Essential amino acid formulations to prevent mitochondrial dysfunction and oxidative stress

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Purpose of review
Both restriction and supplementation of specific amino acids or branched-chain amino acids (BCAAs) are described to improve metabolic homeostasis, energy balance, and health span. This review will discuss the recent findings of the role of amino acid supplements in the regulation of mitochondrial health.

Recent findings
A mixture of essential amino acids (EAAs), BCAA enriched mixture, was found to extend healthy life span in elderly mice and prevent multiple diseases associated with an energy deficit, similarly to caloric restriction or fasting-mimicking diets. A growing body of evidence highlights mitochondria as the central target of this supplement: it promotes mitochondrial biogenesis and the activation of antioxidant defence systems in different physiological (e.g., exercise or ageing) or pathological conditions (e.g., sarcopenia, muscular dystrophy, liver steatosis, or impaired cognition). Based on these results, new formulas have been created enriched with Krebs cycle substrates, behaving more efficiently than BCAA enriched mixture.

Summary
EAA-BCAA balanced supplements might be valuable not only for healthy individuals undergoing to energy deficit (e.g., athletes) during strenuous exercise or training but also against diseases characterized by a dysregulated catabolic state or mitochondrial dysfunction, such as age-related disorders. The associated mechanistic processes should be identified as potential pharmacological targets.

Keywords
ageing, essential amino acids, mechanistic target of rapamycin, mitochondrial biogenesis, nitric oxide

INTRODUCTION

Nutrition is one of the significant determinants of health. Among the three primary macronutrients, proteins – particularly the quantity and quality (i.e., the specific amino acid profile) of proteins – play a crucial role in regulating metabolic health and longevity. Significantly, the restriction (e.g., leucine, methionine, or tryptophan) or supplementation (e.g., leucine, glycine, or arginine) of single amino acids can substantially modulate metabolic health [1]. Chronic supplementation with a specific amino acid mixture enriched in branched-chain amino acids (leucine, isoleucine, valine; BCAAs), referred to as BCAA-enriched mixture (BCAAem) preserved mitochondrial metabolism and enhanced physical endurance in middle-aged mice and increased average lifespan [2]. Also following this seminal study, amino acid formulations containing the BCAAs have assumed particular prominence to counteract muscle wasting in elderly, cachectic or sarcopenic patients [1,3,4▪–6▪].

By activating the mechanistic target of rapamycin complex 1 (mTORC1), BCAAs take part in vital pathways linking nutrition with health and ageing [1,5▪]. Here, we review several relevant topics within the field, to clarify the health effects of essential amino acids (EAAs) through their actions on mitochondrial function and oxidative stress defence systems in various pathological contexts, providing

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KEY POINTS

- EAA-BCAA supplementation in healthy individuals is associated with beneficial effects on muscle growth and energy expenditure.
- Oral EAA-BCAA supplements might be harmful or beneficial in humans, depending on metabolic (anabolic vs. catabolic) state.
- Oral intake of balanced EAA-BCAA mixtures reproduces the mechanisms and beneficial effects of calorie restriction and fasting-mimicking diets.
- The health-promoting effects of oral EAA-BCAA supplements in older patients are related to the enhanced mitochondrial bioenergetics and antioxidant defence in multiple tissues, including skeletal muscles, heart, and brain.
- Novel EAA-BCAA mixtures with tricarboxylic acid cycle substrates show enhanced amelioration of mitochondrial dysfunction in ageing and diverse preclinical models.

new insights into the relationship between what we eat, metabolic disease, and health.

AMINO ACIDS AND ENERGY METABOLISM

The amino acids represent the only source of nitrogen for mammals. There is no specific storage of amino acids in the body. Their carbon backbone can be converted into the mitochondrial tricarboxylic acid cycle (TCA) intermediates or precursors, and subsequently metabolized to CO_2 and H_2O, releasing ATP, or used to produce glucose (glucogenic amino acids). Moreover, specific amino acids can be catabolized to acetyl-CoA or acetoacetate and lead to the production of fatty acids (FAs) or ketone bodies (ketogenic amino acids). Therefore, the amino acids can be converted not only into energy, but also into carbohydrates, lipids, and biochemical intermediates, based on body metabolic demands.

In healthy individuals, a positive correlation between high consumption of animal-derived proteins and the increase in the overall mortality was described [4▪]. Both protein or amino-acid restricted diets can regulate glycaemic control and reduce pancreatic β-cell metabolic stress, suggesting that a relatively modest protein or amino acid restricted regimen may have clinical benefits in healthy individuals [7]. Although basal protein synthesis rates are not different between age-groups [8], protein malnutrition is often observed in elderly people. In contrast, the consumption of a high animal-protein diet or increased amino acid intake in older people has been associated with reduced overall mortality rate, probably due to the positive effect of amino acids on muscle wasting [3].

The EAAs are considered the most relevant nutritional input for protein synthesis, with a crucial role in the control of glucose and lipid metabolism and the maintenance of energy balance [4▪]. In particular, the BCAAs constitute ~35% of the amino acids in muscle proteins, and a tightly regulated system has evolved to control their catabolism. The first step of BCAA oxidation involves the transamination to branched-chain keto acids by cytosolic (mainly in the brain) or mitochondrial BCAA transaminase (BCAT). The second step, the decarboxylation and dehydrogenation by the branched-chain ketoacid dehydrogenase complex (BCKDH) inactivated by phosphorylation by BCKDH kinase (BCKDK) and activated by dephosphorylation by mitochondrial phosphatse 2C (PP2Cm) is the rate-limiting reaction on the inner membrane of mitochondria. Even if oxidation steps are unique to each BCAA, the end-products acetyl-CoA or succinyl-CoA ultimately enter the TCA cycle, where they can be completely oxidized [9]. Of note, the endurance exercise activates the BCKDH complex in experimental animals and humans [10]; accordingly, the muscle-specific deletion of BCATm or BCKDK decreases the endurance capacity after training [11]. Multiple observations relate the amino acid metabolism, particularly the BCAA oxidation, with mitochondrial dysfunction and oxidative stress. For example, mice with a skeletal muscle-specific expression of the active form of the human ATP synthase inhibitor show augmented BCAA catabolism with an augmented production of acetyl-CoA, and this metabolic rewiring leads to lipid synthesis and accumulation [12▪▪]. Consequently, muscle accumulation of the acetyl-CoA inhibits mitochondrial respiratory complex II, through an acetylation-dependent process, leading to an increased radical oxygen species (ROS) production. Notably, the transgenic mice with restrained mitochondrial ATP synthase activity presented an overt metabolic syndrome phenotype, with intrafibre lipid droplets, higher visceral white adipose tissue (WAT) depot, and insulin resistance [12▪▪]. Most important, edaravone – a lipophilic antioxidant drug, approved by Food and Drug Administration to recover from stroke and to treat amyotrophic lateral sclerosis, with a potent mitochondrial enhancer function – increased mitochondrial respiration, restored lipid β-oxidation and ROS imbalance in the ATP synthase restrained mice. Consequently, these mice were protected from obesity and insulin resistance development when fed with a high-fat diet and simultaneously treated with edaravone. Of note, plasma BCAA levels were slightly

reduced compared with untreated animals. Again, the increased uptake of circulating BCAAs from brown adipocytes in mice acutely exposed to a low-temperature environment constitutes another example of the close relationship between the BCAA oxidation and mitochondrial function. Adaptation to cold was markedly impaired in mice with a brown adipose tissue-specific defect of BCAA oxidation: these mice on a high-fat diet gained significantly more body weight than littermate controls, owing to increased adipose tissue and liver mass [13▪▪]. This process requires SLC25A44, the mitochondrial BCAA transporter: SLC25A44 depletion caused a significant reduction in noradrenaline-induced mitochondrial respiration in the presence of valine in human brown adipocytes. Although the SLC25A44-depleted brown adipocytes displayed active mitochondrial respiration, overexpression of *Slc25a44* in mouse inguinal WAT-derived adipocytes or C2C12 myotubes significantly increased mitochondrial valine uptake and oxidation and cellular respiration [13▪▪], confirming the connection between mitochondrial activity and BCAA catabolism.

AMINO ACIDS, MITOCHONDRIA, AND ANTIOXIDANT SYSTEMS

A body of evidence established a correlation between amino acid supplements and mitochondrial activity and function. Mitochondria have crucial roles in the setting of metabolic and age-related disorders, suggesting these organelles as potential therapeutic targets [12▪▪]. Mitochondrial biogenesis, dynamics, and clearance of damaged mitochondria by mitophagy cooperate to assure efficient energy production. Nitric oxide (NO), a short-living signal molecule produced by endothelial NO synthase (eNOS), induce the expression of the master regulator of mitochondrial biogenesis peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α), promoting mitochondrial respiration in various tissues [14]. As superbly reviewed by Farah *et al.* [15], physiological levels of NO favour angiogenesis, blood flow, as well as brown adipocyte differentiation and 'browning' of WAT. Significantly, the eNOS-PGC-1α-mediated mitochondrial renewal mechanisms are at the basis of the beneficial effects of calorie restriction, exercise, and BCAA supplementation [2,14,15].

The BCAA leucine activates mTORC1 and sirtuin 1 (SIRT1)-mediated deacetylation (i.e., activation) of PGC-1α. It increases mitochondrial biogenesis, NAD^+ levels, and FA oxidation in skeletal myotubes [16]. Leucine supplementation attenuates the mitochondrial dysfunction, hyperglycaemia, and the rate of body fat production in high-fat-diet-fed mice [17]. Oral intake of leucine or its metabolite beta-hydroxy-beta-methyl butyrate could aid the maintenance of muscle mass and function in the elderly, but well designed clinical trials are lacking [18].

Multiple reports demonstrate that balanced combinations of different amino acids are safe and more effective than single amino acid supplementation [3,4▪]. Of note, the lifespan-promoting effects of the BCAAem are eNOS-dependent and occur via the mTORC1-SIRT1-PGC-1α pathways, with enhanced mitochondrial biogenesis and function in cardiac and skeletal muscles of aged mice [2]. BCAAem also activates the endogenous defence system against ROS [2]. A virtuous cycle between eNOS and mTORC1 seems to be involved in the BCAAem beneficial effects [2]. The BCAAem was able to mitigate muscular dystrophy in *mdx* mice (the experimental model of Duchenne muscular dystrophy) [19], preserve skeletal muscles from rosuvastatin-induced myopathy [20], and prevent alcohol-induced liver steatosis [21]. More importantly, this original formula has proven health-promoting effects in a variety of clinical settings [3].

We recently investigated two novel amino acid formulations, containing balanced EAA-BCAA stoichiometric ratios and Krebs' cycle precursors and cofactors (i.e., citric, succinic, and malic acid), designed to optimize their effects on mitochondrial bioenergetics (for composition, refer to Table 1) [22–25]. The first formula (termed α5) was able to boost mitochondrial function and ROS scavenging

Table 1. Composition of the essential amino acid mixtures

Essential amino acids	BCAAem	α5	PD-E07
L-Leucine	30.01	31.09	22.45
L-Lysine (chlorhydrate)	19.58	16.90	21.13
L-Isoleucine	15.00	10.36	11.23
L-Valine	15.00	10.36	11.23
L-Threonine	8.40	7.25	13.1
L-Cysteine	3.60	3.11	2.81
L-Histidine	3.60	3.11	2.81
L-Phenylalanine	2.40	2.07	1.87
L-Methionine	1.20	1.04	0.94
L-Tyrosine	0.72	0.62	–
L-Tryptophan	0.48	2.07	0.94
Vitamin B1 (thiamine chlorhydrate)	–	0.004	0.02
Vitamin B6 (pyridoxine chlorhydrate)	–	0.004	0.02
Citric acid	–	8.00	7.65
Malic acid	–	2.00	1.92
Succinic acid	–	2.00	1.92
Leucine : isoleucine : valine ratio	2 : 1 : 1	3 : 1 : 1	2 : 1 : 1

All values are reported as percentage (g/100 g). BCAAem, branched-chain amino acid-enriched mixture.

mechanisms, promoting neuronal stem cell differentiation [22]. In particular, the α5 mixture improved energy metabolism of differentiating neurons derived both from murine neural stem cells and human-induced pluripotent stem cells. We found that α5 promoted the total dendritic length, the mean number of branches, and the number and maturation of the dendritic spines in the differentiating neuronal cells compared to untreated cells. The neuronal spines in treated neurons emerged more durable with stubby and mushroom phenotype and with increased expression of molecules involved in synapse formation. Treated neurons adjusted their mitochondrial dynamics, raising the mitochondrial fusion and, consistently with the increase of cellular ATP content, they strongly activated cellular mTORC1-dependent anabolism. The global transcriptomic analysis further revealed that treatment induces nuclear factor, erythroid 2 like 2 (Nrf2)-mediated gene expression in neurons, and this was associated with a functional increase in the ROS scavenging machinery. The α5 mixture was also observed to induce Krüppel-like factor 15 (KLF15, a transcriptional regulator of BCAA oxidation) and activate a KLF15-eNOS-mTORC1 signalling axis in mouse cardiomyocytes. It exerted mitochondrial biogenic and antioxidant effects, and efficiently prevented the doxorubicin-mediated cardiac damage [23]. We reported that the second mixture (PD-E07) could induce PGC-1α and the activity of the mitochondrial respiratory complexes in skeletal muscle and hippocampus of Senescence-Accelerated Mouse Prone 8 (SAMP8) mice, an accurate model of age-related muscular and cognitive alterations [24]. In particular, 3-months of dietary treatment with PD-E07 in drinking water preserved healthy body condition, muscle weight to body weight ratio, motor endurance, and working memory at 12 months of age, an age characterized by molecular and physical impairments in the untreated SAMP8 mice. The PD-E07 mixture increased the protein levels and the enzymatic activities of mitochondrial complexes and the expression of optic atrophy protein 1 and Nrf2, beyond PGC-1α, in muscles and hippocampi, preventing neuromuscular and cognitive dysfunction in the elderly mice [24]. Both α5 and PD-E07 mixtures have been found to promote mitochondrial biogenesis and protection against oxidative stress more efficiently than BCAAem in cardiomyocytes [25].

Most of the studies evaluating the efficacy of amino acid supplements were conducted in male mice, and no conclusive evidence has been obtained in females. However, nutrients exert sex-specific effects. The sexual dimorphism of mitochondrial oxidative capacities has been observed in various tissues [26]. Female mitochondria are more efficient and generate less ROS than male mitochondria in stress conditions [26]. Sexual dimorphism in regulating BCAA metabolism has also been observed. In particular, hepatic BCKDH expression was reduced in male but not in female obese patients [27▪]. Sex differences in response to amino acid-based nutritional approaches should be further investigated.

Overall, peculiar amino acid mixtures enriched in BCAAs (with or without metabolic enhancers) seem to promote health and longevity by preserving mitochondrial energy efficiency and enhancing the ROS defence system, that is sharing multiple mechanisms with calorie restriction [2]. Calorie restriction – the reduced caloric intake without malnutrition – extends healthy lifespan in diverse animal models, and its effects seem to be sex-independent [28▪,29]. Calorie restriction is challenging in the long-term, mainly in older people, and various fasting-mimicking diets (FMDs) are under scrutiny to promote health benefits while minimizing the burden of chronic restriction [28▪]. Here, the potential of amino acid nutraceuticals able to mimic the calorie restriction or FMD beneficial effects in a regular dietary regimen (Fig. 1).

AMINO ACIDS, GUT MICROBIOTA, AND ENERGY HOMEOSTASIS

An intriguing study demonstrates that host genetics has a minor role in determining microbiome composition in healthy individuals. By contrast, the interperson microbiota variability is associated with environmental factors comprising diet, drugs, and lifestyle [30▪▪]. The effects of calorie restriction and FMDs on microbiota composition might contribute to their beneficial effects on lifespan [31▪,32]. Similarly, the beneficial metabolic effects of exercise are related to changes in gut microbial composition and plasma amino acid profile [33]. In turn, the diverse influences of EAAs in catabolic or anabolic conditions may be partially attributed to differences in the gut microbiota composition [34].

The amino acids can be absorbed by the intestinal epithelium or utilized by bacteria in the intestinal lumen. Gut microbes produce certain EAAs; at the same time, the amino acids serve as essential nitrogen sources to support microbiota growth and survival [35]. Notably, the EAAs affect intestinal bacterial growth in a species-specific manner [35]. The gut microbiota catabolizes the amino acids primarily, but not exclusively, by deamination and decarboxylation; collectively, the catabolic reactions produce a variety of metabolites, including NO and short-chain FAs (propionate, butyrate, and acetate; SCFAs). SCFAs regulate lipid and

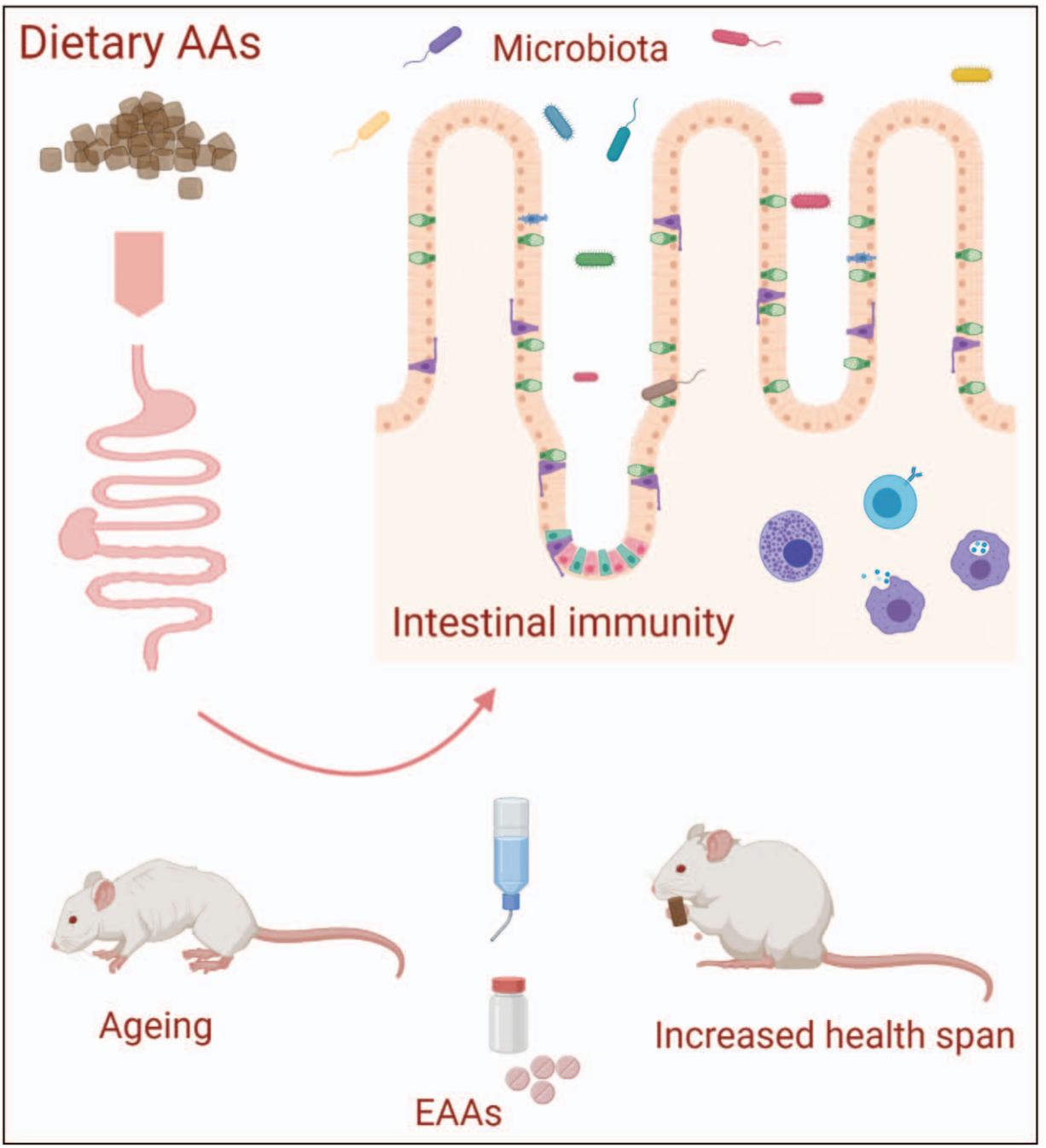

FIGURE 1. Dietary supplementation of essential amino acids has been shown to change gut microbiota and promote healthspan. Essential amino acids regulate glucose and lipid metabolism and energy balance, increase mitochondrial biogenesis, and maintain immune homeostasis. In particular, the essential amino acid supplementation-induced changes of the intestine-associated microbial communities (or gut microbiota) has emerged as a significant regulator of the host metabolism. Diet and host-health influence gut microbiota, and composition of gut microbiota, in turn, controls many aspects of host health, including nutrient metabolism, resistance to infection, and immune signals. Altered communication between the innate immune system and the gut microbiota might contribute to complex diseases. This figure was created with BioRender.com.

glucose metabolism in experimental models and obese individuals [36,37]. They increase energy production modulating mitochondrial function and dynamics, and protect brown fat, liver, pancreas, and muscle from oxidative stress [38]. EAAs and SCFAs share some mechanisms (increase glucagon-like peptide 1 release from L cells, and downregulate genes involved in the intestinal FA transport and lipogenesis) [39]. The effects of amino acid and BCAA intake on gut microbiota

are under active investigation. High plasma BCAA levels were associated with *Prevotella copri* or *Bacteroides vulgatus* enrichment in the gut of insulin-resistant subjects [40▪]. Other studies showed that dietary BCAAem rejuvenated the gut microbiota of aged mice, reducing the *Firmicutes*/*Bacteroidetes* ratio [39], which is known to be elevated in the gut of obese patients [39]. Most significantly, some functional amino acids, and especially BCAAs protect the intestinal barrier and mucosal immunity, with potential benefits on local as well as systemic health [35] (Fig. 1).

AMINO ACIDS IN ENERGY DEFICIT OR HIGHER REQUIREMENT CONDITIONS

The causative or bystander role of high blood BCAA levels in obesity and type 2 diabetes is unclear yet [9,41]. Lean mice with a PP2Cm deletion, leading to moderate BCAA catabolic defect and elevated plasma BCAAs showed enhanced insulin sensitivity and lower body weight [42]. Conversely, the pharmacological BCKDK inhibitor 3,6-dichlorobenzo[b]-thiophene-2-carboxylic acid (BT2) enhanced BCAA catabolic flux, reduced BCAA levels, and attenuated insulin resistance in obese mice [41]. The BCAA oxidation defect is an emerging metabolic hallmark of heart failure in animal models and humans [43]. Treatment with BT2 enhanced BCAA catabolism and improved systolic contractility and diastolic mechanics in mice with transaortic constriction and cardiac dysfunctions [44]. These studies further highlight the complexity of BCAA roles in pathophysiology and suggest the therapeutic potential of targeting enzymes involved in BCAA catabolism in selected conditions.

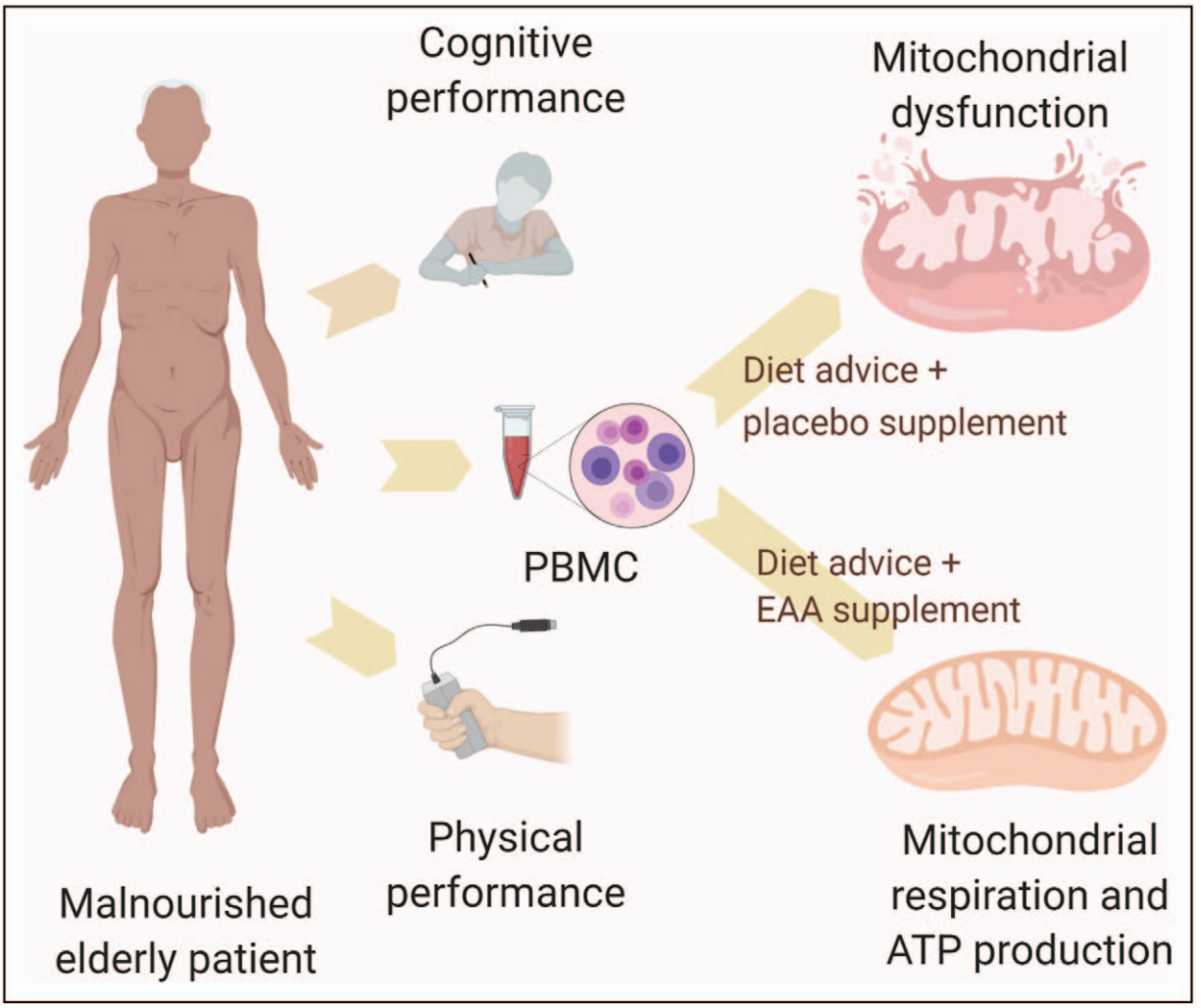

FIGURE 2. Peculiar amino acid formulations, containing essential amino acids with precise stoichiometric ratios, have beneficial effects in elderly patients. Essential amino acid supplements ameliorate mitochondrial function and ATP production in peripheral blood mononuclear cells (PBMC) and improve muscular and cognitive performance in malnourished over 80 patients [6▪]. This figure was created with BioRender.com.

BCAA-based supplements are widely administered to maximize muscle protein synthesis during energy deficit or prevent exercise-induced muscle damage [45,46,47▪]. Balanced EAA-BCAA formulations have shown beneficial effects in numerous pathologic conditions [3,39]. A body of clinical data has proven that these supplements counteract protein disarrangement and preserve energy homeostasis in acute and chronic hypercatabolic conditions without influencing renal function [3]. In particular, BCAAem reduces the incidence of infections in geriatric nursing homes; further, it lowers inflammatory markers and prevents anaemia in haemodialysis patients on a low-protein diet [39]. Short-term parenteral supplementation of BCAAem accelerates the recovery from posttraumatic vegetative or minimally conscious state [39].

For decades, a universally accepted indication of functional amino acid formulations has been the prevention of disability in older people [3], and recent clinical trials add further evidence. Five-week BCAA-enriched supplementation led to improved physical performance, muscle strength, and muscle mass in presarcopenic or sarcopenic patients with normal nutritional status; the effects receded 12 weeks after suspension [5▪]. In a 2 month, open-label randomised trial, Buondonno *et al.* [6▪] administered BCAAem or provided diet advice to 155 elderly malnourished patients. They studied both physical and cognitive performance and further analysed oxidative stress and mitochondrial function in peripheral blood mononuclear cells (PBMC) (Fig. 2). In agreement with our recent preclinical findings [24], they demonstrated the efficacy of BCAAem supplementation on both muscle and cognitive performance and, most significantly, found a strong correlation between clinical improvement and PBMC bioenergetics.

CONCLUSION

Balanced formulations of functional amino acids are valuable tools to manage conditions characterized by a catabolic state, oxidative stress or defects in energy balance, such as age-related physical and cognitive decline, cardiometabolic diseases, trauma, cancer cachexia, and sepsis. Designer amino acid mixtures have proved to promote mitochondrial bioenergetics and ROS defence in metabolically active tissues. Amino acid supplementation shares multiple molecular mechanisms and beneficial effects with calorie restriction and FMD but is safer in the long-term and more manageable than individual amino acid restriction. Future clinical trials might extend its therapeutic indications to renormalize defective energy production.

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Conflicts of interest

There are no conflicts of interest.

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