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Title: Targeting the muscle for the treatment and prevention of hepatic encephalopathy

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Muscle mass loss or sarcopenia is a principle component of malnutrition which prevails in 65-90% of patients with end-stage liver disease [1]. Intuitively, the roots of malnutrition play a precipitating role in muscle catabolism. Undernutrition frequently occurs in cirrhosis since an inadequate diet is compounded by a hypermetabolic energy demand. However, multiple other factors contribute to the pathogenesis of malnutrition including malabsorption of nutrients, metabolic alterations, increased intestinal protein losses, reduced protein synthesis, increased protein catabolism and disturbance of substrate utilization [2,3]. Sarcopenia adversely affects quality of life, leads to longer hospitalizations, increases susceptibility to infections, negatively impacts clinical outcomes pre- and post-liver transplantation and is an independent prognostic factor for survival in patients with cirrhosis [4]. The pathophysiological pathways triggering a reduction in muscle protein synthesis and/or an increase in proteolysis, resulting in loss of muscle mass, remain elusive. Furthermore, in addition to loss of quantity, quality of muscle is also affected during muscle wasting. Altered muscle metabolism and contractile function, concomitant with a reduction in muscle mass, contributes to the onset of frailty as well as functional decline in physical performance, leading to increased morbidity in patients with cirrhosis [5].

The liver is a powerful metabolic organ which in addition to being the center for the metabolism of nutrients (glucose and lipids), also plays a major role in ammonia disposal. Exclusively expressing all the enzymes of the urea cycle, the liver regulates the circulating blood ammonia levels arising from the gut (primary source of ammonia generation). Thus, severe liver impairment, leads to the occurrence of hyperammonemia. At physiological pH, the majority (98%) of ammonia is found in ionic form (NH_4^+) with ~ 2% arising in gas form (NH_3). Both forms are capable of crossing cellular membranes. NH_3 via diffusion and NH_4^+ via K^+-channels and cotransporters since NH_4^+ has very similar ionic properties (ionic radius and diffusion coefficient) to K^+. In addition, specific ammonia transporters have also been identified [6]. Subsequently, following its concentration gradient, ammonia is dispersed throughout the

body, entering all cells, organs and tissues. Elevated concentrations of ammonia (including increased flux (uptake) of ammonia across cell membranes) lead to changes in pH, adjusted membrane potential and altered cell metabolism, which independently and/or collectively lead to a cascade of pathophysiological events [7]. Since ammonia easily crosses the blood-brain barrier, blood-derived ammonia leads to neurotoxic levels of ammonia in the brain which is a fundamental component implicated in the pathogenesis of hepatic encephalopathy (HE).

Aside from primarily affecting the brain, the toxicity of ammonia has also demonstrated to affect other organs and tissues, including muscle. Muscle plays a significant compensatory role in detoxifying ammonia during liver disease since it houses the enzyme glutamine synthetase (GS), an important ammonia removing pathway during the amidation of glutamate to glutamine. Therefore, in the setting of liver disease, reduced capacity to remove ammonia in the liver, aggregated with muscle mass depletion, further reduces the body's capacity to clear ammonia which in turn leads to a higher risk of developing hyperammonemia and HE [8]. However, paradoxically, within the last 5 years, Dasarathy and colleagues have provided solid evidence that elevated levels of ammonia cause detrimental effects to the muscle. It has been shown that elevated ammonia i) upregulates myostatin (an autocrine growth inhibitor) in myotubes through a NF-κB-dependent pathway [9], ii) stimulates muscle autophagy [10] and iii) impairs skeletal muscle contractility and strength [11]. In the recent issue (JHEPAT-D-15-02306R2), Davuluri and colleagues, using human tissue as well as *in vitro* and *in vivo* models of hyperammonemia (including knockout and knockdown of a number of molecular targets), elegantly identified the molecular pathways implicated in the inhibition of muscle protein synthesis in patients with cirrhosis and hyperammonemia [12]. The authors found that ammonia activated the general control nonderepressible 2 (GCN2) kinase (amino acid deficiency sensor) which inactivated the eukaryotic initiation factor 2 (eIF2α) (via phosphorylation of the α subunit) and additionally inactivated mTORC1, resulting in global repression of mRNA translation and hence protein synthesis in the

skeletal muscle. Under physiological conditions, phosphorylation of eIF2α is followed by an adaptive integrated stress response (ISR) that is mediated via upregulation of activating transcription factor 4 (ATF4) which through downstream signalling pathways leads to the reversible dephosphorylation of phospho-eIF2α. Here, authors demonstrated that loss of feedback negative loop of ISR during hyperammonemia, as evidenced by failure in ATF4 induction, results in chronic and persistent low protein synthesis. Since the effects of increased ammonia (pH, membrane potential and metabolism) touch all cells within the body and therefore, are not specific to the brain, the importance of hyperammonemia and its impact on clinical outcomes merits to be thoroughly investigated.

Davuluri and colleagues also demonstrated in this issue (JHEPAT-D-15-02306R2) the therapeutic benefit of L-leucine on muscle protein synthesis that was due to the metabolic adaptation of muscle to hyperammonemia. Leucine, a branched-chain amino acid (BCAA), is capable of activating mTORC1 and activating protein synthesis [13]. Furthermore, the authors demonstrated that L-leucine reversed GCN2-eIF2α phosphorylation, removing protein synthesis inhibition. The authors determined that leucine "starvation", provoked by ammonia, resulted in a compensatory upregulation of the leucine transporter (leucine/glutamine exchanger (SLC7A5)) and therefore leucine supplementation rescued the inhibition of protein synthesis.

Davaluri and colleagues administered 15g of leucine-enriched BCAA (7.5g L-leucine, 3.75g L-isoleucine and 3.75g L-valine) to 6 hyperammonemic patients with cirrhosis and demonstrated a reversal in muscle protein synthesis inhibition. However, muscle mass (quantity or quality) was not evaluated, most likely due to short treatment time of 7 hours. A supporting study by Les et al., 2011 demonstrated leucine-enriched BCAAs (13.5g L-leucine, 9g L-isoleucine and 7.5g L-valine) given as a nutritional supplementation for 56 weeks lead to an improvement in muscle mass (mid-arm muscle circumference) in 46 patients with cirrhosis [14]. To date, only one study has tested the independent effect of L-leucine and, following 10g/day supplementation for 12 weeks to 9 patients with cirrhosis,

thigh circumference was not enhanced [15]. This study puts in question the beneficial effect of L-leucine on optimizing muscle mass. However, following the positive results of Davuluri and colleagues, a time-dependent, dose-response study evaluating the effect of L-leucine on muscle mass merits to be thoroughly conducted.

The beneficial effect of L-leucine on muscle protein synthesis and subsequently muscle wasting may also be a result of lowering ammonia. BCAAs are highly metabolized in the muscle and are capable of replenishing α-ketoglutarate, believed to be depleted during hyperammonemia as α-ketoglutarate is aminated to glutamate which subsequently is amidated to glutamine. These ammonia-removing pathways are stimulated during hyperammonemia in attempt to reduce ammonia. BCAAs have been reported to lower blood ammonia following 3 months of daily supplementation (0.24g/kg; 50% L-leucine, 25% isoleucine and 25% valine) in patients with cirrhosis which was associated with an improvement in HE [16]. The treatment time and dose appear to be vital as 3-hour administration of BCAAs (0.45g/kg; 45.5% L-leucine, 30% isoleucine and 24.5% valine) did not lower blood ammonia in patients with cirrhosis but did increase BCAA-derived ammonia clearance in muscle [17]. It remains to be defined whether the beneficial effect of L-leucine (BCAA) on muscle protein synthesis is due to a lowering of blood ammonia.

Patients with cirrhosis and sarcopenia have a higher risk of developing HE [8] as loss of muscle mass, in addition to liver impairment, further reduces the capacity of ammonia removal, leading to higher risk of hyperammonemia [18]. There is an invested interest in improving extra-hepatic ammonia clearance (targeting GS in the muscle) for the treatment of HE [19]. Both L-ornithine L-aspartate and L-ornithine phenylacetate have demonstrated to lower blood ammonia by generating glutamate and stimulating GS activity in the muscle [20,21]. However, L-ornithine phenylacetate may be more efficient by chelating glutamine released during muscle catabolism and preventing ammonia generation through glutaminase activity. Overall, increasing the muscle's capacity to clear ammonia by either stimulating GS activity and/or optimizing muscle mass are attractive strategies for the treatment and

prevention of HE. A recent meta-analysis revealed that BCAAs have a beneficial effect on HE [22]; however the precise mechanisms remain unclear. Interestingly, exercise has been shown to be advantageous in optimizing muscle mass in patients with liver disease [23] and furthermore, in combination with L-leucine supplementation, exercise demonstrated to a have greater impact of muscle mass which was associated with an improvement in minimal HE compared to L-leucine alone [15]. The independent effect of exercise on HE has not been evaluated.

In conclusion, the integrated relationship between muscle (mass and activity of GS), ammonia and cognitive function remains to be comprehensively investigated. Studies evaluating the beneficial effect of improving muscle mass on ammonia clearance and the treatment and prevention of HE are lacking. The data presented by Davuluri and colleagues highlights the important contribution of hyperammonemia on the inhibition of muscle protein synthesis and muscle mass wasting in cirrhosis. This depicts the toxicity of ammonia lies beyond the brain and provides compelling evidence that long-term treatment of hyperammonemia can be beneficial for the muscle and the brain in cirrhosis, improving clinical outcomes pre- and post-liver transplantation.

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