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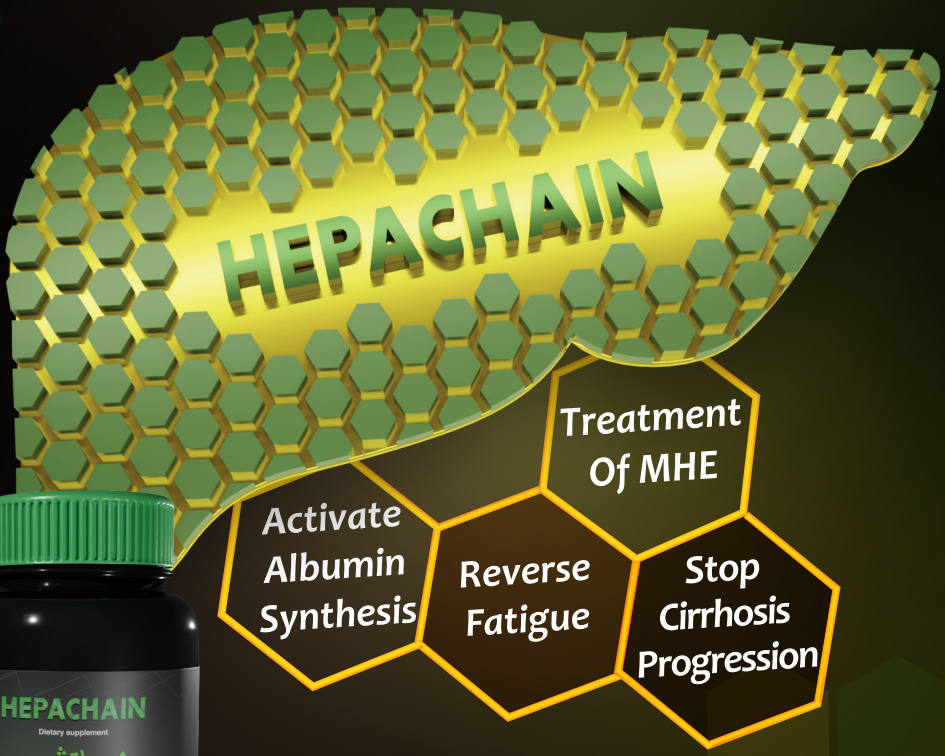

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Emerging role for branched-chain amino acids metabolism in fibrosis

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ABSTRACT

Fibrosis is a common pathological feature of organ diseases resulting from excessive production of extracellular matrix, which accounts for significant morbidity and mortality. However, there is currently no effective treatment targeting fibrogenesis. Recently, metabolic alterations are increasingly considered as essential factors underlying fibrogenesis, and especially research on metabolic regulation of amino acids is flourishing. Among them, branched-chain amino acids (BCAAs) are the most abundant essential amino acids, including leucine, isoleucine and valine, which play significant roles in the substance and energy metabolism and their regulation. Dysregulation of BCAAs metabolism has been proven to contribute to numerous diseases. In this review, we summarize the metabolic regulation of fibrosis and the changes in BCAAs metabolism secondary to fibrosis. We also review the effects and mechanisms of the BCAAs intervention, and its therapeutic targeting in hepatic, renal and cardiac fibrosis, with a focus on the fibrosis in liver and associated hepatocellular carcinoma.

1. Introduction

Fibrosis is a common pathological feature of many chronic diseases. It can occur in many organs, including liver, kidney, heart, lung and skin, which leads to permanent scarring, organ dysfunction and failure, and eventually death. The burden of fibrotic diseases is considerable, affecting around 25% of the world population, and accounting for significant morbidity and mortality [1,2]. However, there are currently no effective therapies for controlling fibrogenesis in fibrotic diseases.

Despite the lack of effective treatment, substantial progress in understanding the underlying mechanisms has been made. Fibrosis is characterized by the imbalanced extracellular matrix (ECM) homeostasis when tissue injury progresses over a prolonged period of time, favoring ECM production over degradation [3]. Specifically, during fibrogenesis, tissue injury driven by a variety of stimuli, such as toxins and infections, initiates the proinflammatory responses associated with

Abbreviations: ACE-I, angiotensin-converting enzyme inhibitor; BCAAs, branched-chain amino acids; BCAT, branched chain amino transferase; BCKA, branched chain α-ketoacid; BCKDC, branched chain amino acid dehydrogenase complex; BCKDH, branched chain amino acid dehydrogenase; BCKDK, branched chain α-ketoacid dehydrogenase kinase; BTR, BCAAs-to-tyrosine ratio; CCl_4, carbon tetrachloride; CKD, chronic kidney disease; CLD, chronic liver disease; Co-SMAD, common mediator SMAD; DEN, diethylnitrosamine; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FAO, fatty acid oxidation; GLS, glutaminase; GPx, glutathione peroxidase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IPF, idiopathic pulmonary fibrosis; IR, insulin resistance; I-SMAD, inhibitory SMAD; JAK, Janus kinase; JNK, Jun N-terminal kinase; KLF6, Krüppel-like factor 6; LLC, large latent complex; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NFY, Nuclear factor Y; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OGG1, 8-oxoguanine DNA glucosylase 1; PA, plasminogen activator; PAI, PA inhibitor; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; PPAR, peroxisome proliferator-activated receptor; PPM1K / PP2Cm, protein phosphatase 1 K, mitochondrial; R-SMAD, receptor-regulated SMAD; SMAD, suppressor of mothers against decapentaplegic; SOD, superoxide dismutase; STAT, signal transducer and activator of transcription; TCA, tricarboxylic acid; TGF-β1, transforming growth factor β1; TGFβR, TGF-β receptor; TRAF, tumor necrosis factor receptor–associated factor; UUO, unilateral ureteral obstruction; α-KG, α-ketoglutarate; α-SMA, α-smooth muscle actin.

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the recruitment of local and invading immune cells (e.g., macrophages) [4]. The immune cells produce cytokines, chemokines and other mediators, which facilitate activation of cells of mesenchymal origin, including fibroblasts, myofibroblasts, fibrocytes and possibly (still in dispute) cells derived through epithelial-to-mesenchymal transition [5, 6]. These activated effector cells have the capacity to synthesize ECM components like collagen and fibronectin, and to release proinflammatory mediators to further stimulate effector cells, hence these cells are the key players in fibrotic tissue remodeling [7–9].

Considering the central role of the effector cells and the fact that fibroblasts and myofibroblasts are key effector cells in most organs, illuminating the molecular mechanisms that underlie the differentiation, proliferation and activation of myofibroblasts has become the focus of antifibrotic studies [4]. Macrophages [10], integrins [11], cytokines [12] and gut microbiota [13] have been found to be involved in these processes. Recently, metabolic regulation has been increasingly recognized as an important field of antifibrotic research. Glycolysis, fatty acid oxidation and fatty acid synthesis are the major metabolic pathways found to be involved in fibrogenesis, and targeting these pathways has yield beneficial effects in preclinical studies [14–18], hence a number of drugs targeting these pathways have been tested in clinical trials [1]. However, studies on amino acid metabolism in fibrosis therapy are scarce, and only glutaminolytic reprogramming has been explored in depth [19–21]. Amino acids are not only substrates for protein synthesis, but also regulatory molecules in metabolism. Among them, branched-chain amino acids (BCAAs), including leucine, isoleucine and valine, play important roles in the mediation of nutrition metabolism, energy homeostasis and immune response via a variety of signaling pathways [22]. In this article, we mainly review the metabolic regulation of fibrosis and the research progress on the roles of BCAAs metabolism in fibrotic diseases of different organs, including liver, kidney and heart.

2. Metabolic regulation of fibrosis

2.1. TGF-β1 signaling and ECM homeostasis

The molecular mechanisms of fibrosis are very complex and multifactorial. Among them, transforming growth factor β1 (TGF-β1) signaling plays a major role (Fig. 1) [23]. TGF-β1 is synthesized intracellularly through a series of steps and ultimately secreted to the ECM as the form of the large latent complex (LLC) [24–27]. Large amounts of LLCs are deposited extracellularly for rapid activation by the microenvironmental factors, such as proteases, reactive oxygen species (ROS) or altered pH [28]. Once TGF-β1 is activated, it binds to TGF-β receptor II (TGFβRII), causing a conformational change which leads to the phosphorylation and activation of TGFβRI [29,30]. The activated TGFβRI can regulate downstream cellular responses through both canonical, i.e.

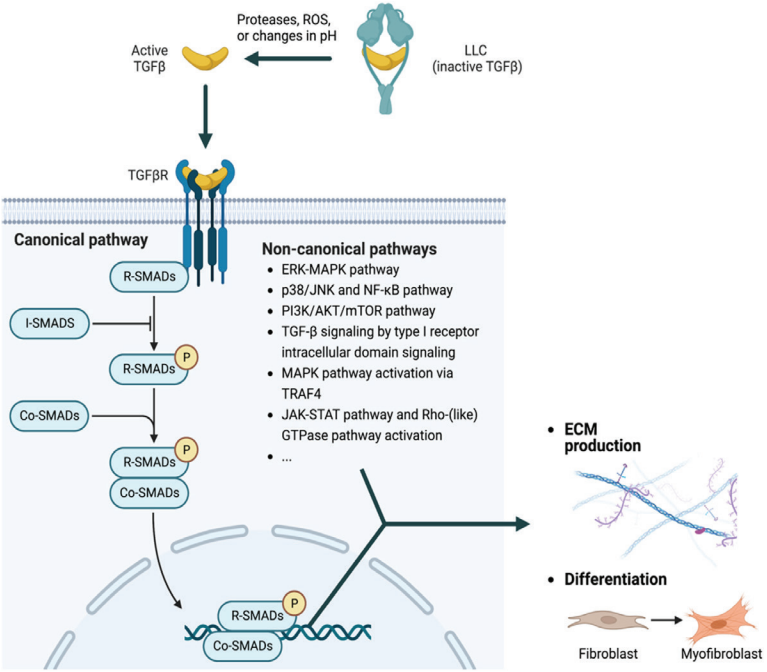

Fig. 1. Overview of TGF-β signaling. Upon activation, TGFβ binds to TGFβR and regulates downstream canonical and non-canonical pathways, facilitating ECM production and fibroblast differentiation into myofibroblasts. ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; JNK, Jun N-terminal kinase; LLC, large latent complex; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K, phosphoinositide 3-kinase; SMAD, suppressor of mothers against decapentaplegic; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor-β; TGFβR, TGFβ receptor; TRAF, tumor necrosis factor receptor–associated factor. (Created with BioRender.com).

suppressor of mothers against decapentaplegic (SMAD) signaling, and non-canonical pathways. The activated TGFβRI can directly phosphorylate the receptor-regulated SMADs (R-SMADs, including SMAD1–3, 5 & 8), which bind the common mediator SMADs (Co-SMADs, including SMAD4) forming heterooligomeric complexes. These complexes then translocate into the nucleus and modulate target gene transcription. The inhibitory SMADs (I-SMADs, including SMAD6&7) can interact with TGFβRI to antagonize the activity of R-SMADs by interfering the docking of R-SMADs and inducing the degradation of R-SMADs via the ubiquitin proteasomal pathway [31,32]. TGF-β1 signaling promotes production of ECM and the differentiation of fibroblasts into myofibroblasts, thus it is widely perceived as the major profibrotic pathway [33–35].

Following tissue injury, the ECM promotes the restoration of the tissue by stabilizing tissue structure, preserving local cytokines and growth factors, and enabling the migration of effector cells into injured tissue [36]. Thus, the ECM is now recognized as a dynamic factor affecting the progression of fibrosis. The constituents of ECM include collagen, fibronectin, glycosaminoglycans, proteoglycans, laminins, elastin, and several other glycoproteins, among which collagen type I is the major component [37]. The production and degradation of collagen have been thoroughly studied. Under stimulation like activated TGF-β1, fibroblasts and myofibroblasts secret triple-helix molecules into the extracellular space where peptidases cleave the terminal segments resulting in insoluble monomeric collagens [38]. The monomeric molecules make up microfibrils which combine with each other to form collagen fibril, and then collagen fibers generated by a bunch of collagen fibrils undergo crosslinking with each other or other components to form ECM [38]. ECM production is excessively upregulated in fibrosis, which leads to increased stiffness of organs [39]. On the other hand, a collagen fibril is usually larger than a single cell, hence ECM must first be degraded by extracellular proteases, among which the matrix metalloproteinases (MMPs) are predominant and the most well studied [40]. MMPs are regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs) which compete the protease site [41]. Cleaved collagen is then internalized by various cells (including fibroblasts, chondrocytes and osteoblast-like cells) and completely degraded in the lysosomes [42].

2.2. Metabolic alterations in fibrogenesis

During wound healing, activated fibroblasts undergo metabolic alterations to perform specific functions. Because of the deleterious effects of direct inhibition of TGF-β signaling, targeting the downstream metabolic disorders, including dysregulated metabolism of carbohydrates, lipids and amino acids may represent a more effective antifibrotic therapeutic approach [1].

Glycolysis is increased in fibroblasts to sustain energy-consuming functions. On the one hand, there is a positive-feedback loop between glycolysis and TGF-β signaling. TGF-β directly induces glycolytic enzymes such as hexokinase and phosphofructokinase and indirectly activates specific proglycolytic pathways [43,44]. Conversely, augmented glycolysis produces more by-products such as lactate which acidifies the extracellular pH, leading to the activation of TGF-β signaling [45]. Elevated glucose levels have also been shown to increase the TGF-β signaling [46]. On the other hand, glycolysis promotes ECM production via directly supplying energy for multiple steps and providing amino acids for synthesis and indirectly activating fibroblasts [47–49]. Glycolytic inhibitors have been shown to be efficacious in the preclinical studies of various fibrotic disorders [14,15,50,51]. Besides, loss of fatty acid oxidation (FAO) and subsequent lipid accumulation have also been demonstrated to be closely related to impaired ECM degradation [16,52]. The peroxisome proliferator-activated receptor (PPAR) signaling pathway, which promotes fatty acid oxidation and directly facilitates ECM degradation through CD36 [17], has been found downregulated in the effector cells of multiple fibrotic diseases [53–55]. Based on the abundance of preclinical studies on carbohydrate and lipid metabolism in fibrosis, several drugs targeting these metabolic pathways have been in clinical investigations [1]. However, for amino acid metabolism in fibrosis, only glutaminolysis has been studied in detail. Elevated glutaminolysis by upregulated glutaminase (GLS) was observed in TGFβ1-induced fibroblasts, which facilitates the conversion of glutamine to glutamate and hence increases the stability of collagen by mTOR [19]. Knockout of GLS1 in fibroblast or application of GLS1 inhibitor protected mice from lung fibrosis [21].

Although drugs targeting glucose and lipid metabolism display less toxicity than direct antagonism of TGF-β1 signaling, their off-target effects remain an obstacle to their clinical utilization [1]. For instance, glycolytic inhibitors may exert ECM production facilitation via inhibition of capillary expansion [56,57]. In addition, a variety of adverse events were observed in the long-term use of PPAR agonists, including hepatic, cardiac, muscular and carcinogenic toxicity [58,59]. Despite the paucity of relevant studies, targeting amino acid metabolism represents a promising opportunity for fibrosis therapy. Firstly, the ultimate purpose of treating fibrosis is to reduce the excessive deposition of ECM in which amino acids are the predominant constituents [1]. Additionally, amino acids also play important regulatory roles in ECM production. For instance, glutamine is capable of regulating collagen mRNA expression in fibroblasts [60]. Hence, ECM homeostasis cannot be maintained without ordered amino acid metabolism. Moreover, combination of drugs targeting different metabolic pathways might be an alternative approach due to potential synergies [1]. Delving into amino acid metabolism and developing related drugs can provide more options for research of drug combination, particularly in those fibrotic diseases with significant changes in amino acid metabolism, considering that metabolic alterations are type and cell dependent in fibrosis. For BCAAs, they serve as building blocks for ECM production directly, or indirectly by supplying nitrogen for alanine, aspartate, glutamate, glutamine synthesis [61]. Specially, ECM secreting cancer-associated fibroblasts whose BCAAs catabolism is remarkably upregulated survived under BCAAs deprivation by internalizing ECM proteins in pancreatic ductal adenocarcinomas [62]. Therefore, the abnormalities in the BCAAs metabolism gradually emerged as an indispensable part of metabolic alterations in fibrogenesis, fueling the hope that they may offer therapeutic strategies in the future.

3. BCAAs metabolism in fibrosis

3.1. Overview of BCAAs metabolism

Due to their small, branched and hydrophobic R groups, BCAAs are the key components in most proteins, accounting for approximately 18% of amino acids and 63% of hydrophobic amino acids in most species [63]. The molar ratio of leucine, isoleucine and valine is usually around 2.2:1.0:1.6, reflecting their linked nature of metabolism [63]. BCAAs cannot be synthesized in animals and thus must be obtained through food digestion. Under physiological conditions, the intake and excretion of BCAAs maintain a delicate balance. BCAAs in the body are mainly composed of circulation pool and tissue (liver and skeletal muscle protein) pool [64]. BCAAs in the circulation maintain a relatively stable level: they are primarily derived from dietary intake and tissue protein breakdown, and are primarily consumed by oxidative breakdown and tissue protein synthesis (excretion through urine is trivial) [64].

All reactions of BCAAs catabolism almost occur in the mitochondrial matrix (Fig. 2). The initial two steps of BCAAs catabolism are similar. Three BCAAs are first reversibly transaminated with nitrogen receptors (usually α-ketoglutarate, α-KG) by branched chain amino transferases (BCATs), yielding branched chain α-ketoacids (BCKAs) and glutamate [65]. BCATs are encoded by two genes: *BCAT1* (also known as *cBCAT* because it encodes a cytoplasmic protein) which is mainly expressed in the brain and *BCAT2* (also known as *mBCAT* because it encodes a mitochondrial protein) which is expressed in many tissues [66]. Unlike most amino acids which are catabolized in the liver, BCAAs are firstly

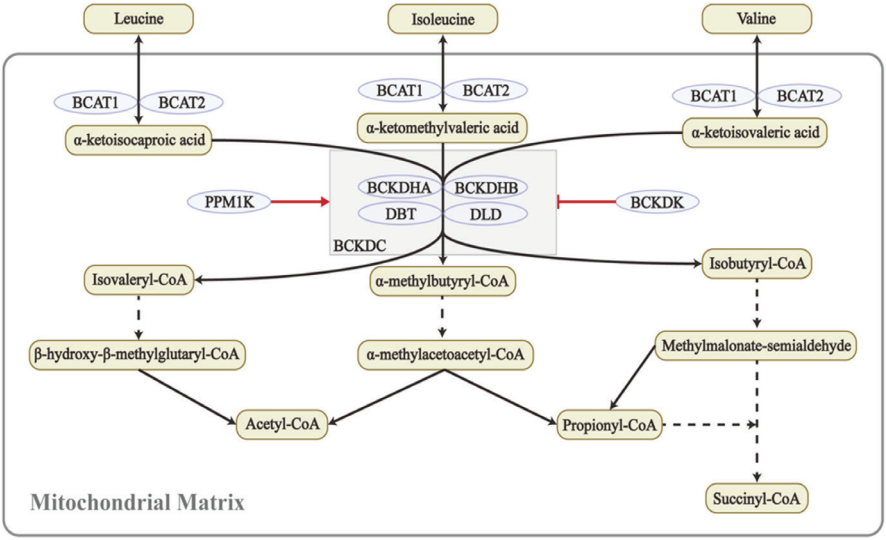

Fig. 2. Overview of the BCAAs catabolism. In the mitochondrial matrix, all BCAAs are first reversibly transaminated by BCAT and then irreversibly decarboxylated and dehydrogenated by BCKDC which is regulated by PPM1K and BCKDK. The remaining steps resemble fatty acid oxidation, yielding propionyl-CoA and/or acetyl-CoA. BCAT, branched chain amino transferase; BCKDC, branched chain amino acid dehydrogenase complex; *BCKDHA* and *BCKDHB* encode E1 subunit of BCKDC (branched chain α-ketoacid decarboxylase); BCKDK, branched chain α-ketoacid dehydrogenase kinase; *DBT* encodes E2 subunit of BCKDC (lipoamide acyltransferase); *DLD* encodes E3 subunit of BCKDC (lipoamide dehydrogenase); PPM1K, protein phosphatase 1 K, mitochondrial.

catabolized in peripheral tissues like skeletal muscle because BCATs are barely expressed in hepatocytes [67]. The BCKAs are then irreversibly decarboxylated and dehydrogenated by the branched chain amino acid dehydrogenase (BCKDH) complex, which is located on the inner surface of the inner membrane of mitochondria [63]. The activity of BCKDH complex is high in liver, moderate in kidney and heart, and limited in skeletal muscle [68]. The BCKDH complex consists of three parts: a thiamin-dependent decarboxylase (BCKDH-E1, encoded by *BCKDHA* and *BCKDHB*), a lipoate-dependent dihydrolipoyl transacylase (BCKDH-E2, encoded by *DBT*) which transfers the acyl groups to CoA, and a FAD-dependent dihydrolipoyl dehydrogenase (BCKDH-E3, encoded by *DLD*) which transfers the released electrons to NAD^+ [69,70]. The BCKDH complex is the rate-limiting enzyme for BCAAs catabolism. It is tightly regulated through phosphorylation (inhibition) of BCKDH-E1 by BCKA dehydrogenase kinase (BCKDK) and through dephosphorylation (activation) of BCKDH-E1 by protein phosphatase 1 K, mitochondrial (PPM1K or PP2Cm) [70,71]. Although the subsequent steps of the catabolism are unique to each BCAA, they all resemble fatty acid oxidation. Ultimately, the carbons of BCAAs either enter the tricarboxylic acid (TCA) cycle or are transformed into CO_2. In detail, valine enters the TCA cycle as propionyl-CoA (gluconeogenic), while leucine enters the TCA cycle as acetyl-CoA (ketogenic), and isoleucine enters the TCA cycle as both propionyl-CoA and acetyl-CoA [72].

BCAAs are not only nutrient substrates for protein synthesis, but also play important roles in the mediation of nutrition metabolism, energy homeostasis and immune response via a variety of signaling pathways [22]. Several diseases have been shown to be associated with abnormal BCAAs metabolism. For instance, in maple syrup urine disease, BCAAs are excessively accumulated to exert neurotoxic effects because of mutations in BCKDH [73]. Besides, elevation in circulating BCAAs has been proven to be associated with an increased risks of diabetes, cancer and heart failure [63]. Abnormalities in BCAAs metabolism have also been found in multiple fibrotic diseases, which will be discussed in the next sections.

3.2. Fibrosis and dysregulated BCAAs metabolism in liver diseases / hepatocellular carcinoma

Liver fibrosis is characterized by chronic liver injury induced accumulation of ECM mainly composed of collagen type I and type III, which leads to the presence of fibrous scar and successive cirrhosis [74]. There are two sources of chronic liver injuries: hepatotoxic injury (e.g., chronic hepatotropic virus infection like hepatitis B virus (HBV) and HCV, alcohol abuse, non-alcoholic fatty liver disease (NAFLD), autoimmune liver diseases, and hereditary diseases), and cholestatic injury [75]. The molecular mechanisms leading to liver fibrosis are similar: chronic liver injuries progressively damage the epithelial or endothelial barrier, causing the release of inflammatory cytokines and subsequent recruitment of inflammatory cells which produces TGF-β, which in turn activates hepatic myofibroblasts resulting in the excessive secretion of ECM components [6]. Myofibroblasts are the major source of ECM, but they do not exist in healthy liver. The source composition of myofibroblasts was highly variable, but the vast majority are derived from activated hepatic stellate cells (HSCs) and activated portal fibroblasts [76]. HSCs have a significant role in liver fibrosis. While under physiological conditions HSCs are quiescent and reside in the space of Disse, which mainly function to store vitamin A, upon tissue injury, HSCs are activated and undergo phenotypic transition, migrating to the site of injury and producing ECM [77,78].

Liver fibrosis plays an important role in a variety of liver diseases. Liver fibrosis occurs in most types of chronic liver diseases (CLDs), and the progression of CLDs is determined by the development of liver

fibrosis [79]. The presence of fibrous scar disrupts the architecture of the liver, which results in the loss of hepatocytes and normal hepatic functioning, eventually leading to liver failure [80]. In addition, it is worth noting that, unlike most other organs, liver fibrosis is vigorously associated with liver cancer development, which is the third leading cause of cancer-related death worldwide in 2020 [81]. On the one hand, more than 80% of hepatocellular carcinomas (HCCs) (comprising more than 3/4 of liver cancer [81]) develop in fibrotic livers, usually following the progression sequence of hepatitis, fibrosis, cirrhosis, and HCCs (Fig. 3) [82]. On the other hand, approximately one-third of patients with compensated cirrhosis will eventually develop HCCs [83]. Moreover, there is accumulating evidence that fibrosis is a vital component of the hepatic premalignant environment, actively contributing to the development of HCCs mainly through the ECM and activated HSCs. In addition to providing mechanical tissue support, ECM proteins are able to bind specific receptors and store growth factors, leading to the activation of the downstream signaling facilitating cancer development [84]. Furthermore, activated HSCs could also promote the development of HCCs through production of cytokines and growth factors, and the interaction with other hepatic cells, thereby reducing tumor immunosurveillance and accelerating tumor growth [83]. In conclusion, targeting fibrosis has great clinical significance in the prevention and treatment of liver diseases, especially including HCCs.

The liver, in which many amino acids, glucose and glutathione are synthesized, plays a pivotal role in amino acid metabolism in mammals. In humans, abnormal hepatic amino acid metabolism leads to many diseases, hence targeting these abnormalities has important medical significance [85]. The liver is the only organ to catabolize all amino acids in circulation except BCAAs due to the aforementioned lack of hepatic BCATs, whereas the remaining decomposition steps can be actively carried out in hepatocytes, resulting in propionyl-CoA and/or acetyl-CoA which are further converted into ketone bodies in response to the physiological needs [85]. In patients with liver diseases such as cirrhosis and hepatic encephalopathy characterized by hyperammonemia, serum BCAAs levels decrease to compensate impaired hepatic urea cycle because glutamate produced by the transamination of BCAAs with α-KG has the ability to combine with ammonia to form glutamine to remove excessive ammonia mainly in muscles and the brain [86,87]. Therefore, targeting BCAAs metabolism may have beneficial therapeutic effects on liver diseases.

Altered BCAAs metabolism has been described in murine models of liver fibrosis. It was found that in liver fibrosis and cirrhosis rats treated by thioacetamide, the concentrations of BCAAs in serum all increased at different time points (1, 2, and 3 months), while the concentrations of BCAAs in liver remained stable in the first two months but increased slightly in the third month [88]. Consistent with the previous study, serum BCAAs levels tended to increase during early stages in streptozotocin and high fat diet induced nonalcoholic steatohepatitis (NASH) mice, especially with a large increase in isoleucine that may be due to improved glucose tolerance [89].

However, the situation is not exactly the same in human research. Increased concentrations of BCAAs in livers of cirrhotic patients were also observed compared with donor tissues [90]. In addition, this increase was more significant in cases with more severe fibrosis (NASH vs. alcohol-related liver damage) [90]. But the circulating levels of BCAAs were found to decease in patients with liver cirrhosis [91]. This negative correlation between fibrosis stage and plasma concentrations of BCAAs was later confirmed in 137 patients with NASH [92]. It is also worth noting that this change may be sex dependent. In a cross-sectional cohort study of 112 patients, only female patients with NAFLD displayed increased plasma BCAAs with increased fibrosis [93]. Further research is urgently needed to explain these discrepancies. Additionally, retrospective analysis of 86 patients with NAFLD revealed that BCAT1 was overexpressed and hypomethylated [94].

Changes in plasma BCAAs levels may have the potential to diagnose the degree of liver fibrosis. In clinical practice, liver biopsy is the gold standard method of evaluating the degree of fibrosis[95]. Despite of the significance of liver biopsy, application of this technique is limited because of its expensiveness, potential sampling errors, and its invasive nature [96]. Hence there is an urgent need for noninvasive and convenient biomarkers for liver fibrosis. Metabolic functions of liver are affected by the progression of fibrosis. A recent study in 101 patients showed that the BCAAs-to-tyrosine ratio (BTR) value in serum was significantly lower in patients with chronic hepatitis or liver cirrhosis

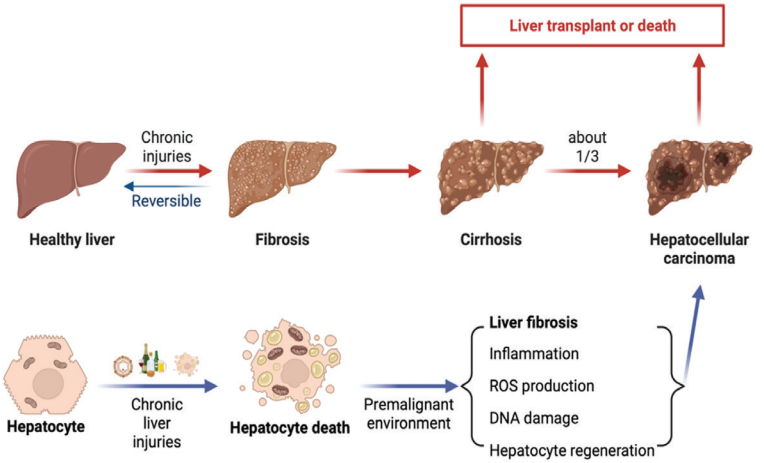

Fig. 3. The progression and development of hepatocellular carcinoma. Most hepatocellular carcinomas develop from liver fibrosis associated with inflammation, increased ROS production, DNA damage and hepatocyte regeneration, which together form the hepatic premalignant environment. ROS, reactive oxygen species. (Created with BioRender.com).

[91]. Subsequent research revealed that the BTR value strongly correlated with the liver fibrosis markers in patients infected with HCV [97–99]. In addition, the BTR value was negatively correlated with the fibrosis stages studied in 137 patients with NASH [92]. Therefore, these studies preliminarily demonstrate a potential to use BCAAs as biomarkers in the diagnosis of liver fibrosis.

3.3. Dysregulated BCAAs metabolism and fibrosis in kidney and heart diseases

The kidney plays an essential role in regulating the amino acid homeostasis by synthesizing, degrading, filtrating, reabsorbing and excreting amino acids and peptides [100]. Under physiological condition, most amino acids can be catabolized and some amino acids and their derivatives can be synthesized in the kidney through various pathways, and almost all filtered amino acids are reabsorbed into the blood by the proximal tubules. Renal dysfunction causes abnormal alterations of amino acid concentrations in blood, which may further lead to extrarenal diseases such as muscle atrophy and cardiovascular diseases [101]. Therefore, clarifying the metabolism of BCAAs in kidneys is crucial for the prevention and treatment of renal disease. It has been proposed that renal BCAAs metabolism is defined by BCAAs uptake in absorptive stage and significant leucine excretion in post absorptive stage [102]. But during chronic renal failure, metabolic acidosis boosts BCAAs oxidation in tubule cells through elevated amount and activity of BCKDH by lowering BCKDK [103].

Similar to liver fibrosis, the pathological features of kidney fibrosis include excessive accumulation of ECM, proliferation of interstitial fibroblasts which replace renal parenchyma, and infiltration of inflammatory cells causing tubular cell apoptosis and necrosis which ultimately lead to tubular dilation and atrophy [104]. There are controversies regarding the role of BCAAs in kidney fibrosis. In a mouse model of systemic lupus erythematosus induced renal fibrosis, tissue concentrations of BCAAs increased significantly compared with the control [105]. Moreover, Piret et al. revealed the downregulated expression of BCAAs catabolic enzymes in kidney fibrosis. Krüppel-like factor 6 (KLF6) is able to suppress the expression of genes encoding BCAAs catabolic enzymes through occupation of the promoter region. Kidney fibrosis was ameliorated in *Klf6*-knockdown mice and exacerbated in *Klf6*-overexpression mice [106]. However, Zhao et al. found that BCAAs levels in urine samples and the concentrations of leucine and isoleucine in renal tissue extracts were reduced in unilateral ureteral obstruction (UUO) rats, and administration of the exogenous BCAAs significantly attenuated kidney fibrosis in these rats [107]. Further research is still needed to determine the role of BCAAs in kidney fibrosis.

The heart has an exceptionally high demand for large amounts of continuous ATP production to satisfy contractile function. As a result, assorted materials including fatty acids, glucose, ketones and amino acids can be catabolized in heart, in which mitochondrial oxidative phosphorylation dominates [108]. In most heart diseases, compromised energy metabolism is an indispensable contributor [108]. BCAAs are by far the best identified amino acids in cardiac amino acid oxidation. Although BCAAs only contribute to less than 2% of ATP production in heart, they play a marked role in insulin and mTOR signaling [109]. It has been shown that in patients with heart failure, impaired cardiac BCAAs oxidation induced elevated concentrations of BCAAs in plasma [110], and that accumulation of BCAAs in human heart facilitated cardiac hypertrophy probably via mTOR signaling [111].

Cardiac fibrosis refers to various alterations in the interstitial myocardial collagen network which appear in response to harmful stimuli including cardiac ischemic insults, systemic diseases, or drugs [112]. Fibrosis disrupts the architecture of the myocardium and promotes the development of cardiac dysfunction and arrhythmias such as atrial fibrillation [113], in which the clinical outcome of patients is affected [112]. Therefore, targeting fibrotic pathways could improve the diagnosis and treatment of patients with heart failure. Studies on the role of BCAAs metabolism in cardiac fibrosis are currently scarce. In diabetic cardiomyopathy mice, the concentrations of BCAAs in serum and cardiac tissue were notably increased [114]. In cardiomyopathy patients whose catabolism of BCAAs was impaired compared with the controls, formation of cardiac fibrosis was increased [115].

4. Roles of BCAAs in therapeutic response

BCAAs supplementation and targeting BCAAs catabolism are two ways to target BCAAs metabolism. In principle, they are both valid methods to affect the BCAAs pool in the body, but there are differences at least in four aspects. Firstly, intervening oral intake of BCAAs is efficacious because BCAAs cannot be synthesized in vivo as members of essential amino acids. It was reported that each BCAA may have distinct metabolic effects [116]. Targeting individual BCAAs metabolism is easier to achieve for BCAAs supplementation in which the ratio of BCAAs is readily to modified compared with targeting BCAAs catabolism whose rate-limiting enzyme has catalytic activity for all BCAAs. Secondly, targeting BCAAs catabolism has the ability to act on local BCAAs metabolism by developing pharmaceutically modified drugs or gene-editing techniques, whereas BCAAs supplementation mainly exerts systemic effects. Thirdly, BCAAs supplementation is inexpensive and has manageable toxicity because BCAAs are common endogenous substances. By contrast, developing drugs targeting BCAAs catabolism might be expensive, time-consuming and potentially risky. In addition, targeting BCAAs catabolism may have other benefits with regard to glucose or lipid metabolism. For example, selective inhibition of BCAT1 led to reduced glycolysis in macrophages [117]. Therefore, BCAAs supplementation and targeting BCAAs catabolism have their own applications and both have the potential in the treatment of fibrosis.

4.1. Effects of BCAAs intervention on fibrosis

There are currently no antifibrotic drugs targeting metabolic pathways, not to mention targeting BCAAs metabolism. However, preclinical studies on the intervention of BCAAs in the treatment of fibrosis have been prospering, especially in liver fibrosis. Numerous preclinical studies on the effects of dietary supplementation of BCAAs have been conducted in rodent fibrosis models. Supplementation with BCAAs effectively attenuates liver fibrosis in rat models. In carbon tetrachloride (CCl_4) induced liver cirrhosis rats, the protective role of BCAAs supplementation was proved by histopathological examination and molecular biological methods. Liver fibrosis visualized by Azan staining [118–120] and Masson trichrome staining [121] was less evident in the BCAAs-treated group than in the controls. The expression of α-smooth muscle actin (α-SMA) was suppressed at both mRNA [121] and protein [122] levels. However, despite less fibrosis was observed by Azan staining, no significant difference of α-SMA and TGF-β mRNA levels was found between groups [119]. Moreover, hydroxyproline, a sensitive marker of liver fibrosis, was significantly decreased in the CCl_4 +BCAAs group compared with the CCl_4 group [122]. Apart from CCl_4 treatment, BCAAs also ameliorated fibrosis in a rat model of hepatocellular carcinoma with liver cirrhosis treated by diethylnitrosamine (DEN). Compared with the control group, the mean fibrotic area, the level of α-SMA protein, and the expression of mRNA for fibrosis markers (e.g., TGF-β1, Col1α2, Col3α1, TIMP-1, and TIMP-2) decreased in the BCAAs-treated group [123]. In addition, Sirius staining demonstrated that liver fibrosis was alleviated by BCAAs treatment in choline-deficient amino acid-defined fed rats [124].

As for the effects in the mouse model, BCAAs supplementation significantly improved liver fibrosis (less fibrosis area, less hydroxyproline, and lower expression of fibrosis markers mRNA) in atherogenic and high-fat diet induced NASH mice [125], while no statistical difference was observed between the control and BCAAs-treated groups in choline-deficient high-fat induced NASH mice [126]. BCAAs supplementation reduced fibrotic area in Azan-stained sections,

hydroxyproline content in liver, and the expression of α-SMA in the DEN-treated *db/db* mice which were genetically altered to generate phenotypes of obesity and diabetes mellitus [127]. Meanwhile, in the platelet-derived growth factor C transgenic mice which develop liver fibrosis and tumors, the area of fibrosis and the expression of fibrosis markers mRNA were both significantly decreased in the BCAA-treated group [125]. Although the protective role of BCAAs has been fully validated in murine models, single treatment with BCAAs did not display any improvement in fibrosis in patients with liver cirrhosis, which reflects the complexity of the mechanisms of liver fibrosis [128].

As mentioned above, single treatment with BCAAs did not exert any antifibrotic effects in human liver. This may be due to the heterogeneous pathogenesis of liver fibrosis. Therefore, it could be a better therapeutic strategy to target multiple pathways simultaneously. Daou et al. developed an endogenous metabolic modulator LIVRQNac composed of leucine, isoleucine, valine, arginine, glutamine, and N-acetylcysteine. LIVRQNac treatment effectively inhibited the TGF-β-driven induction of α-SMA and proliferation rate in primary human stellate cell model in a dose-dependent manner, whereas single components of BCAAs did not exert inhibitory effects in this model [129]. The authors believe this may be due to the additive and even synergistic effects brought by the combination of certain amino acids whose metabolism has broad impact on other pathophysiological features in NASH except fibrosis [129]. Similar results were found in vivo. The combination treatment with BCAAs and ACE-I obviously improved the progression of serum fibrosis markers, while individual treatment with either BCAAs or ACE-I was not as impactful as the combination therapy [128]. The authors think it may be possible that the combination suppressed the neovascularization in liver, given that fibrosis and angiogenesis develop synergistically [128]. In conclusion, the potential synergistic effects displayed in the above two preliminary studies demonstrate the complexity of the pathological process of fibrosis. BCAAs supplementation as part of the treatment of human liver fibrosis could be a more rational choice. Further research is required to discover efficacious combinations and elucidate potential mechanisms.

In contrast to the beneficial effects of BCAAs supplementation in liver fibrosis, existing research suggests that BCAAs supplementation may be detrimental to renal fibrosis. BCAAs supplementation strongly increased the mRNA expression of SMA and collagen in a 5/6 nephrectomy rat model [130]. These discoveries are in accordance with the clinical practice of restricting protein intake to slow down chronic kidney disease (CKD) progression [131]. The effect of BCAAs supplementation on cardiac fibrosis is still controversial. In mice subjected to experimental myocardial infarction, the high leucine diet inhibited myocardial collagen content [132]. By contrast, severe myocardial fibrosis was reported in the myocardium (assessed by Masson staining) after BCAAs treatment [133]. In short, unlike the abundance of studies related to liver fibrosis, further extensive research is still needed to determine the role of BCAAs supplementation in renal and cardiac fibrosis.

4.2. Mechanisms of BCAAs intervention on liver fibrosis

To date, preclinical studies on liver fibrosis have proposed three mechanisms by which BCAAs supplementation alleviates fibrosis: mTORC1-dependent inhibition of TGF-β signaling, less oxidative stress, and improved insulin resistance (Fig. 4).

BCAAs supplementation was shown to inhibit TGF-β signaling in murine models of liver fibrosis. The expression of mRNA of TGF-β1 and SMAD3 was up-regulated and the expression of mRNA of SMAD7 was down-regulated in livers of CCl_4 induced mice [122]. Meanwhile, the

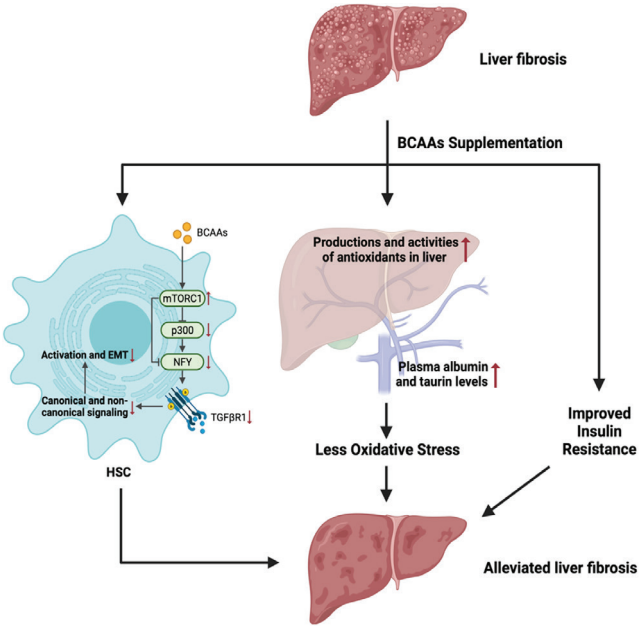

Fig. 4. Mechanisms of alleviated liver fibrosis by BCAAs treatment. The existing research suggests that BCAAs supplementation ameliorates liver fibrosis via inhibiting mTORC1-dependent TGF-β signaling, reducing oxidative stress, and improving insulin resistance. (Created with BioRender.com).

expression of hepatic TGFβR1 (mRNA and protein) and p-SMAD3L (protein) was increased in the Ath+HF group and repressed in the Ath+HF+BCAAs group considerably in mice [125]. Furthermore, BCAAs supplementation decreased expression of SMAD4, Col1α2, and TIMP-2 which regulates the activity of MMPs by inhibiting TGF-β1 in livers of DEN treated rats [123]. Apart from SMAD signaling, BCAAs treatment also caused the reduced phosphorylation of p38 (the marker for SMAD-independent p38 mitogen-activated protein kinase signaling) in the human HSC line Lx-2, which is the major source of ECM and a critical target for anti-hepatic fibrosis therapies [134].

The inhibition of TGF-β signaling after treatment with BCAAs was found to be mTORC1-dependent in HSC. BCAAs, particularly leucine, play their regulatory role mainly through the mTOR signaling pathway. mTOR is a conserved serine-threonine kinase and it interacts with other proteins to form two complexes: mTOR complex 1 (mTORC1) and mTORC2, of which only mTORC1 integrates inputs from amino acids [135]. Amino acids can somehow promote the Rags (a class of small GTPases) to load GTP to form heterodimers, enabling the interaction of the heterodimer with the raptor (regulatory associated protein of mTOR) of mTORC1 [136]. mTORC1 is then translocated from cytoplasm to lysosomal surface and conducts its downstream signaling by phosphorylating a plethora of targets, leading to the activation of protein and lipid synthesis and energy metabolism, and the inhibition of autophagy and lysosome biogenesis [137,138]. The influence of BCAAs on TGF-β1 signaling and its underlying signaling pathway were evaluated in Lx-2. BCAAs repressed TGF-β1 signaling by targeting TGFβR1 through inhibition of NFY and p300, and this inhibition is mTORC1-dependent [125]. Nuclear factor Y (NFY) binds to CCAAT motifs in the promoter region of various genes, including TGFβR1. NFYA is a regulatory subunit of NFY, it can be acetylated by the histone acetyltransferase p300. BCAAs decreased the expression of NFYA and p300 and thus the promoter activity of TGFβR1 in TGF-β1 induced Lx-2 cells [125]. Mutations at the NFY binding site attenuated the inhibitory effects of BCAAs [125]. In addition, overexpression of Rheb (Ras homolog) and knockdown of raptor showed that activation of mTORC1 suppressed TGF-β1 signaling. The inhibitory effects of BCAAs existed only when raptor was expressed normally [125]. In summary, BCAAs exerted antifibrotic effects by activating the negative feedback regulation from mTORC1 to TGF-β1 signaling in HSCs.

BCAAs were found to alleviate liver fibrosis through less oxidative stress. Oxidative stress is characterized by increased ROS production. ROS disrupts DNA, proteins, and lipids, which induce apoptosis and necrosis of hepatocytes, resulting in aggravated inflammatory response. During the process, fibrogenesis is facilitated by increased secretion of profibrogenic cytokines, especially TGF-β. There are a number of antioxidant mechanisms in human body to counteract the deleterious effects of ROS, including the enzymatic and non-enzymatic antioxidant defenses, such as albumin, superoxide dismutase (SOD), glutathione peroxidase (GPx), and 8-oxoguanine DNA glucosylase 1 (OGG1) [139]. In livers of CCl_4 induced rats, BCAAs supplementation significantly decreased hepatic 4-hydroxynonenal (4-Hne) and 8-hydroxydeoxyguanosine (8-OHdG) levels and improved hepatic iron overload [120]. 4-Hne, 8-OHdG and hepatic iron all produce oxidative stress in the liver. This reduction of ROS is probably due to the activation of antioxidant mechanisms. BCAAs supplementation improved serum albumin concentration in rats [120,121] and in patients [140]. The BCAAs supplementation resulted in the increased SOD protein levels [120], activity of SOD and GPx [122], and the upregulated expression of mRNA and protein of OGG1 [121] in livers of CCl_4 induced rats. Moreover, taurine supplementation could prevent fibrosis by reducing oxidative stress. COD catalyzes the rate-limiting step of taurine biosynthesis, but its gene expression is suppressed by TGF-β. However, BCAAs antagonized the suppressive effect on *CdoI* gene expression and increased plasma taurine levels in patients [141].

BCAAs also ameliorate liver fibrosis by improving insulin resistance (IR). IR status directly facilitated liver fibrosis through the activation of HSCs proliferation in obese diabetic rats [142]. Studies have also revealed that IR is closely related to fibrosis development in patients with CLDs, including chronic hepatitis C and NAFLD [143,144]. IR is described as the co-existence of high blood glucose and insulin levels, which both contribute to fibrosis. On the one hand, hyperglycaemic conditions were found to stimulate TGF-β signaling in the kidney and heart [45,46]. On the other hand, insulin and insulin-like growth factor 1 promote phosphatidylinositol 3-kinase and extracellular signal-regulated kinase signaling, leading to the activation of HSCs and subsequent fibrosis [145,146]. BCAAs supplementation decreased serum insulin level without changing blood glucose in CCl_4 induced rats [120]. Khedr et al., however, discovered that BCAAs supplementation decreased circulating glucose and increased insulin levels in CCl_4 induced rats [122]. Moreover, increased hepatic glucose production through gluconeogenesis is a typical feature of IR, and ROS-induced gluconeogenesis through FoxO1 pathway was mitigated in the presence of BCAAs-supplementation in a human liver cancer cell line [120]. Additionally, combined treatment with BCAAs and angiotensin-converting enzyme inhibitor (ACE-I) slowed down the progression of fibrosis with significantly improved IR status in patients with liver cirrhosis [128].

4.3. Effects of targeting BCAAs catabolism on fibrosis

Recent studies have preliminarily demonstrated the potential for the treatment of fibrosis by targeting BCAA catabolism. KLF6, a transcription factor, which is widely expressed has important roles in hepatic, renal, and cardiac fibrosis. The direct regulation of BCAAs catabolic enzymes by KLF6 was substantiated by classification and pathway enrichment analysis [106]. Kidneys in mice overexpressing KLF6 were more prone to develop fibrosis, while genes encoding BCAAs catabolic enzymes were remarkably downregulated [106]. Meanwhile, renal fibrosis was significantly mitigated in mice with a PT-specific knockdown of *Klf6* [106]. In addition, results from data mining of previous reports manifest that suppressed BCAAs catabolic enzymes were closely related to renal fibrosis in multiple rodent models and CKD patients [106]. Moreover, pyridostigmine ameliorated cardiac fibrosis in diabetic cardiomyopathy mice partly through lowered BCAAs concentrations in cardiac tissue and serum by upregulation of BCAT2 and PP2Cm, and a downregulation of the p-BCKDHA/BCKDHA and BCKD kinases, which improved the catabolism of BCAAs [114]. Furthermore, *Phellinus linteus* polysaccharide (PLP) extracts inhibited liver fibrosis in thioacetamide induced rat model through regulation of oxidative stress, heatshock, and metabolic pathways, in which BCKDHA showed higher expression in the PLP group [147].

5. Concluding remarks

Fibrosis results from imbalanced ECM homeostasis in response to chronic injuries and leads to progressive architectural remodeling of tissues and malfunction in multiple organs. In spite of the considerable morbidity and mortality of fibrotic diseases worldwide, there are currently no effective clinical tools to alleviate or reverse fibrosis. In recent years, correcting metabolic dysregulations has been increasingly recognized as a promising antifibrotic strategy, including targeting abnormal amino acids metabolism. BCAAs are an important class of essential and functional amino acids which play a crucial role in the regulation of substance and energy metabolism, and abnormalities in their metabolism have been shown to be associated with several diseases. Although controversial, abnormal changes in BCAAs metabolism have been identified in hepatic, renal, and cardiac fibrosis, and monitoring changes in plasma BCAAs levels has the potential to diagnose the degree of liver fibrosis associated with the HCC progression. Preclinical studies on the intervention of BCAAs in the treatment of fibrosis have been thriving, especially in liver fibrosis. BCAAs supplementation significantly ameliorated liver fibrosis in rodent models, but exhibited

preliminary deleterious effects in renal and cardiac fibrosis. Specifically, BCAAs treatment has been shown to improve liver fibrosis through mTORC1-dependent inhibition of TGF-β signaling in HSCs, less oxidative stress, and improved insulin resistance. However, in patients with liver fibrosis, BCAAs can only be effective when combined with other treatment, which reflects the complexity of the mechanism of fibrosis. So far, studies on BCAAs metabolism in fibrosis are still preliminary, and the conclusions are not fully consistent. Future research should pay more attention to the enzymes related to BCAAs metabolism in effector cells that play a key role during fibrogenesis, providing potential targets for fibrosis therapy.

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Author's contributions

TW and MW designed this review and drafted the manuscript. TW performed the figures. XZ, SR and HX confirmed the topic, critically instructed the writing and furtherly revised the manuscript. FN, SZ and XH were responsible for revising and polishing the manuscript. All authors read and approved the submitted version of the manuscript.

Declaration of Competing Interest

There are no financial or other interests with regard to the submitted manuscript that might be construed as a conflict of interest.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2022.106604.

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Branched-Chain Amino Acids as Pharmacological Nutrients in Chronic Liver Disease

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Branched-chain amino acids (BCAAs) are a group of essential amino acids comprising valine, leucine, and isoleucine. A low ratio of plasma BCAAs to aromatic amino acids is a physiological hallmark of liver cirrhosis, and BCAA supplementation was originally devised with the intention of normalizing amino acid profiles and nutritional status. However, recent studies on BCAAs have revealed that, in addition to their role as protein constituents, they may have a role as pharmacological nutrients for patients with chronic liver disease. Large-scale, multicenter, randomized, double-blinded, controlled trials on BCAA supplementation have been performed in Italy and Japan, and results demonstrate that BCAA supplementation improves not only nutritional status, but also prognosis and quality of life in patients with liver cirrhosis. Moreover, accumulating experimental evidence suggests that the favorable effects of BCAA supplementation on prognosis may be supported by unforeseen pharmacological actions of BCAAs. This review summarizes the possible effects of BCAAs on albumin synthesis and insulin resistance from clinical and basic viewpoints. We also review the newly discovered clinical impact of BCAAs on hepatocellular carcinoma and the prognosis and quality of life of patients with liver cirrhosis. (HEPATOLOGY 2011;54:1063-1070)

The liver is a central organ for regulating metabolism, and a variety of metabolic disorders are frequently seen in patients with chronic liver disease.[1,2] Decreased serum ratio of branched-chain amino acids (BCAAs) to aromatic amino acids (AAAs) is a hallmark of liver cirrhosis and is caused by several factors, including reduced nutritional intake, hypermetabolism, and ammonia detoxification in skeletal muscle.[3] Low serum BCAA/AAA ratio reduces biosynthesis and secretion of albumin in hepatocytes,[4] and is also associated with the prognosis of patients with chronic liver disease.[5]

BCAAs have aliphatic side chains with a branch point, and comprise valine (Val), leucine (Leu), and isoleucine (Ile) (Fig. 1). BCAAs are not only a constituent of protein, but also a source of glutamate, which detoxifies ammonia by glutamine synthesis in skeletal muscle.[3] Clinical studies have demonstrated that intravenous administration of BCAA improves hepatic encephalopathy with hyperammonemia.[6] Although dairy products and vegetables contain high BCAA content, increased consumption of these foods does not affect plasma BCAA levels in patients with cirrhosis.[7] The guidelines of the American Society for Parenteral and Enteral Nutrition and the European Societies for Clinical Nutrition and Metabolism currently recommend BCAA supplementation only for patients with cirrhosis with chronic hepatic encephalopathy unresponsive to pharmacotherapy.[8,9] A series of subsequent clinical trials and *in vitro* and *in vivo* studies suggest the possibility of more expansive utility of BCAA supplementation in liver disease.

Abbreviations: BCAA, branched-chain amino acid; BCATm, mitochondrial BCAA aminotransferase; DC, dendritic cell; GLUT, glucose transporter; IGF, insulin-like growth factor; IL, interleukin; Ile, isoleucine; Leu, leucine; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; MSUD, maple syrup urine disease; mTOR, mammalian target of rapamycin; NK, natural killer; PI3K, phosphatidylinositol 3-kinase; QOL, quality of life; Val, valine.

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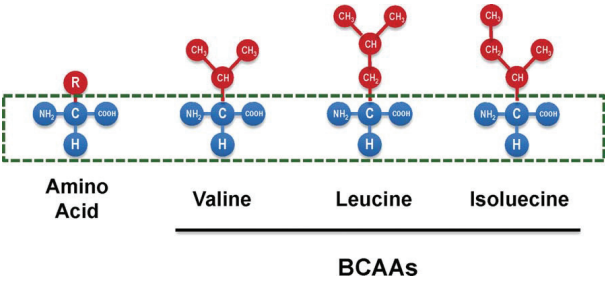

Fig. 1. Chemical structure of BCAAs. The dotted rectangle indicates the basic amino acid structure. The generic BCAA has an aliphatic side chain with a branch point. R, residue.

The liver carries out four main functions in protein metabolism: formation of plasma proteins, amino acid interconversion, deamination of amino acids, and urea synthesis (for ammonia excretion). Among the many other functions of the liver, it is responsible for the metabolism of hormones that have discordant effects on protein metabolism, including insulin, androgens, and glucagon. It is thus not surprising that cirrhosis is associated with altered circulating amino acid profiles, with decreased serum BCAA levels seen in patients even with compensated cirrhosis.[10] It is widely believed that the changes in amino acid metabolism not only occur as an epiphenomenon of liver disease but also play a role in the pathogenesis of many of the complications of cirrhosis, such as encephalopathy,[11] hypoalbuminemia with edema, and insulin resistance.[12-14] The potential of BCAA supplementation to alter the metabolic basis and frequency of complications of cirrhosis is suggested by studies indicating that BCAAs may inhibit hepatocarcinogenesis and improve immune function and oxidative stress *in vitro* and *in vivo*.[15-19] Clinical studies have further demonstrated that BCAA supplementation may improve the quality of life (QOL) and prognosis in patients with liver cirrhosis.[16,20,21]

Nutritional aspects of BCAAs on hepatic encephalopathy, liver regeneration, or hepatic cachexia have been well reviewed.[22,23] In this article, we review the recently identified pharmaceutical aspects of BCAAs on pathological conditions and complications associated with chronic liver disease from both the clinical and basic research viewpoints. We also summarize side effects of BCAA supplementation (Supporting Text).

Albumin Synthesis

BCAAs, particularly Leu, activate the mammalian target of rapamycin (mTOR) and subsequently up-regulates the downstream eukaryotic initiation factor 4E-binding protein-1 and 70-kDa ribosomal protein S6 kinase, which regulate messenger RNA (mRNA) translation and synthesis of albumin in cultured rat hepatocytes (Fig. 2).[4,12,24] Leu also stimulates the nuclear import of polypyrimidine-tract–binding protein, which binds to albumin mRNA and increases its translation in HepG2 cells (Fig. 2).[25] Consistent with these *in vitro* studies, BCAA supplementation has been found to activate the mTOR signaling cascade and increase albumin synthesis in animal models of cirrhosis.[26]

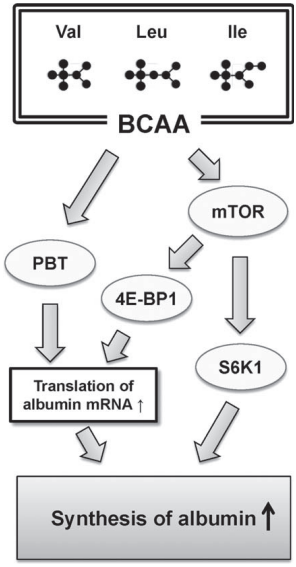

Fig. 2. Molecular mechanisms for BCAA-induced albumin synthesis. BCAA activates the mTOR and subsequently up-regulates the downstream molecules, eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) and 70-kDa ribosomal protein S6 kinase (S6K1), which regulate mRNA translation and synthesis, respectively. BCAAs also stimulate the nuclear import of polypyrimidine-tract-binding protein (PBT), which binds with albumin mRNA and increases albumin translation.

Muto et al. conducted a multicenter, randomized, controlled trial in which 622 patients with cirrhosis were administered BCAAs at 12 g/day for 2 years. In that study, serum albumin levels in the BCAA group were significantly higher than in the nutrient intake-matched control group.[16] However, in another randomized, controlled study by Marchesini et al., BCAA treatment did not result in a significant increase in serum albumin levels.[15] Although the reason for this discrepancy remains unclear, a possible explanation is the difference in the BCAA/AAA ratio among the participants in the two studies. Approximately 45% of enrolled patients were Child-Pugh class A in the former study,[16] whereas all the patients were Child-Pugh class B or C in the latter study.[15] The BCAA/AAA ratio decreases along with progression of liver cirrhosis.[27] Because the BCAA/AAA ratio is positively correlated with the synthesis and secretion of albumin,[4] and the response to BCAA treatment,[27] a low BCAA/AAA ratio may be a reason for the discrepancy in results between the studies. In addition, the majority of other randomized, controlled trials have demonstrated that BCAA supplementation results in a significant increase in serum albumin levels in patients with cirrhosis (Supporting Table 1). The aggregate of the evidence suggests that BCAA administration may increase serum albumin levels in patients with liver cirrhosis.

Insulin Resistance

BCAAs are thought to affect glucose metabolism.[28] Recently, She et al. knocked out the gene of mitochondrial BCAA aminotransferase (BCATm), which catalyzes the first step of BCAA catabolism, leading to a significant elevation in the serum BCAA level. In $BCATm^{-/-}$ mice, fasting blood glucose and fasting serum insulin levels were decreased by 33% and 67%, respectively, and the Homeostasis Model Assessment for Insulin Resistance index was significantly lower than that of wild-type mice.[14] Similarly, treatment with Leu or Ile has been reported to improve insulin sensitivity in mice fed a high-fat diet.[29,30]

Supplementation with BCAAs enhances glucose metabolism in skeletal muscle, adipose tissue, and liver; however, the molecular mechanisms in each organ are different. In skeletal muscle, BCAAs promote glucose uptake through activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase C and subsequent translocation of glucose transporter 1 (GLUT1) and GLUT4 to the plasma membrane (Fig. 3).[13,31] In adipose tissue, Leu enhances insulin-induced phosphorylation of Akt (protein kinase B) on Ser473 and Thr308

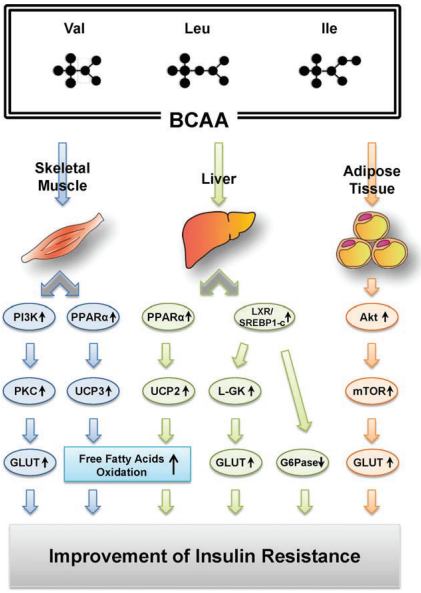

Fig. 3. Distinctive molecular pathway for BCAA-induced improvement of insulin resistance in insulin target organs. BCAAs improve glucose metabolism by acting on insulin target organs such as skeletal muscle, adipose tissue, and the liver. However, the molecular mechanisms in each organ differ. In the skeletal muscle, BCAAs promote glucose uptake through activation of PI3K and protein kinase C and subsequent translocation of GLUT1 and GLUT4 to the plasma membrane. In the adipose tissue, BCAAs, especially Leu, augment insulin-induced phosphorylation of Akt and mTOR, and consequently increase the glucose uptake. In the liver, BCAA activates the liver X receptor α (LXR)/sterol regulatory element binding protein-1c (SREBP1-c) pathway and subsequently up-regulates liver-type glucokinase (L-GK) and GLUT2. In addition, LXR/SREBP-1c activation suppresses hepatic expression of glucose-6-phosphatase (G6Pase), which catalyzes the final steps of gluconeogenesis. BCAAs also increase peroxisome proliferator-activated receptor (PPAR) α expression and subsequent uncoupling proteins 2 (UCP2) in liver and UCP3 in muscle. Up-regulation of UCP2 and UCP3 expression increases oxidation of free fatty acids and improves insulin resistance.

and mTOR on Ser2448, ultimately increasing glucose uptake (Fig. 3).[32] In the liver, BCAAs up-regulate the liver X receptor α (LXRα)/sterol regulatory element binding protein-1c (SREBP1c) pathway and subsequently activate liver-type glucokinase and GLUT2. In addition, BCAA suppresses hepatic expression of glucose-6-phosphatase, which catalyzes the final steps of gluconeogenesis (Fig. 3).[33] Recently, BCAA supplementation has been reported to improve insulin resistance by increasing oxidation of free fatty acids. BCAAs increase peroxisome proliferator-activated receptor α

expression and subsequent expression of uncoupling proteins 2 in liver and uncoupling proteins 3 in muscle (Fig. 3).[34,35] These recent studies have revealed distinct cross-talk mechanisms between BCAAs and the insulin signaling cascade in insulin target organs.

Previous clinical studies have reported that BCAA infusion decreases plasma glucose levels in patients with advanced liver cirrhosis.[36] Furthermore, oral BCAA supplementation reduces both blood glucose[37,38] and insulin resistance in patients with chronic liver disease.[18,39] However, these studies had small sample sizes and/or were lacking in adequate controls. A randomized, controlled trial is required to definitively evaluate the effects of BCAA supplementation on insulin resistance in cirrhosis.

Hepatocellular Carcinoma

Clinical studies have reported that long-term oral supplementation with BCAAs is associated with decreased frequency of development of hepatocellular carcinoma (HCC) and HCC recurrence after treatment with radiofrequency ablation in patients with cirrhosis.[17,40] Recent animal studies have also suggested an antihepatocarcinogenic activity of BCAAs.[41,42] Animals used in these studies were, however, obese diabetic mice with insulin resistance.[41,42] Because insulin resistance is closely linked to hepatocarcinogenesis,[43] it is possible that BCAAs may inhibit hepatocarcinogenesis through amelioration of insulin resistance. Indeed, suppression of hepatocarcinogenesis is accompanied with significant reduction in insulin resistance in BCAA-treated animals.[41,42] A randomized, controlled trial demonstrated that BCAA supplementation reduces the frequency of development of HCC, but the effect was only evident in patients with cirrhosis who are obese and have hepatitis C virus infection (approximately 30% reduction in the development of HCC in 3 years).[17] Because patients who are obese and infected with hepatitis C virus frequently have insulin resistance,[44,45] these findings also support the hypothesis that BCAAs suppress hepatocarcinogenesis through amelioration of insulin resistance.

Insulin is a carcinogenic factor with mitogenic and cell proliferative effects through activation of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase pathway.[46] Insulin also cross-reacts with insulin-like growth factor 1 (IGF-1) receptor and further activates the Raf/MAPK kinase/MAPK cascade.[47] Moreover, excess insulin binds to IGF-binding proteins, resulting in increased levels of free serum IGF-1 (Fig. 4).[48] Thus, insulin resistance/hyperinsulinemia enhances hepatocarcinogenesis through multiple pathways. Possible mechanisms for BCAA-induced inhibition of HCC development include: (1) BCAA activation of the insulin signaling cascade through up-regulation of PI3K[2,13,18] with reduction of serum insulin levels (Fig. 4) and (2) inhibition of the IGF/IGF-1R axis by suppressing the expressions of IGF-1, IGF-2, and IGF-1 receptor mRNA (Fig. 4).[41]

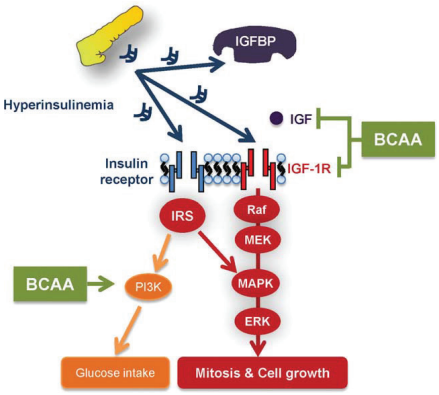

Fig. 4. Molecular mechanisms of the association between hyperinsulinemia and HCC and of BCAA-induced inhibition of hepatocarcinogenesis. As an adaptive response to insulin resistance, pancreatic beta cells secrete excess insulin. Insulin activates mitosis and cell growth through activation of the insulin receptor substrate (IRS)/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway. Insulin also cross-reacts with IGF-1 receptor (IGF-1R) and further activates the Raf/MAPK kinase (MEK)/MAPK cascade. Furthermore, excess insulin binds to IGF-binding proteins (IGFBP), resulting in increase in the level of free serum IGF-1. BCAA activates the insulin signaling cascade via up-regulation of PI3K and improves glucose uptake and reduces the serum insulin levels. BCAA also suppresses the IGF/IGF-1R axis through down-regulation of IGF-1, IGF-2, and IGF-1R mRNA expressions, leading to inhibition of mitosis and cell growth.

Besides activation of intracellular insulin and IGF-1 signaling cascade, insulin causes angiogenesis,[42] migration of HCC,[49] and epithelial mesenchymal transition of hepatocytes.[50] Because BCAAs reduce insulin resistance, BCAAs may suppress angiogenesis, migration, and epithelial mesenchymal transition of hepatocytes. BCAAs are also known to attenuate insulin resistance-induced expression of endothelial growth factor and eventually suppress hepatic neovascularization.[42] Thus, the diverse effects of BCAAs on insulin resistance may suppress hepatocarcinogenic activity.

In addition, BCAAs are reported to affect immune function *ex vivo* and *in vivo* studies (Supporting Table

2). In patients with cirrhosis, BCAAs increase liver-associated lymphocyte counts and restore phagocytic function of neutrophils and natural killer activity of lymphocytes.[51] Moreover, BCAA treatment may suppress hepatic oxidative stress by modulating the redox state of albumin.[52,53] Serum albumin is divided into two forms, reduced and oxidized albumin, depending on the redox state at Cys34,[54,55] and the oxidized/reduced albumin ratio increases in patients with cirrhosis.[56,57] BCAA supplementation increases ratio of reduced albumin[52] and decreases iron-related oxidative stress in patients with cirrhosis,[53] suggesting that BCAAs may reduce the iron-induced oxidative stress through a qualitative alteration of serum albumin. Thus, BCAAs may suppress hepatocarcinogenesis partly by improvement of immune function and reduction of oxidative stress.

Mortality and Clinical Decompensation

Some reports suggest that oral BCAA supplementation improves survival in a rat model of cirrhosis and in decompensated patients with cirrhosis.[58-60] Marchesini et al. first performed a randomized, controlled trial exploring the usefulness of BCAAs in patients with cirrhosis.[15] One year of BCAA treatment significantly reduced the occurrence of the primary outcome (a composite of death, number of hospital admissions, and duration of hospital stay) compared to that in the lactalbumin-treated group.[15] Although this study shows the effectiveness of BCAA supplementation, the complications that contributed to the reduction of outcome incidence was not identified because of a small number of enrolled patients (n = 59 in BCAA group) and high dropout rate (15% in the BCAA group) due to poor compliance with the BCAA supplement.

Since 1996, a BCAA supplement formulation (L-Val:L-Leu:L-Ile = 1.2:2:1; Ajinomoto Pharmaceuticals, Tokyo, Japan) has been approved for use in cirrhosis in Japan. The supplement is in the form of small uniform granules, which reduces BCAA-induced stimulation of taste buds and contributes to improved compliance. Using these BCAA granules, Muto et al. performed a large (n = 314 in the BCAA group) randomized, controlled trial.[16] None of the patients discontinued the study because of poor compliance. A preplanned safety analysis revealed that BCAA granules significantly reduced the occurrence of the overall primary outcome (hepatic failure, variceal bleeding, development of liver cancer, and death from any cause) compared to that in the control diet group. Among individual events of primary outcome, the occurrence of hepatic failure was significantly less in the BCAA group compared to the control diet group (hazard ratio 0.45; 95% confidence interval 0.23-0.88; $P = 0.016$). On the basis of the results, the Data and Safety Monitoring Board concluded that the harm associated with the increased occurrence of primary outcome in the control diet group outweigh any potential benefits and the study was discontinued 10 months early due to safety concerns. Beneficial effects of BCAAs on clinical decompensation, including development of hepatic failure, are also reported in patients with cirrhosis accompanied with HCC.[61-63] Thus, the treatment with BCAA supplementation is now recommended in the guidelines for the treatment of liver cirrhosis by the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis from the Ministry of Health, Labour and Welfare of Japan.[64]

Quality of Life

Generally, the overall health status and QOL of patients with liver cirrhosis is poor.[65,66] Patients with cirrhosis frequently complain of fatigue and sleep disturbances. There is, however, no standard approach to the management of these symptoms in the absence of overt hepatic encephalopathy.[67] In a randomized study, BCAA-enriched supplements have been reported to improve weakness and easy fatigability compared to ordinary food.[20] BCAA-enriched supplementation has also been reported to improve the Epworth Sleepiness Scale score.[21] In large-scale randomized controlled trials, BCAA supplementation was found to significantly improve the Short Form-36 scores of general health perception compared to control groups.[15,16]

Although it is still unclear how BCAA supplementation provides relief from fatigue and sleep disturbances in patients with cirrhosis, there are at least three possible mechanisms. First, fatigue and sleep disturbances could be caused by minimal hepatic encephalopathy, and BCAA may ameliorate these symptoms by improving this condition.[68] Second, increased serum tryptophan levels are known to impair the QOL in various conditions involving malnourishment, including liver cirrhosis.[69] Tryptophan is a precursor for the neurotransmitter 5-hydroxytryptamine, which is associated with fatigue and sleep disturbances.[70] Because BCAAs compete with tryptophan for transport into the brain, these symptoms may be alleviated by supplementation with BCAAs.[71] Third, impaired cerebral blood flow is associated with fatigue and sleep disturbance[72] and is decreased in patients with liver cirrhosis.[73,74] BCAA supplementation is known to improve cerebral blood flow, possibly resulting in lessened fatigue and sleep disturbances.[75,76]

Muscle cramps are also associated with poor QOL in patients with liver cirrhosis,[77] and the frequency of muscle cramps has been reported to be dramatically reduced by BCAA supplementation over a period of 3 months (7.4 ± 2.0 versus 0.3 ± 0.5 times/week).[78] Muscle cramps are caused by a variety of factors, including diuretic treatment, reduction of circulating volume, and deficiency of vitamin E and taurine.[79] Amino acid imbalance decreases taurine production, and therefore, BCAA may inhibit muscle cramps, possibly through improvement of the imbalance and consequent restoration of taurine production.[78,79]

Conclusion

In this article, we have reviewed evidence for potential pharmaceutical properties of BCAAs on various physiological and clinical events associated with chronic liver disease. Evidence for beneficial effects of BCAA supplementation has yet to be fully validated, and improvement for low compliance of BCAA supplementation is still required. However, there is substantial evidence that depletion of serum BCAA levels is involved in the progression of liver disease and the development of clinically important sequelae. Pharmacological supplementation with BCAAs may be a promising therapeutic strategy for patients with liver cirrhosis.

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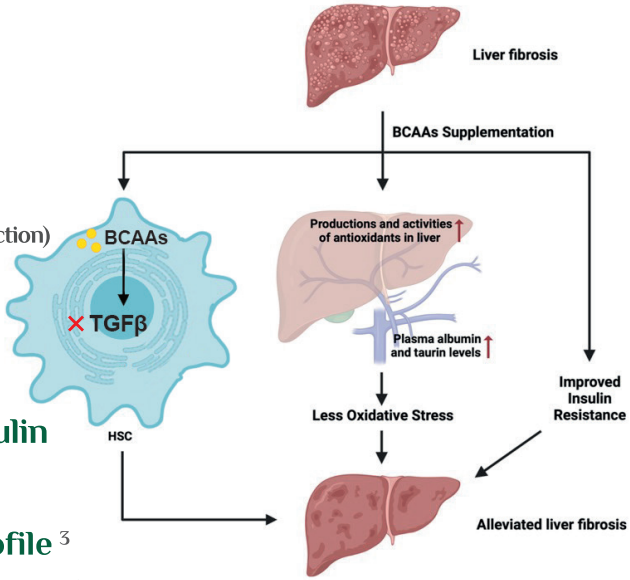

Dose: One tablet daily

to be taken with the evening meal

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