and be implicated in metabolic alterations such as anabolic and insulin resistance [70]. This would be due to alterations in protein folding, a process that, driven by hydrophobic interactions, is responsible for their acquisition of functional structure [71]. As reported in a meta-analysis, several studies have shown an effect of EAAs supplementation on the improvement of muscle strength, although the risk of bias in the studies analysed does not allow definitive conclusions to be drawn [7]. However, our observations are in line with these studies by demonstrating a magnitude of increase in the HGS value significantly greater in the supplemented group than in the not supplemented one, which also resulted in a significantly higher final absolute value in this group. Yet, the exact mechanisms by which EAAs increase muscle strength have not been clarified, and the conclusions remain speculative. For this reason, our hypothesis to explain the increase in HSG values in the supplemented group is that, as previously reported, a putative mechanistic explanation would be due to the possible effect of EAAs in improving tissue hydration by (a) stimulating MPS with (b) consequent increasing intramuscular protein concentrations, (c) leading to an osmotic change which, in turn, (d) draws water back into the cell (Fig. 3).

There is evidence that an alteration in intra- and extracellular fluid distribution, with particular reference to elevated ECW due to increased extracellular osmolarity, represents an inflammatory stimulus that directly induces the secretion of flogosis markers such as tumour necrosis factor and interleukins 1, 6 and 8 [72]. It is plausible to speculate, therefore, that determining a change in intracellular osmolarity could perpetuate a mechanism capable of attenuating the inflammatory stimulus. In our study, we observed that hs-CRP levels (a validated inflammatory biomarker) were reduced more in the supplemented group than in the not supplemented one, also resulting in significantly lower absolute values. These results are in line with a previous retrospective study showing that supplementation of EAAs with the same formula used in our study resulted in a significant reduction in CRP levels in post-acute inflamed elderly subjects [73]. Similarly, we observed that the PhA values increased to a greater extent in the supplemented group, resulting in significantly higher absolute values. From a strictly mathematical point of view, PhA is a direct measure of the relationship between Xc and R and expresses,

biologically, the quality and functionality of cell membranes, as well as reflecting BCM values [74]. More in detail, PhA depends on various factors (including cell content, body fluids, membrane integrity and permeability) [75], thus providing information on both cell integrity and size [76, 77], as well as intra- and extracellular fluid distribution [78, 79], following an inverse relationship [80, 81]. Based on these concepts, PhA is considered a valid surrogate marker of inflammation [75], whose increased values correlate with reduced levels of inflammatory markers, including hs-CRP, also following specific dietary interventions, such as VLEKT [82]. Considering, therefore, inflammation from a BIA point of view, it is possible to describe it as a process which, by causing a change in the intra/extracellular hydro-electrolyte balance induced by the release of related substances (e.g. cytokines) [83], is reflected in a reduction of Xc and R values as a consequence of reduced membrane capacity and expansion of extracellular fluids, respectively. This, in turn, results in a reduction of PhA [75]. These mechanisms allow us, once again, to speculate on the hypothesis that the change in intracellular osmolarity brought about by EAAs as a result of the increased MPS described above and reflected in changes in intra/extracellular fluid distribution, may represent a possible additional mechanism for their inflammation-mitigating effect (Fig. 3). Actually, however, it should be mentioned that additional mechanisms have been proposed to explain the effect of EAAs supplementation on the reduction of inflammatory parameters. These mechanisms are essentially based on the effects of EAAs on the regulation of innate and adaptive immunity [84, 85], influencing the activity of immune cells rather than their number [73]. In particular, EAAs are able to stimulate lymphocytes both directly and indirectly. Directly, EAAs promote protein synthesis by stimulating mTOR in lymphocytes, which is necessary for their differentiation, activation and function [86]. Indirectly, they act (i) by stimulating the synthesis of glutamine, an amino acids which contributes to numerous functions including T-cell proliferation, protection from apoptosis, inhibition of cytokine synthesis and modulation of the balance between Treg and Th cells [87], (ii) stimulating the synthesis of insulin-like growth factor-1, whose receptors are present on almost all immune cells and which regulates lymphocyte function [88], and (iii) reducing cortisol levels following reduced inflammation, which results in increased lymphocyte numbers [89].

Limitations and strengths

Our study has a number of strengths but also limitations. Firstly, being a retrospective study, the population included in the statistical analysis is not large. However, considering that various parameters show clear gender differences, including HGS, PhA values and hs-CRP levels, we included in the pre/post fruit reintroduction phase statistical analysis only women of reproductive age who were in the same BMI class and who had not changed their physical activity levels during the observation, in order to make the sample more homogeneous. Similarly, the decision to include only women in the study also lies in recent observations reporting a gender difference in the effects of VLEKT which, although significant in terms of weight and fat mass reduction, as well as in the improvement in inflammatory status for both sexes, appear to be more pronounced in men [90]. These observations thus suggest, in a broader sense, the importance of considering VLEKT as a valid tool also in precision nutrition. Similarly, such differences may also exist in the response to supplementation. These data are currently not yet available, so no firm conclusions can be drawn. However, it is possible to speculate on the hypothesis that, even if there was no greater response to supplementation with EAA in males, this might represent an added value in the female population in order to balance out the lesser response to the metabolic effects of VLEKT, at least at the muscular level. Furthermore, in order to reduce possible interpretation bias, particularly related to BIA, all tests were performed at the same stage of the menstrual cycle in all participants. Also, the observations of this study were not extended to the subsequent steps of the re-education phase. This was dictated both by a (i) functional motivation linked to the fact that the prolongation of the observation period could have increased the drop-out rate, leading to a reduction in the sample size, and by a (ii) conceptual motivation, linked to the fact that, in our opinion, supplementation with EAAs would have more reason to be carried out in the phase of fruit reintroduction, rather than in the subsequent phases, for the reasons described above. Moreover, despite the extensive literature on the effects of supplementation with EAAs, the absence of evidence that has investigated changes in BC, muscle strength and inflammatory status in the phases following the ketogenic one of VLKET with or without supplementation allows only speculative conclusions to be drawn, with possible mechanisms of action proposed. However, the originality of this study represents an important strength that could inspire further investigations in this direction on a larger number of subjects, in order to possibly confirm our observations. Finally, no other markers of inflammation or other immunity-related blood parameters were analysed, which makes it impossible to delineate precise mechanisms of action for the inflammation-reducing effect of EAAs supplementation for our study. However, hs-CRP is the most studied marker of inflammation in numbers of clinical settings, and the study design involving a comparison group reinforces the observed results.

Conclusion

In conclusion, this study shows for the first time how, in concomitance with fruit reintroduction during the first step of the post-VLEKT re-educational phase, supplementation with 8 g daily EAAs improves BCM, muscle strength and inflammatory status in young women. These results suggest the supplementation with EAAs in this non-ketogenic phase as a strategy to maintain or prolong the beneficial results of VLEKT over time, determining a therapeutic continuum during a transition phase that could be relevant in the long term. If confirmed by studies on a larger number of subjects, of different age group and gender, these results could guide towards an update of the KeNuT protocol, including supplementation with EAAs in this phase.

Abbreviations

BC	Body composition
BCM	Body cell mass
BCMI	Body cell mass index
BIA	Bioelectrical impedance analysis
BMI	Body mass index
EAAs	Essential amino acids
ECW	Extracellular water
FFM	Fat-free mass
FM	Fat mas
HGS	Hand-grip strength
hs-CRP	High sensitivity C reactive protein
ICW	Intracellular water
KeNuT	Ketogenic nutritional therapy
MM	Muscle mass
MNT	Medical nutrition therapy
MPS	Muscle protein synthesis
mTOR	Mammalian target of rapamycin
PhA	Phase angle
R	Resistance
SMM	Skeletal muscle mass
TBW	Total body water
VLCKD	Very low-calorie ketogenic diet
VLEKT	Very low-energy ketogenic therapy
WC	Waist circumference
Xc	Reactance

Author contributions

The authors' responsibilities were as follows G.A., G.M., A.C., and L.B.: were responsible for the concept and design of the study and interpreted data and drafted the manuscript; G.A., G.M., A.C., and L.B.: conducted statistical analyses; L.V,. V.D.O., M.C., S.G., and S.S.: provided a critical review of the manuscript. All authors contributed to and agreed on the final version of the manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the guidelines outlined in the Declaration of Helsinki, which provides ethical principles for medical research involving human subjects. Additionally, the Ethics Committee of the Federico II University of Naples reviewed the study procedures and granted a positive opinion on the study protocol (reference no. 50/20).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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