Special Article

Immunomodulatory role of branched-chain amino acids

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Abstract

Branched-chain amino acids (BCAA) have been associated with immunomodulation since the mid-1970s and 1980s and have been used in the nutritional therapy of critically ill patients. Evidence shows that BCAA can directly contribute to immune cell function, aiding recovery of an impaired immune system, besides improving the nutritional status in cancer and liver diseases. BCAA may also play a role in patients with sepsis or trauma, contributing to improved clinical outcomes and survival. BCAA, especially leucine, are activators of the mammalian target of rapamycin (mTOR), which in turn, interacts with several signaling pathways involved in biological mechanisms of insulin action, protein synthesis, mitochondrial biogenesis, inflammation and lipid metabolism. Although many studies both *in vitro* and in human and animal models have provided evidence for the biological activity of BCAA, findings have been conflicting and the mechanisms of action of these amino acids are still poorly understood. This review addresses several aspects related to BCAA, including their transport, oxidation, and mechanisms of action and their role in nutritional therapy and immunomodulation.

Key-words: Branched-chain amino acids; Leucine; mTOR; immunomodulation; nutritional therapy.

Introduction

Nutritional therapy (NT) is one of the main pillars for the recovery and maintenance of nutritional status in critically ill patients,[1] since it provides macro and micronutrients as energy substrates and metabolic co-factors, respectively, so decreasing muscle and tissue degradation, increasing protein synthesis and mitochondrial function, and enhancing muscle function and mobility.[2] NT also reduces the risk of malnutrition, which directly impacts the digestive and absorptive capacity due to the alteration of the structure of the gut barrier, contributing to an impaired systemic and intestinal immune function.[3] In this sense, early enteral nutrition (EN) can restore gut integrity, thereby reducing inflammation and increasing immunocompetence, can reduce insulin resistance, improving glucose control, and can reduce disease severity.[4,5] Parenteral nutrition (PN) provides an appropriate feeding in a timely manner and plays a key role in supporting patients with intolerance to EN.[6]

Considering that the prevention of protein-energy malnutrition and other related functional disorders, such as impaired cell-mediated immunity and atrophy of the primary and secondary lymphoid organs,[7] relies on optimal nutrition support, the quantity and the quality of nutrients provided in EN and PN are important to meeting the individual needs of patients.[8] Amongst the most important nutrients in this regard are amino acids including glutamine, arginine and branched-chain amino acids (BCAA).[9]

In the mid-1970s and 1980s, the first studies that evaluated the immunomodulatory capacity of BCAA – leucine, isoleucine, and valine – appeared (see reference [7] for a review of the early research). An important role in maintenance of immune function was attributed to these amino acids, given that leucine is an activator of the mammalian target of rapamycin (mTOR) signaling pathway,[10] that regulates several biological processes, such as autophagy, ribosome biogenesis, cell growth and proliferation, by monitoring the availability of nutrients, mitogenic signals, energy status, oxygen levels and growth factors.[11–14]

Despite the importance of BCAA in modulating the function of the immune system, the relevant mechanisms of action of these amino acids have still not been completely elucidated. Thus, the present review aims to synthesize available knowledge on the transport, oxidation and biomolecular mechanisms by which BCAA participate in inflammatory and immunological responses, as well as, the role of BCAA in NT.

Transport of branched-chain amino acids across cell membranes

BCAA transport is carried out by neutral amino acid Na^+-independent transporters, known as "solute carriers" (SLC),[15] categorized into subfamilies containing different types of transporters, among them, the SLC7 family, which includes the L-type amino acid transporters (LAT) 1 (LAT1 or SLC7A5) and 2 (LAT2 or SLC7A8) and the SLC43 family, which includes LAT 3 (or SLC43A1) and LAT4 (or SLC43A2).[16]

LAT 1, 2, 3 and 4 are all able to transport BCAA into the cells. LAT1 and LAT2 are expressed in brain, spleen, liver, skeletal muscle, stomach and placenta,[17,18] while LAT3 is expressed in liver, skeletal muscle and pancreas,[19] and LAT4 is expressed in kidneys and small intestine.[20] Recent studies also show that LAT4 is highly present in human peripheral blood leukocytes[16] and that LAT1 is an important transporter of BCAA in activated human T cells.[15]

Although the transport is Na^+-independent, both LAT1 and LAT2 depend on the type II transmembrane glycoprotein CD98 (4F2hc, SLC3A2) for the exchange of amino acids across the plasma membrane, to which they bind, forming a high-affinity heterodimer, facilitating the translocation of LAT1 and LAT2 to the plasma membrane.[21] In contrast LAT3 and LAT4 are facilitators of the diffusion of amino acids, not requiring specific binding to surface molecules.[22] In addition, it is important to note that leucine transport is dependent on glutamine. First, glutamine is transported into the cells by the Na^+-dependent alanine-serine-

cysteine-preferring transporter 2 (ASCT2),[23] belonging to the SLC1 family (SLC1A5), and then is transported out of the cells by LAT1, that uses intracellular glutamine as an efflux substrate to regulate the uptake of extracellular leucine into the cells.[24]

Studies show that leucine increases gene expression of ACST2 and the cationic amino acid transporter 1 (CAT1), as well as other proteins involved in BCAA transport, such as 4F2hc and rBAT of the heteromeric amino acid transporters (HAT), emphasizing the importance of leucine in the regulation of transport of other neutral and cationic amino acids.[25]

Oxidation of branched-chain amino acids

Overview of the pathway of oxidation of BCAA

The oxidation of BCAA is self-regulated since the differing concentrations in the cytosol and in mitochondria acts to maintain the equilibrium in the degradation of BCAA for energy production.[26,27]

Figure 1 shows the steps in the catabolism of BCAA. The first step is a reversible transamination process, which results in the generation of the respective branched-chain α-keto acids (BCKA), that is, leucine to α-ketoisocaproate (KIC), isoleucine to α-keto-β-methylvalerate (KMV) and valine to α-ketoisovalerate (KIV).[28,29] This reaction is catalyzed by branched-chain amino acid aminotransferase (BCAT), subcategorized into two isoforms: the cytosolic (BCAT1 or BCATc), which is found in brain and in immune cells, such as activated $CD4^+$ T-cells, and the mitochondrial (BCAT2 or BCATm), also seen in immune cells and in most human tissues, especially in the skeletal muscle, stomach, pancreas and kidneys.[30,31] It is worth mentioning that both BCAT1 and BCAT2 are absent or have low activity in the liver, restricting this tissue to oxidative decarboxylation of BCAA.[32]

The amino group (NH_3) originating from the transamination process catalyzed by BCAT is incorporated into α-ketoglurate to form glutamate. In turn the amino group from glutamate can be transferred to pyruvate to generate alanine, an important process in skeletral muscle, catalyzed by pyruvate amino transferase.[33] Addition on an amino group to glutamate generates glutamine, a reaction catalyzed by glutamine synthetase.[33,34]

The second step in BCAA catabolism is the irreversible oxidative decarboxylation of the respective BCKA, whereby the carbon skeletons of KIC, KMV and KIV are converted to isovaleryl-CoA, 3-methylbutyryl-CoA, and isobutyryl-CoA, respectively.[30] This reaction is catalyzed by the branched-chain α-keto acid dehydrogenase (BCKD) complex, composed of several copies of branched-chain α-keto acid decarboxylase (E1), dihydrolipoamide acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) enzymes.[35] E1 uses a coenzyme-A reduced substrate for decarboxylation, while E2 uses lipoic acid as the acceptor of the decarboxylated substrate and transfers it to acetyl-CoA by reducing the lipoamide to dihydrolipoamide. E3, on the other hand, constitutes the lipoamide dehydrogenase, transferring its hydrogen to nicotinamide adenine dinucleotide (NAD) through flavin adenine dinucleotide (FAD).[35] The BCKD complex is regulated by the phosphorylation of lipoamide kinase (inhibition) and lipoamide phosphorylase (activation).[36]

The third step in the catabolism of BCAA corresponds to the ATP generation process. Dehydrogenation of acyl-CoA esters and production of unsaturated α-acyl-CoA is catalyzed by isovaleryl-CoA dehydrogenase or methyl acyl-CoA branched chain dehydrogenase.[37]

As a result of this pathway, leucine is considered to be ketogenic, as it forms acetyl-CoA and acetoacetate, while valine is glucogenic, and can be converted into succinyl-CoA. Both isoleucine and valine are metabolized to succinate via methyl-malonyl-CoA. Isoleucine can also form acetoacetate and therefore can be considered as a glucogenic and ketogenic amino acid.[38,39]

An alternative route of leucine metabolism involves the enzyme α-ketoisocaproate dioxygenase (KICD), in which KIC is converted to β-hydroxy-β-methyl butyrate (HMB) in the cytosol of liver cells.[40] HMB is related to the reduction of skeletal muscle injuries, attenuation of muscle protein degradation, increased muscle protein synthesis, the growth hormone-insulin growth factor-1 (GH-IGF-1) axis activity and modulation of IGF-1 expression in muscle.[41–43]

Oxidation of BCAA by the intestinal mucosa

Both BCAT and BCKD are present in the intestinal mucosa. However, while BCAT greatly contributes to transamination of BCAA, BCKD is only able to decarboxylate approximately 30% of the BCKA produced. It is suggested that this difference in metabolic activity between these two enzymes allows the gut to gradually use BCKA formed throughout the digestion and absorption process as energetic substrates, avoiding the loss of free BCAA during feeding.[35]

Oxidation of BCAA by the brain

The brain also participates in oxidation of BCAA due to its high activity of BCAT and BCKD enzymes; however, the transamination rate significantly exceeds the oxidation rate.[44] The influx of BCAA into the brain positively contributes to the control of glutamate synthesis through transamination and re-amination of BCKA, preventing high concentrations of this neurotransmitter from becoming toxic in the central nervous system (CNS).[44–46] BCAA are also related to central fatigue delay and the reduction of hepatic encephalopathy symptoms, by decreasing influx of sulfonated amino acids through the blood-brain barrier.[47–49]

Oxidation of BCAA by the liver

As previously mentioned, the liver shows an absence or low activity of BCAT and as a result, unlike other amino acids, BCAA are not primarily catabolized in this organ.[37] However, the liver has a high BCKD activity, acting significantly in the decarboxylation process of BCKA.[32] The BCKD complex activity is regulated by a phosphorylation-dephosphorylation cycle, in which the BCKD kinase is responsible for the inactivation of the complex, and the BCKD phosphatase, through a dephosphorylation process, is responsible for the reactivation of the complex.[50] BCKD kinase is inhibited by KIC, so that when tissue accumulation of KIC occurs, the BCKD complex is activated, thus promoting the oxidation of BCAA.[50] The liver also plays a key role in the conversion of KIC to β-hydroxy-β-methyl butyrate (HMB): although the majority of KIC is converted into isovaleryl-CoA, approximately 5-10% of the non-decarboxylated KIC is converted to HMB by KICD. This reaction is regulated by BCKD itself and by KICD activity.[51]

Both alanine and glutamine generated through the transamination process contribute to ammonia transportation in the body, carrying this metabolite from muscle to the liver and kidneys to be metabolized and excreted, respectively, avoiding its accumulation.[34,52,53] Considering that ammonia is toxic and its buildup is detrimental to the activity of several organ systems, such as the CNS,[54,55] the hyperammonemia state, common in liver diseases, contributes to the development of hepatic encephalopathy and the worsening of clinical conditions.[56,57] In addition, in chronic hepatopathies, there is a reduction in the plasma concentration of BCAA and an increase in the plasma concentration of the aromatic amino acids (AAA) tryptophan, phenylalanine, and tyrosine.[58] This imbalance in BCAA/AAA ratio, also called Fischer's ratio, is associated with the development of hepatic encephalopathy and progression of liver diseases.[59,60]

Oxidation of BCAA by skeletal muscle

Skeletal muscle is the major site for BCAA oxidation. The oxidation of BCAA in skeletal muscle is regulated by the activity of BCKD, which is found in 5 to 8% in its active form at rest and 20 to 25% in its active form during exercise.[61,62] Furthermore, BCKD activity is regulated by the concentration of BCAA and their BCKA in muscle fibers, by depletion of muscle glycogen during and after exercise, by a decrease in pH and by the alteration in the ATP:ADP ratio.[63]

Skeletal muscle captures BCAA from the bloodstream in order to oxidize them during prolonged exercise to generate energy.[64] This process only seems to occur when there is exogenous supply of BCAA (i.e., it is dose-dependent) or a reduction of muscle glycogen stores.[65] Studies show that leucine supplementation decreases glycogen degradation in both the muscle and the liver, since, as previously mentioned, increased serum concentrations of leucine and KIC inhibit BCKD kinase activity, enhancing BCAA oxidation and promoting a lower utilization of hepatic glycogen.[66,67] In addition, elevated intracellular levels of leucine decreased pyruvate dehydrogenase activity, an important convergence point of the glycolytic pathway and the tricarboxylic acid (TCA) cycle, therefore promoting the conversion of pyruvate to alanine which, in turn, acts as a precursor in hepatic gluconeogenesis.[33,67]

Besides serving as a carbon source for energy production, BCAA also act as regulators of muscle protein turnover, inhibiting catabolism and increasing anabolism mediated by the mTOR.[50,68,69] The maintenance of muscle protein synthesis is also influenced by the synergistic effect of insulin and leucine, since this amino acid promotes an increase in the serum concentration of insulin, which in turn exerts a permissive effect on protein synthesis in the presence of amino acids[70,71] and plays a key role in maintenance of glucose homeostasis, via the glucose-alanine cycle, by enhancing recycling of glucose.[68,72]

A study reported that leucine ingestion (1 mmol/kg of lean body mass) alone is not able to promote a rise in peripheral insulin concentration or a change in glucose

concentration[73]. However, when administered with glucose (25 g), leucine significantly attenuated the glucose response compared to the same amount of glucose ingested without leucine, thereby confirming the synergism between insulin and leucine.[73]

Oxidation of BCAA by adipose tissue

In vitro and *ex-vivo* studies show that adipose tissue is able to metabolize BCAA, although it is not a quantitatively significant site of BCAA catabolism. Although studies show that BCKD is the rate-determining enzyme in BCAA oxidation in muscle and liver, in adipose tissue this enzyme does not appear to perform the same function.[74] It is noteworthy that in obese humans, the expression of BCAT2 and BCKD is reduced, mainly in visceral adipose tissue.[75]

Adipose tissue plays an important role in homeostatic regulation of BCAA concentrations.[76] In the mitochondria of adipocytes, the oxidized BCAA (i.e. the BCKA) are converted into anaplerotic TCA cycle intermediates, acetyl-CoA and succinyl-CoA, which favors the generation of citrate and subsequently the process of lipogenesis.[77] In contrast to undifferentiated adipocytes, which use glucose and glutamine to generate acetyl-CoA as a lipogenic substrate, differentiated adipocytes use BCAA, mainly leucine and isoleucine, representing up to 30% of the lipogenic acetyl-CoA pool. As adipocyte differentiation evolves, BCAT is progressively activated.[75]

Oxidation of BCAA by immune cells

In vitro studies have demonstrated that immune cells have a high activity of BCAT and BCKD, using isoleucine, valine and, mainly, leucine as substrates, and show increased uptake of these amino acids, mainly during the S phase of the cell cycle.[78] Moreover, in

response to mitogens, the consumption of BCAA by T cells is enhanced, with a reported increase of 270% in transport, 195% in transamination and 122% in leucine oxidation.[79]

In lymphocytes from peripheral human blood, the rate of leucine transamination is significantly higher than the oxidation rate, suggesting that decarboxylation of KIC by BCKD plays a role in regulating the oxidation rate of leucine in these cells.[80] In addition, *in vitro* studies show that leucine appears to be a key regulator of its own oxidation and also the oxidation of other amino acids, since high levels of leucine decreases the extracellular concentrations of valine, isoleucine and their α-keto acids.[81] On the other hand, a low concentration of isoleucine, valine and glutamine may exert an inhibitory effect on the transamination and oxidation of leucine.[80]

During their activation, T cells undergo metabolic reprogramming, increasing the demand for amino acids to sustain the high rate of protein synthesis. These cells decrease oxidative decarboxylation of pyruvate, while the formation of lactate regenerates the NAD+ necessary to continue generating energy. Also, BCAA transamination triggered by BCAT1 increases in activated T cells.[82]

In liver-associated lymphocytes - large granular and agranular lymphocytes - BCAA, especially valine, increase cell proliferation by stimulating lymphopoiesis, in addition to increasing natural killer (NK) and lymphokine-activated killer activity.[83]

Hematopoietic stem cells (HSC) are especially sensitive to amino acid deprivation, and, in order to maintain their integrity, rely on a complex system promoted by non-haematopoietic cells in bone marrow. It has been found that valine and leucine deprivation during the growth of human HSC is significantly detrimental and results in impairment of the number of white and red blood cells.[84,85]

Oxidation of BCAA in other tissues

The distribution of BCAT was also determined in other tissues by enzymatic assays and western blotting analysis. The activity of the BCAT2 is high in gastric cells, pancreas and salivary glands,[35] whereas the BCAT1 has its greatest activity in the ovaries and placenta.[86] In addition to the aforementioned tissues, BCKD has high activity in gastric cells and kidneys.[32]

Intracellular signaling mechanisms of branched-chain amino acids: The mTOR target

Belonging to the phosphatidylinositol 3-kinase-related kinases (PIKK) superfamily, mTOR is a conserved protein kinase divided into two distinct complexes, mTORC1 and mTORC2. mTORC1 contains mTOR, the regulatory protein associated with mTOR (Raptor), the G-protein β-subunit-like protein (GβL or mammalian lethal with Sec13 protein 8 – mLST8), the proline-rich Akt substrate of 40 kDa (PRAS40) and the DEP domain-containing mTOR-interacting protein (Deptor).[87] mTORC2 contains mTOR, Deptor, GβL, the rapamycin insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein 1 (mSIN1) and the protein observed with Rictor (Protor).[88]

Upstream regulation of mTORC1

The major sensor involved in mTORC1 activity is the tuberous sclerosis complex (TSC), that is a heterodimer of TSC1 (or hamartin) and TSC2 (or tuberin).[89] When stimulated by growth factors, mTORC1 is upstream regulated by the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) pathway or by extracellular-signal-regulated kinase 1 and 2 (ERK1/ERK2), that increase the phosphorylation of TSC2, leading to the inactivation of TSC1-TSC2 complex with subsequent activation of mTORC1.[90] The TSC1 and TSC2 proteins form a suppressor complex that function as a GTPase-activating protein (GAP) for

the Ras homolog enriched in brain (Rheb), a small GTPase that, when bound to GTP, directly interacts with the mTORC1.[91–94]

The cell energy status is regulated by mTOR activity, by a different pathway mediated by the AMP-activated protein kinase (AMPK). AMPK is activated in response to low ATP:ADP ratio, phosphorylating the TSC2, thus increasing the GAP activity of this complex towards Rheb, leading to a reduced mTORC1 activation. Moreover, in response to energy depletion, AMPK can directly phosphorylate Raptor, likewise reducing mTORC1 activity.[95]

The increased intracellular concentration of amino acids, including leucine, promotes the activation of mTORC1 as well (Figure 2). The activation of mTORC1 promoted by leucine is mediated by the Rag guanine triphosphatases (GTPases) that function as heterodimers with active complexes of GTP-bound RagA or B complexed with GDP-bound RagC or D.[96] The GTP loading of RagA/B proteins binds to Raptor and promotes the translocation of mTORC1 to the lysosome, where the GTP-bound-Rheb stimulates the kinase activity of mTORC1.[97] The Rag GTPase complex is also dependent of the interaction with the Ragulator complex (LAMTOR1, 2, 3 4 and 5) through the C-terminal domains of RagA/B and RagC/D, which serves as a guanine nucleotide exchange factor for RagA and RagB, promoting their GTP binding in exchange of GDP and recruiting the Rag GTPases to lysosomes.[98,99] It is worth mentioning that the absence of amino acids shifts the Rag into an inactive form of GDP-bound RagA/B and GTP-bound RagC/D, inactivating mTORC1.[97]

The negative regulation of the Rag GTPases is stimulated by two GAP complexes: GATOR1 which acts on RagA and RagB, and Folliculin (FLCN)-folliculin interacting protein 2 (FNIP2) acting on RagC and RagD.[100] GATOR1 is negatively regulated by the GATOR2 complex, consisting of interacting proteins such as Sestrin2, a leucine sensor upstream of mTORC1.[101] Studies show that leucine binds to Sestrin2, disrupting the Sestrin2-GATOR2 interaction, thus activating mTORC1.[102–104]

As with Sestrin2, the leucyl-tRNA synthetase (LRS) also mediates leucine signaling in the mTORC1 pathway.[105] In the presence of amino acids, LRS is translocated to the lysosome, where, acting as a GAP, it facilitates the GTP hydrolysis of RagC/D, resulting in the formation of GDP-RagC/D and activation of mTORC1.[105,106] In this sense, while Sestrin 2 is a negative regulator of mTORC1, LRS is a positive regulator of mTORC1.[107]

Upstream regulation of mTORC2

So far, the mechanisms involved in mTORC2 activation are not well described. However, it has recently been shown that phosphatidylinositol (3,4,5)-tris-phosphate (PIP_3), generated downstream of PI3K, promotes mTORC2 activity through mSin1 binding, a component present exclusively in the mTORC2, essentially for the activation of this complex.[108]

Stimulated by amino acids, mTORC2 plays a role in Akt (Serine473), protein kinase C (PKC) α and serum and glucocorticoid-inducible kinase (SGK) activation.[109,110] (Figure 3) PKCα regulates multiple cellular functions, such as adhesion, secretion, proliferation, differentiation and apoptosis,[111] while SGK alters the activity of several enzymes, such as inducible nitric oxide synthase (iNOS), regulates cell survival and proliferation, muscle mass maintenance and the organization of the cytoskeleton.[112] mTORC2 plays a role in the organization of the actin cytoskeleton, as well as in the regulation of PKC.[110]

It has also been show that Rictor plays an essential role in T cell amino acid sensing, since in the absence of this component, $CD4^+$ T cells proliferate normally, even in limiting leucine. This finding suggests that Rictor controls an amino acid-sensitive checkpoint that permits T cells to determine whether the microenvironment contains sufficient resources for cell generation.[113]

Protein synthesis: S6K/eIF pathway

The activation of mTORC1 by both insulin and amino acids leads to the phosphorylation of the ribosomal S6 kinase (S6K) and the eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1) promoting protein synthesis and cell growth.[114] 4E-BP1 is an inhibitory phosphoprotein that, when hypophosphorylated, is bound to eIF4E and hinders the recruitment and assembly of the eIF4F complex.[115] The eIF4F complex consists in four major components: the eIF4E, a protein that binds to the 5'-cap structure of the mRNA; the eIF4A, a canonical DEAD-box helicase; the eIF4G, a scaffold protein that binds eIF4E, eIF4A and the mRNA transcript itself; and the poly(A)-binding protein (PABP), which binds to the 3' poly(A) tail of eukaryotic mRNA initiating the translation process.[116,117] Phosphorylation of the inhibitory 4E-BP1 protein enhances the formation of the elF4F complex promoting translation initiation. As well, activated S6K phosphorylates the eIF4B, a regulator of eIF4A, increasing the affinity of eIF4A for ATP and its helicase activity, thus providing an extra stimulus for the initiation of translation.[117–119]

There are two S6K proteins, S6K1 and S6K2, both belonging to the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family.[120] Both S6K1 and S6K2 have important regulatory domains: the acidic N-terminus that contains the TOS (TOR signaling) motif; the kinase domain that contains the T-loop; a linker region that contains the TM and HM sites; and a basic C-terminus containing an autoinhibitory pseudosubstrate domain.[121] S6K1 phosphorylation (Thr389 residue) on multiple C-terminal sites facilitates mTORC1-mediated phosphorylation of the HM site (Thr389) in the linker domain and phosphoinoisitide dependent kinase 1 (PDK1)-mediated phosphorylation of the T-loop site (Thr229).[122,123]

Leucine is the most potent amino acid known to activate the mTOR/S6K pathway.[124] Studies show that acute provision of leucine enhances the mTOR and S6K activation in the

heart of rats and in human myotubes independently of insulin stimulation.[125,126] It is also been reported that the intake of an essential amino acids mixture together with leucine may potentiate leucine-mediated mTOR signaling in skeletal muscle, due to increased S6K phosphorylation.[127] KIC can also stimulate 4E-BP1 phosphorylation.[128]

Insulin cascade: IRS-1 pathway

The insulin receptor is a transmembrane tyrosine kinase that coordinates intracellular signaling cascades promoting the biological action of insulin.[129] Insulin binding to its receptor promotes the phosphorylation of tyrosine residues of the cytosolic insulin receptor substrates (IRS) 1 and 2. This leads to the activation of the PI3K/Akt pathway, responsible for the majority of the metabolic actions of insulin, and to the activation of Ras/mitogen-activated protein kinase (MAPK) pathway, that regulates the expression of many genes and links with the PI3K pathway to control cell growth and differentiation.[130]

Akt phosphorylates and inhibits the Rab-GTPase-activating protein, triggering the activation of Rab small GTPases involved in the cytoskeletal re-organization that culminates in the translocation of the glucose transporter GLUT4 to the plasma membrane.[130] Besides Akt, the atypical protein kinase C isoforms ζ and λ (PKCζ/λ) also promote GLUT4 translocation, so controlling glucose transport into the cell.[131] At the same time, Akt also phosphorylates and inhibits TSC2, leading to the activation of mTOR pathway, with subsequent activation of S6K and eIF4E.[130]

Both hyperactive mTOR and S6K1 negatively regulate Akt by inducing IRS-1 serine phosphorylation, disrupting the interaction between insulin and its receptor leading to degradation.[69] In this context, when high levels of BCAA persistently activate mTORC1, insulin resistance is induced through a negative feedback loop of IRS-1.[69] In addition, mTORC2 promotes the negative feedback of IRS-1 and prevents its inactive form

accumulation in the cytosol, through the stabilization of the substrate-targeting subunit of the CUL7 E3 ligase complex (Fbw8), which in turn, mediates the ubiquitination and degradation of IRS-1.[132] However, it is poorly understood whether physiological levels of BCAA may induce mTOR activation and the subsequent IRS-1 and IRS-2 serine phosphorylation. Some studies suggest that impaired insulin sensitivity mediated by S6K is only affected by transient high-protein diets (<6 wk), since in longer interventions (6 wk -18 wk), high-protein diets did not affect S6K1 expression.[69]

Despite the evidence that supplementation with BCAA or intake of a BCAA-rich diet improves metabolic health,[133] levels of BCAA tend to be increased in insulin-resistant obesity and type 2 diabetes mellitus (T2DM).[134] Recent studies suggest that these effects may depend on ethnicity.[135] Epidemiological studies have suggested that elevated levels of plasma BCAA are associated with insulin resistance, substantial secretory pressure on the pancreatic β-cells and onset of T2DM in European whites, Chinese, and South Asians, highlighting the decreased expression of enzymes that metabolize BCAA and an increase in muscle protein degradation as the possible mechanisms involved.[135] Other recent evidence has suggested a significant inverse correlation between plasma BCAA and insulin clearance and a contribution of BCAA to the development of obesity-associated insulin resistance in overweight individuals submitted to high saturated fat diet.[135,136]

It is well known that diabetes can significantly increase the risk of developing certain cancers[137,138] and, in this context, since oncological patients have a substantial reduction in muscle mass (cachexia),[139] BCAA supplementation has been shown to be an important strategy for inhibiting protein catabolism, stimulating protein synthesis and promoting muscle repair.[140] However, considering previous reports that elevated plasma levels of BCAA are related to the development of insulin resistance and diabetes, this effect should be considered prior to any therapeutic strategies involving BCAA administration.[141] Under

hyperinsulinemic conditions, BCAA act as a suppressor of insulin-induced over-activation of PI3K/Akt by both inducing a negative feedback loop though mTORC1/S6K1 activation and by suppressing mTORC2 kinase activity toward Akt, exhibiting growth inhibitory effects by inducing apoptosis.[142]

Inflammation: NF-kB pathway

The nuclear transcription factor kappa B (NF-κB) comprises five members in mammals: c-REL, RELB, NF-κB1 (or p105), NF-κB2 (or p100) and RELA (or p65).[143] RELA is responsible for most NF-κB transcriptional activity due to the presence of a strong transcriptional activation domain.[144] The NF-κB pathway is activated by several stimuli, such as tumor necrosis factor α (TNF-α), interleukin (IL)-1, lipopolysaccharide (LPS), heat shock proteins (Hsp) and T-cell activators.[145] These activators induce the activation of the I-kappaB-kinase (IKK) enzyme complex (IKKα, IKKβ and IKKγ), which phosphorylates the inhibitory IκBα proteins inducing their ubiquination and proteasome-dependent degradation. This allows the translocation of NF-κB to the nucleus.[146] NF-κB is responsible for the upregulated synthesis of immune-related cytotoxic factors such as iNOS, cyclooxygenase (COX)-2 and proinflammatory cytokines which contribute to the eradication of infectious agents.[147,148] Dysregulated and prolonged activation of NF-κB can trigger tissue damage, multiple organ failure, and even death. Therefore, the inhibition of this factor may be a key target in patients with sepsis. NF-κB has also been recognized as an important target in the treatment of several types of cancer, resulting in apoptosis of cancer cells and in the reduced levels of the vascular endothelial growth factor (VEGF) produced in leukemia, glioma, and rheumatoid arthritis.[56,142,149]

The mTOR signaling pathway plays a key role in the inflammatory response mediated by NF-κB.[150] It has been demonstrated that IKKβ phosphorylates and suppresses TSC1, thus

416 activating the mTOR pathway, which enhances angiogenesis resulting in tumor development,
417 besides being associated with VEGF production in multiple tumor types.[151] Moreover, it has
418 been reported that mTOR reciprocally activates IKK and NF-κB, when IKKα is induced to
419 interact with mTORC1.[152]

420 Some studies show that TNF-α may also stimulate Akt activity from interaction with
421 the IKKβ, leading to activation of NF-κB, which contributes to cell proliferation, tumor
422 growth and angiogenesis.[153] TNF-α binding to its receptor TNFR1 leads to the recruitment of
423 TNFR1-associated death domain (TRADD) protein, followed by the recruitment of FAS-
424 associated death domain (FADD) protein, the TNF receptor-associated factor 2 (TRAF2), and
425 the receptor-interacting protein 1 (RIP1) kinase.[154,155] The TRAF2-RIP1 association promotes
426 the activation of IKKβ.[156]

427 LPS also stimulates the mTOR pathway. LPS induces the NF-κB pathway through
428 binding to toll-like receptors (TLR), which in turn, activate mTOR via the PI3K/Akt
429 pathway.[157,158] Activated mTOR associates with the adapter protein MyD88, allowing the
430 activation of the interferon regulatory factor (IRF)-5 and IRF-7, key transcription factors
431 involved in regulation of the expression of genes encoding proinflammatory cytokines and
432 type I interferons (IFN).[157] It has also been demonstrated that activation of the mTOR/IκB-
433 α/NF-κB pathway increases the expression of iNOS and COX-2, thus promoting a
434 vasodilatory and proinflammatory effect that contributes to LPS-induced hypotension and
435 inflammation.[148] Considering that some autoimmune diseases are driven by type I IFN and
436 LPS-induced-endotoxemia involves proinflammatory cytokines, the short-term inactivation
437 of mTOR may be an interesting strategy to control the progression and symptoms of certain
438 pathological conditions.[148,157]

439 Paradoxically, the inflammatory insult produced by LPS may decrease mTOR, 4E-
440 BP1 and eIF4G phosphorylation, leading to impaired protein synthesis, so decreasing lean

body mass.[159] Nevertheless, the mechanism involved in this response is not well understood, since the activation of Akt is not affected by LPS.[159] Concomitantly, LPS may impair the anabolic response to amino acids, notably mTOR-dependent muscle protein synthesis activated by leucine.[160] Animal studies show that even a high dose of leucine is not able to increase muscle protein synthesis in sepsis, probably due to a sepsis-induced leucine resistance mechanism, which could explain, at least in part, the findings of muscle wasting in septic patients that was not reversed by BCAA supplementation.[159] A plausible hypothesis to explain this resistance to leucine may involve the depletion of intramuscular glutamine due to its release by skeletal muscle during sepsis, impairing the transport of leucine into the muscle, or the ability of the muscle to "detect" leucine, since, as previously mentioned, transport of leucine is highly dependent on the intracellular concentration of glutamine.[161,162]

Ageing: SIRT1 pathway

Sirtuin 1 (SIRT1) is a NAD^+-dependent deacetylase that regulates calorie restriction-mediated longevity and is associated with the insulin signaling pathway, regulating various cellular processes including ageing.[163,164] As a member of the sirtuin family, this deacetylase is also associated with increased mitochondrial biogenesis, maintenance of the cellular energetic state, increased activity of antioxidant defense systems and decreased synthesis of reactive oxygen species.[165,166]

SIRT1 and mTOR have both been related with age-associated diseases such as T2DM, obesity, cardiovascular disease, cancer and neurodegenerative diseases:[165] activation of SIRT1 exerts a therapeutic effect in some of these diseases whereas inhibition of mTOR confers the protective effect.[167]

It has been proposed that SIRT1 activity is primarily regulated by telomerase and there is evidence that SIRT1, which is a deacetylase enzyme, acts as a nutrient-responsive

growth suppressor by regulating mTOR signaling.[168,169] The interconnection of SIRT1 and mTOR is related to the promotion of stress sensing pro-survival signals. This seems to not directly involve mTOR, but rather the interaction of SIRT1 with TSC1-TSC2 exerting an inhibitory effect on mTOR, similar to that performed by TSC2.[167] Autophagy is an important mechanism of control in response to stress and aging; autophagy is negatively regulated by mTOR, while SIRT1 activates autophagy by deacetylating several essential components of the autophagy machinery.[170–172] Studies have shown that inactivation of SIRT1 impairs starvation-induced autophagy, resulting in the activation of NF-κB and subsequent inflammation.[173] In contrast, SIRT1 deficiency promoted mTOR signalling that could not be reversed even under cellular stress caused by leucine starvation and other stress inducible stimuli.[167]

Many studies have found associations between BCAA or leucine supplementation and attenuation of high fat diet-induced mitochondrial dysfunction, insulin resistance, and obesity[165] or muscular metabolic changes and mitochondrial biogenesis.[174] However, these studies do not mention the role of mTOR in these processes, instead linking these events only to the increased expression of SIRT1 mediated by the AMPK signaling pathway, leading to a reduced acetylation of the peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC1α) and the forkhead box protein O1 (FoxO1).[175] Notwithstanding this, it is hypothesized that the enhanced mitochondrial biogenesis promoted by BCAA could be mediated by a positive feedback mechanism between the nitric oxide-generating system and mTOR, since NO regulates mTOR activity and its downstream proteins.[166]

Lipid metabolism: PPARγ pathway

Another example of the regulatory potential of mTOR is found in nuclear receptor signaling involving peroxisome proliferator-activated receptor γ (PPARγ), considered to be

the major regulator of adipocyte differentiation and glucose and lipid metabolism in mature adipocytes.[176] In adipose tissue, mTOR activation is related to an increase in fat mass in obesity, while inhibition of mTOR is associated with a reduction in fat mass in situations of caloric restriction and fasting.[177] mTOR is known to modulate the activity of PPARγ by translating signals of nutrient availability.[178] Activation of mTORC1 induces the phosphorylation of 4EBP-1, releasing eIF4E, required to increase the translation of CCAAT-enhancer-binding proteins (C/EBP)-a and -d, seen in adipose tissue.[179] This transcription factor is necessary for establishing the adipogenic cascade since C/EBP-d increases the amount of C/EPB-a and PPARγ.[180] When sufficient levels of PPARγ are produced, this transcription factor promotes adipogenesis and lipid synthesis through increased expression of lipogenic genes.[181] In adipocytes, mTOR is responsible for the control of protein synthesis, the morphogenesis of adipose tissue, and the synthesis and secretion of leptin.[182]

Studies show that leucine has a positive effect on adipocyte differentiation, by regulating PPARγ and C/EBP-a via the mTOR pathway.[183] However, the results from manipulation of dietary leucine are inconsistent,[184] inasmuch as a study showed that an increase in dietary leucine has no effect on lipid metabolism,[185] while leucine deprivation significantly reduced body weight and abdominal adipose mass and improved metabolic health.[186–188] It is evident that more studies are needed to further investigate the role of BCAA on lipid metabolism.

The mechanisms described above are illustrated in figure 4.

Branched-chain amino acids in nutritional therapy and immunomodulation

Research into the association between BCAA and immunomodulation began in the 1970s and 1980s, when researchers evaluated critically ill patients receiving total parenteral nutrition (TPN) and observed that an increase in the total amount of BCAA in the formula

516 resulted in elevation of lymphocyte counts in blood and reduced mortality, mainly in the
517 postoperative period and in cases of sepsis.[189–191] Furthermore, when comparing plasma
518 amino acid concentrations, surviving patients were found to have significantly higher
519 concentrations of BCAA and lower concentrations of aromatic and sulfur amino acids than
520 those who did not survive sepsis, indicating a preserved liver function and better maintenance
521 of energy metabolism.[189] Considering that hepatic failure is a common cause of death in
522 septic patients, this result demonstrates the beneficial effect of BCAA supplementation to
523 improve patient survival.[192]

524 Bower et al.[193] made both quantitative and qualitative analyses of TPN for septic
525 patients. They used three formulas with different proportions of BCAA. The standard control
526 formula contained 25% BCAA (660 mg/mL of leucine, 560 mg/mL of valine and 510 mg/mL
527 of isoleucine), while the other two formulas contained 45% BCAA, one with a predominance
528 of leucine (1576 mg/mL leucine, 789 mg/mL valine and 789 mg/mL isoleucine), and another
529 with a predominance of valine (263 mg/mL leucine, 1838 mg/mL valine and 1050 mg/mL
530 isoleucine). However, in the first phase of the study, the valine-rich solution was insufficient
531 to maintain a plasma concentration of amino acids and protein turnover, while the leucine-
532 rich solution proved to be more effective in this context. It is possible that this positive result
533 obtained with the administration of the leucine-rich solution was mediated by the
534 secretagogue effect of leucine on insulin, as mentioned earlier. Nevertheless, nitrogen
535 balance was marginally improved with both solutions, although the cumulative nitrogen
536 balance was not altered.

537 Vente et al.[194] observed that a BCCA enriched solution (50.2% of total amino acid
538 content) compared to a standard TPN solution (BCAA at 15.6% of total amino acid content)
539 was ineffective in improving nitrogen metabolism in septic and traumatized patients.
540 Considering that the control group was not deprived of BCAA, the increase of BCAA did not

confer additional benefits on the proliferation of lymphocytes. Even so, the presence of BCAA is indispensable for cellular growth and proliferation, as mentioned in a study by Chuang et al.[195], who found that lymphocyte proliferation was dependent on 15 amino acids, among them BCAA, and the absence of any of these amino acids directly impacted on the proliferation of these cells impairing the response.

In addition to directly impacting the functions of immune cells, BCAA are related to the liver function. BCAA may be used as energy sources, thus improving nitrogen balance, besides having a beneficial effect on the anorexia associated with hepatocellular failure, since they promote muscle protein balance.[196] However, such an effect on lean body mass is dependent on adequate protein intake and quality.[197] The supplementation with BCAA, especially when associated with a high-fiber, high-protein diet is considered a safe intervention in patients with cirrhosis, contributing to the increase of muscle mass and not raising the levels of ammonia or glucose, besides not being associated with the development of hepatic encephalopathy.[198] The European Society for Clinical Nutrition and Metabolism (ESPEN) recommends a protein intake of 1.2–1.5 g/kg of body weight/day for patients receiving EN or PN, as well as oral BCAA supplementation to improve clinical outcome in advanced cirrhosis.[199] The addition of 35-45% BCAA and the reduction of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and sulfur amino acids in total PN for cirrhotic patients with hepatic encephalopathy is also recommended.[200] Furthermore, BCAA supplementation may reduce ascites symptoms in these patients, as a result of increased total serum protein and serum albumin levels.[201] A study conducted by Nakamura et al.[202] evaluated the effect of supplementation with 12 g/day BCAA (2.8 g of isoleucine, 5.7 g of leucine and 3.4 g of valine per day) on neutrophil phagocytic function, NK cell activity, plasma albumin concentrations and Fisher's ratio in patients with hepatic cirrhosis. After three months of supplementation, Fisher's ratio was significantly increased; however, there

566 was no statistical difference in plasma albumin concentrations. The authors also observed a
567 significant improvement in neutrophil phagocytic function and lymphocyte NK cell activity,
568 concluding that BCAA supplementation could reduce the risk of bacterial and viral infections
569 in patients with decompensated cirrhosis.

570 Taking into consideration the fact that NK cells also play an important role in tumor
571 defense, BCAA supplementation for patients with cirrhosis could reduce the risk of
572 developing various liver cancers.[202] Several studies indicate that BCAA supplementation is
573 associated with a lower incidence of hepatocellular carcinoma (HCC) in patients with
574 cirrhosis and may improve nutritional status and the quality of life.[203–205] Moreover, in
575 patients undergoing therapeutic interventions for treatment of HCC, BCAA supplementation
576 prevents the decrease in serum albumin level, in addition to reducing postoperative
577 complications.[206]

578 Besides the beneficial effects on HCC, BCAA supplementation is related to the
579 improvement of the inflammatory state and the nutritional status of patients with
580 gastrointestinal cancer. Sun et al.[207] investigated the potential benefits of parenteral
581 administration of a solution enriched with 30% BCAA in undernourished patients with
582 gastrointestinal cancer undergoing surgery. Decreased leukocyte count and plasma C-reactive
583 protein (CRP), alkaline phosphatase and gamma-glutamyl transferase (γ-GT) levels were
584 observed. The enriched solution also maintained stable albumin and prealbumin
585 concentrations and a positive nitrogen balance. In this study, the authors observed a
586 significant decrease in the incidence of postoperative comorbidities.

587 Cancer patients often present in an advanced state of malnutrition, leading to intense
588 metabolic stress and catabolic states, characterized by increased energy consumption,
589 negative nitrogen balance, increased glutamine utilization and altered amino acid
590 metabolism. In an attempt to compensate for the increase in energy expenditure and

591 glutamine consumption, there is an increase in BCAA oxidation in skeletal muscle. Studies
592 show that glutamine supplementation improves nitrogen balance and recovery from infection
593 in various clinical conditions, such as burns, radiation injury, severe surgical stress, sepsis,
594 and cancer.[208] The increased availability of glutamine also promotes an increase in glutamate,
595 which is a component of glutathione, the main non-enzymatic intracellular antioxidant.[209] In
596 this sense, the immunomodulatory and antioxidant role of BCAA, whether directly or
597 indirectly, may be of great importance for the prevention and treatment of other conditions
598 involving inflammatory and oxidative states.[210]

599 On the other hand, the effect of BCAA supplementation on nutritional status in well-
600 nourished individuals is poorly elucidated. Verhoeven et al.[211] found no changes on skeletal
601 muscle mass and strength as well as no improvements in indexes of whole-body insulin
602 sensitivity (oral glucose insulin sensitivity index and the homeostasis model assessment of
603 insulin resistance), blood glycated hemoglobin content, or the plasma lipid profile in healthy
604 elderly men after supplementation with 7.5 g of leucine per day. However, it is noteworthy
605 that in this study, there was a decline of 18-25% of basal plasma valine concentration in the
606 supplemented group suggesting an amino acid imbalance. Thus, further studies are needed to
607 understand the effects of BCAA supplementation on nutritional status in well-nourished
608 patients.

609

610 **Future Perspectives**

611 BCAA supplementation has been widely used over the years, especially in clinical
612 conditions of hepatopathy, trauma, cancer and sepsis, in which there is impairment of
613 immune function, loss of lean mass, increased inflammation and oxidative stress. In this
614 sense, BCAA can significantly contribute to the reversal of these symptoms, the recovery of
615 homeostasis and the prevention of patient's worsening, thus improving clinical outcomes and,

more substantially, reducing mortality. Nevertheless the literature lacks studies that elucidate in a clear and integrated way the roles and mechanisms of action of BCAA, since, in certain situations, the results found in clinical practice do not corroborate the biomolecular mechanisms of BCAA action and vice versa. The major research areas that require attention that are identified by this literature review are:

1. Better elucidation of the pro-inflammatory and adipogenic roles promoted by mTOR in association with the NF-kB and PPARγ pathways, respectively.
2. Better elucidation of the synergistic action of leucine and insulin, especially in at-risk groups, as well as better understanding of the role of the overactivation of mTOR and S6K1 that results in insulin resistance.
3. Whether the beneficial effects of BCAA supplementation on protein synthesis outweigh any possible harmful effects on inflammation and adipogenesis.
4. Finally, considering that the activation of BCAA-stimulated mTOR is mainly related to pro-inflammatory effects, to better understand the mechanisms underlying the beneficial effects of BCAA in clinical practice, especially in situations of sepsis and inflammation.

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Author's contributions

Literature searching and initial manuscript preparation was performed by AB and AYC. The manuscript was revised by JT and finalized by PCC and MMR.

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Figures legends

Figure 1. Oxidation of BCAA

In the first step leucine, isoleucine and valine are converted into their respective keto acids. Then the formed keto acids are irreversibly decarboxylated. In the third step BCAA enter into the ATP generation process. BCAT=branched-chain amino acid aminotransferase; BCKD=branched-chain a-keto acid dehydrogenase; KICD= α-ketoisocaproate dioxygenase; HMB=β-hydroxy-β-methyl butyrate.

Figure 2. Upstream regulation of mTORC1 by BCAA

Leucine binds to Sestrin2, disrupting its interaction with GATOR2, which in turn, negatively regulates GATOR1. Ragulator promotes the GTP-RagA/B proteins binding, promoting the translocation of mTORC1, through Raptor, to the lysosome. LRS is translocated to the lysosome, where it promotes the formation of GDP-RagC/D, negatively regulated by FNIP2, resulting in mTORC1 activation. BCAA=branched-chain amino acids; Leu=leucine; Ile=isoleucine; Val=valine; mTORC1= mammalian target of rapamycin complex 1; LRS= leucyl-tRNA synthetase; FNIP2= Folliculin (FLCN)-folliculin interacting protein 2; Raptor= regulatory protein associated with mTOR; GβL= G-protein β-subunit-like protein; PRAS40= proline-rich Akt substrate of 40 kDa; Deptor= DEP domain-containing mTOR-interacting protein.

Figure 3. Upstream regulation of mTORC2 by BCAA

mTORC2 is activated by PIP_3 acting on mSIN1, leading to the activation of PKCα, SGK and Akt (Ser473). BCAA=branched-chain amino acids; mTORC2= mammalian target of rapamycin complex 2; Deptor= DEP domain-containing mTOR-interacting protein; GβL= G-

protein β-subunit-like protein; Rictor= rapamycin insensitive companion of mTOR; mSIN1= mammalian stress-activated protein kinase interacting protein 1 (mSIN1); Protor= protein observed with Rictor.

Figure 4. Intracellular mechanisms of BCAA modulation of the mTOR pathway

mTOR activation is associated with several pathways, including S6K, IRS-1, NF-κB, SIRT-1 and PPARγ. TSC1 and TSC2 are the major sensors involved in mTORC1 activity and regulation. TNF-α= tumor necrosis factor α; TNFR= tumor necrosis factor receptor; TRADD= associated death domain; FADD= FAS-associated death domain; TRAF2= TNF receptor-associated factor 2; RIP1= receptor-interacting protein 1; LPS=lipopolysaccharide; TLR4= toll-like receptor 4; MyD88= cytoplasmatic adapter protein; TRAF6= TNF receptor-associated factor 6; TAK1=TGF-β-activated kinase 1; IKK= I-kappaB-kinase enzymatic complex; IκBα= I-kappaB-kinase-α; NF-κB= nuclear transcription factor kappa-B; IR= insulin receptor, IRS-1= insulin receptor substrate 1; PI3K= phosphatidylinositol 3-kinase; Akt= protein kinase B; TSC= tuberous sclerosis complex; Rheb= Ras homolog enriched in brain; mTORC1= mammalian target of rapamycin complex 1; S6K= ribosomal S6 kinase; 4E-BP1 = eukaryotic initiation factor 4E-binding protein 1; eIF4F= eukaryotic initiation factor 4F complex; SIRT1= sirtuin 1; C/EPB=CCAAT-enhancer-binding proteins; PPARγ=peroxisome proliferator-activated receptor γ; mTORC2= mammalian target of rapamycin complex 2; Fbw8= substrate-targeting subunit of the CUL7 E3 ligase complex; BCAA= branched-chain amino acids; LAT= L-type amino acid transporter; GLN= glutamine.

1324 **Table 1. Summary of human studies of branched-chain amino acids evaluating nutritional and immunological status**

Number of patients	Clinical condition	Supplementation protocol	Nutritional status effects	Immunological effects	References
35	Operative injury of moderate severity	Solutions with 678 mg/100 ml (22%), 1 g/100 ml (35%) and 3 g/100 ml (100%) of BCAA in TPN for 5 days	Nitrogen equilibrium or positive nitrogen balance; reduction of body weight loss; body weight gain (only in 100% group); increase in aspartate aminotransferase levels (only in 22% an 35% group)	Not evaluated	Freund et al. (1979)[190]
23	Major general surgery, polytrauma, or sepsis	0.7 mg/kg body weight/day of BCAA in TPN for 7 days	Improvement of nitrogen retention	Elevation of absolute lymphocyte count; improved plasma transferrin levels; reversal of anergy to recall skin test antigens	Cerra et al. (1983)[191]

37	Surgical stress (major operation, injury or sepsis)	3.15 g/100 ml (45%) of BCAA (leucine-rich) in TPN for 10 days	Improvement of nitrogen retention; maintenance of plasma concentration of amino acids and protein turnover	Not evaluated	Bower et al. (1986)[193]
101	Septic and traumatized patients	17.3 g/L (50.2%) of BCAA in TPN for 7 days	No statistical differences between groups	Increase in neutrophil counts	Vente et al. (1991)[194]
72	Cirrhotic patients	110 g/d of BCAA orally for one month	Increase in muscle mass and a decrease in fat mass	Not evaluated	Ruiz-Margáin et al. (2016)[198]
10	Decompensated cirrhosis	12 g of BCAA orally for three months	Increase of Fisher's ratio	Improvement of neutrophil phagocytic function and NK cell activity	Nakamura et al. (2007)[202]
267	Cirrhotic patients	5.5–12 g/d of BCAA for over 2 years	Reduced risk for HCC and survival prolongation of patients with cirrhosis	Not evaluated	Kawaguchi et al. (2014)[203]

46	Cirrhotic patients with unresectable HCC	13.5 g/d of protein (BCAA amount not specified) in a meal for 5 weeks	Improvement of BCAA/tyrosine ratio, prealbumin and ALT levels and area under the concentration curve for glucose	Not evaluated	Harima et al. (2010)[204]
270	Cirrhotic patients with HCC	12.45 g/d of BCAA orally for 2 weeks	Recovery of serum albumin level and reduced postoperative complications in patients undergoing transarterial chemoembolization or radiofrequency ablation	Not evaluated	Ishihara et al. (2014)[206]
64	Malnourished surgical patients with gastrointestinal	30% (w/w) of BCCA in TPN for 6 days	Maintenance of albumin and prealbumin concentrations and positive nitrogen balance	Decrease in leukocyte count, CRP, alkaline phosphatase and γ-GT levels	Sun et al. (2008)[207]

	cancer				
30	Healthy elderly men	2.5 g of leucine orally for 3 months	No changes in skeletal muscle mass and strength; no improvements in indexes of whole-body insulin sensitivity, blood glycated hemoglobin content and plasma lipid profile	Not evaluated	Verhoeven et al. (2009)[211]
35	Cirrhotic patients with HCC	5.56 g/d of BCAA in enteral nutrition for one year	Higher event-free survival rate and increasing in serum albumin level	Not evaluated	Kuroda et al. (2010)[212]
211	Cirrhotic patients	12 g/d of BCAA orally for over 6 months	Lower HCC occurrence rate and higher event-free survival rate; reduced incidence of liver-related events in patients with Child-Pugh A cirrhosis	Not evaluated	Hayaishi et al. (2011)[213]

56	HCC patients after hepatic resection	4.74 g/d of BCAA orally for 6 months	Reduced early recurrence after hepatic resection	Not evaluated	Ichikawa et al. (2013)[214]

Abbreviations: BCAA= branched-chain amino acids; TPN= total parenteral nutrition; NK= natural killer; CRP= c-reactive protein; γ-GT= gamma-glutamyl transferase; ALT= alanine aminotransferase; HCC= hepatocellular carcinoma

GβL	G-protein β-subunit-like protein
HAT	heteromeric amino acid transporters
HCC	hepatocellular carcinoma
HMB	β-hydroxy-β-methyl butyrate
HSC	hematopoietic stem cells
IFN	interferon
IKK	I-kappaB-kinase enzymatic complex
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
IRS	insulin receptor substrate
KIC	α-ketoisocaproate
KICD	α-ketoisocaproate dioxygenase
KIV	α-ketoisovalerate
KMV	α-keto-β-methylvalerate
LAT	L-type amino acid transporters
LPS	lipopolysaccharide
LRS	leucyl-tRNA synthetase
MAPK	Ras/mitogen-activated protein kinase
mLST8	mammalian lethal with Sec13 protein 8
mSIN1	mammalian stress-activated protein kinase interacting protein 1
MSUD	maple syrup urine disease
mTOR	mammalian Target of Rapamycin
mTORC1	mammalian Target of Rapamycin complex 1
mTORC2	mammalian Target of Rapamycin complex 2
NAD	nicotinamide adenine dinucleotide
NF-κB	nuclear transcription factor kappa B
NK	natural killer
NT	nutritional therapy
PABP	poly(A)-binding protein
PDK1	phosphoinoisitide-dependent kinase-1

PGC1α	peroxisome proliferator-activated receptor-gamma coactivator 1α
PI3K	phosphatidylinositol 3-kinase
PIKK	phosphatidylinositol 3-kinase-related kinases
PIP3	phosphatidylinositol (3,4,5)-tris- phosphate
PKC	protein kinase C
PN	parenteral nutrition
PPARγ	peroxisome proliferator-activated receptor γ
PRAS40	proline-rich Akt substrate of 40 kDa
Protor	protein observed with Rictor
Raptor	regulatory protein associated with mTOR
Rheb	Ras homolog enriched in brain
Rictor	rapamycin insensitive companion of mTOR
ROS	reactive oxygen species
S6K	ribosomal S6 kinase
SGK	serum and glucocorticoid-inducible kinase
SIRT1	sirtuin 1
SLC	solute carriers
T2DM	type 2 diabetes mellitus
TCA	tricarboxylic acid cycle
TLR	Toll-like receptor
TNF	tumor necrosis factor
TPN	total parenteral nutrition
TSC	tuberous sclerosis complex
VEGF	vascular endothelial growth factor