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Perspective

Endogenous Metabolic Modulators: Emerging Therapeutic Potential of Amino Acids

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SUMMARY

Multifactorial disease pathophysiology is complex and incompletely addressed by existing targeted pharmacotherapies. Amino acids (AAs) and related metabolites and precursors are a class of endogenous metabolic modulators (EMMs) that have diverse biological functions and, thus, have been explored for decades as potential multifactorial disease treatments. Here, we review the literature on this class of EMMs in disease treatment, with a focus on the emerging clinical studies on AAs and related metabolites and precursors as single- and combination-agents targeted to a single biology. These clinical research insights, in addition to increasing understanding of disease metabolic profiles and combinatorial therapeutic design principles, highlight an opportunity to develop EMM compositions with AAs and related metabolites and precursors to target multifactorial disease biology. EMM compositions are uniquely designed to enable a comprehensive approach, with potential to simultaneously and safely target pathways underlying multifactorial diseases and to regulate biological processes that promote overall health.

INTRODUCTION

Multifactorial diseases have many underlying aspects, causing the identification of successful treatments to be challenging. Patients with multifactorial diseases may be required to take several medications, increasing the risk of drug-drug interactions and unrelated adverse effects. Amino acids (AAs) and related molecules often serve as regulators of metabolism in disease. Thus, the assessment of an individual's metabolic activity can be used for disease prognosis and treatment. There is extensive clinical research with many AAs and related molecules in both health and disease modification, which also informs on precedence of safe use. Advances in network biology have created the potential to design disease-specific endogenous metabolic modulator (EMM) compositions to safely address multiple aspects of multifactorial diseases with a single therapy.

MULTIFACTORIAL DISEASES BENEFIT FROM MULTITARGETED INTERVENTION

Single-target pharmaceutical approaches have incompletely addressed the need for treatments of multifactorial disease, such as with management of liver abnormalities associated with nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) (Friedman et al., 2018; Patel et al., 2017; Oliveira et al., 2019; Safadi et al., 2014). In addition to lifestyle modifications, the treatment of diseases such as NASH or type 2 diabetes mellitus frequently requires polypharmacy, which is the use of multiple medications to treat an individual's health condition (Patel et al., 2017). Polypharmacy, which is particularly common among the elderly, often increases the risk of drug-drug interactions and reduces medication adherence (Patel et al., 2017; Qato et al., 2016). Therefore, there remains a need for multifactorial therapeutic options that can safely address multiple disease pathways.

EMMs are a broad set of molecular families that include AAs, fatty acids, and other lipids, bile acids, ketone bodies, hormones, and other molecules. In health, EMMs drive a myriad of biological pathways to maintain optimal functioning across multiple fundamental biologies. In disease, EMMs have the potential to correct disrupted human metabolism by acting on a variety of disease nodes and metabolic pathways. A class of EMMs includes AAs and related metabolites like creatine, a nitrogenous organic acid involved in energy

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transfer (Taegtmeyer and Ingwall, 2013) and precursors such as N-acetyl cysteine (NAC), which is directly converted to cysteine after oral administration (Zafarullah et al., 2003). AAs and related metabolites and precursors play a central role in metabolic regulation and homeostasis (Gaggini et al., 2018; Kinny-Koster et al., 2016; Tarlungeanu et al., 2016). With the appropriate combinations of these EMMs, targeted modification of multifactorial diseases may be achieved. Thus, EMMs administered in specific combinations as interventions may be a viable alternative to polypharmacy with existing therapeutic options.

Here, we describe the emerging concept of EMM compositions, focusing on combinations of AAs and related metabolites and precursors in formulations designed for oral administration. EMM compositions can be specifically designed to have multitargeted effects on multifactorial pathways in health and disease. The foundation for designing EMM compositions for therapeutic use involves a rigorous and unbiased compilation of knowledge platforms, including structural, functional, and safety data from EMM clinical studies, cell-based research, disease metabolite analysis, and advancements in combination therapeutic design. Together, these insights can be combined to create multitargeted, innovative EMM compositions with the potential to impact afflicted biologies in multifactorial diseases.

AAS: PART OF A KEY CLASS OF EMMS

Most familiar as the building blocks of protein, AAs play multifaceted roles as substrates and regulators of metabolism (Broer and Broer, 2017; Efeyan et al., 2015). Arginine, for example, is metabolized by four enzymatic pathways into a variety of biochemically diverse compounds, including urea, creatine, polyamines, agmatine, and nitric oxide (Morris, 2016). As another example, based on a comprehensive whole-body metabolic analysis, branched-chain AAs (BCAAs) have been shown to be metabolized to form ketoacids, tricarboxylic acid cycle intermediates, substrates, and bioactive metabolites like beta-aminoisobutyric acid and 3-hydroxyisobutyrate (Neinast et al., 2019).

We analyzed existing biological interaction networks to comprehensively demonstrate the widespread connectivity of AAs in human physiology. Human metabolic network data from Recon3D showed AA connectivity to a broad range of pathways and metabolites (Figure 1). Our analysis revealed that AAs are directly connected to 39 diverse subsystems and pathways, each with unique metabolic features (Figure 1A). We confirmed the vast AA involvement in fundamental cellular processes, including protein synthesis, biomass regulation, and transport mechanisms that also involve symporter and antiporter molecules. AAs have tightly interconnected metabolic pathways, as is demonstrated by connections within groups of AAs: glycine, serine, alanine, and threonine; cysteine and methionine; and valine, leucine, and isoleucine. Beyond the canonical AA systems, AAs are also directly involved in several distinct metabolic pathways, like fatty acid oxidation, metabolism of glutathione, nicotinamide adenine dinucleotide, and sphingolipids. Through these 39 subsystems, AAs are connected to 1,393 metabolites within three chemical reactions (Figures 1B and 1C). First-degree metabolites are directly involved in AA reactions as central metabolites (Figure 1B). AA connections specifically related to the top five first-degree metabolites (NADPH, ATP, NADP+, ADP, and NAD+) are both structurally and functionally linked in the modulation of core downstream metabolic pathways. Significant regulation of human metabolites within a small number of reactions emphasizes the ubiquitous roles of AAs. The network biology approach demonstrated by this analysis is built from decades of AA metabolism research. The interconnected pathways of AA physiology may be implicated in multifactorial disease biology and provide the basis for novel disease-specific therapeutic applications of EMMs.

A well-established example of AA signaling is in nutrient sensing through mammalian target of rapamycin complex 1 (mTORC1), a master regulator of cell growth and metabolism. Leucine and arginine activate mTORC1 via complex signaling (Wolfson et al., 2016; Chantranupong et al., 2016). When AAs are replete, active mTORC1 promotes protein synthesis and reduces autophagy (Broer and Broer, 2017). In contrast, when AA levels are low, kinase general control nondepressible 2 (GCN2) is activated to inhibit translation in anticipation of inefficient protein synthesis (Efeyan et al., 2015). Other examples include tryptophan and phenylalanine regulation of glucose homeostasis via activation of a G protein-coupled receptor, GPR142 (Wang et al., 2016; Husted et al., 2017), and glutamate activation of heterotrimeric taste receptors (T1Rs) to mediate mTORC1 signaling (Husted et al., 2017; Wauson et al., 2013). Through these direct signaling pathways and many others, AAs can impact systemic homeostatic pathways, via mediators such as fibroblast growth factor 21 (FGF21), peroxisome proliferator-activated receptor-α (PPAR-α), and AMP-activated protein kinase (AMPK) (Broer and Broer, 2017; Laeger et al., 2014; Dalle Pezze et al., 2016). In addition, there

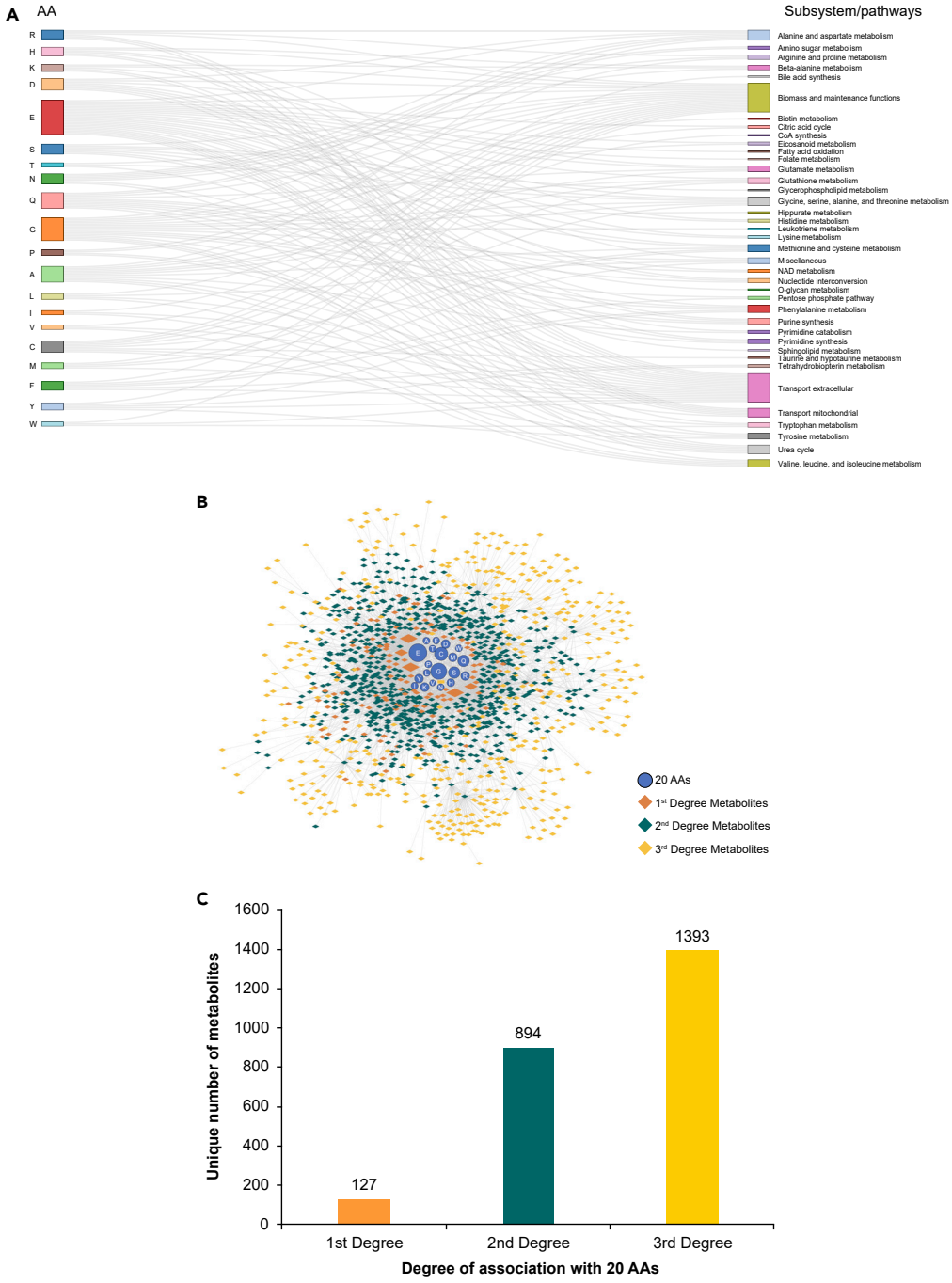

Figure 1. Network Depiction of AA Interactions Built From Recon3D

(A) Human metabolic network data from Recon3D. AAs in the network are shown on the left, and subsystems/pathways are shown on the right. The height of each box on the left representing each AA corresponds to the number of subsystems/pathways in which they are directly involved. The height of each box to the right represents each subsystem/pathway and corresponds to the number of connected AAs.

(B) Human metabolic map highlighting AA connectivity to metabolites using Recon3D. Nodes correspond to reactants and products in Recon3D and are connected by edges (reactions) to construct a network graph. AAs are shown as blue nodes, and the first-degree metabolites, shown in orange, are directly involved in reactions with AAs. The sizes of the 20 AAs indicate the total number of first-degree metabolites associated with each AA, with the top five first-degree metabolites increased in size to reflect their connectivity. Second- and third-degree metabolites are two or three reaction edges away from AAs, respectively. For visualization purposes, seven highly structurally connected metabolite hubs (H^+,

Figure 1. *Continued*
H_2O, CO_2, phosphate, O_2, H_2O_2, O_7P_2) and transport subsystems were removed on the basis of a lack of significant functional connection within the network.
(C) Distinct metabolites connected to AAs from first-, second-, and third-degree reactions.
A, alanine; AA, amino acid; CoA, coenzyme A; C, cysteine; D, aspartate; E, glutamate; EMM, endogenous metabolic modulator; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; NAD, nicotinamide adenine dinucleotide; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

is an emerging role of AA metabolism in the human gut microbiome through production of AA metabolites with local and systemic effects on the host (Dodd et al., 2017; Nicholson et al., 2012; Oliphant and Allen-Vercoe, 2019). Thus, AAs are systemic, ubiquitous, and essential.

METABOLOMICS AND DISEASE METABOLITE ANALYSIS MOTIVATE EMM INTERVENTIONS

Metabolomics is the large-scale study of small molecules (i.e., metabolites) within cells, biofluids, tissues, or organisms and their interactions within a biological system, known as the metabolome. Once thought to be limited to biomarker discovery and detection, metabolomics is now being used to identify metabolites that are active drivers of biological processes and potential interventions (Guijas et al., 2018; Rinschen et al., 2019). Within the metabolomics field, metabolite analysis allows for the classification of individuals into subgroups based on combinations of specific metabolites that make up an individual's metabolic profile (Hillesheim and Brennan, 2019). In multifactorial diseases, perturbations in more than one metabolite are common, aligned with the multifactorial nature of disease and potential for intervention with EMMs toward these perturbations. Disease metabolite analysis is useful to characterize dysregulated disease nodes. Thus, disease metabolite analysis is foundational to multitargeted EMM composition design.

Both NAFLD and cirrhosis have well-established disease metabolic profiles that have been the basis for single-agent AA interventions. In NAFLD, glutamate-serine-glycine index (plasma concentration of glutamate/[serine + glycine]) changes were correlated with hepatic insulin resistance and fibrosis levels (Gaggini et al., 2018). Another example of metabolic profiling in NAFLD/NASH is defined by elevated plasma BCAA concentrations in patients with type 2 diabetes, NAFLD, or obesity (Iwasa et al., 2015). Elevated BCAAs may represent an adaptive physiologic response to hepatic stress in patients with NAFLD that involves protein breakdown and BCAA catabolism downregulation (Lake et al., 2015). The Fischer ratio (BCAA/aromatic AA) was identified as a late-stage liver disease metabolic profile in the 1970s (Campollo et al., 1992; Fischer et al., 1974). More recently, a reduced Fischer ratio has been associated with advanced liver disease among patients with cirrhosis and hepatic encephalopathy (HE) and with mortality prediction in patients with end-stage liver disease (Fischer et al., 1974; Campollo et al., 1992; Kinny-Koster et al., 2016). Disease metabolic profiling studies point to EMMs that have been tested as clinical interventions, reinforcing the use of metabolic profiling as a potential tool to identify EMMs as interventions to target multifactorial diseases (Lake et al., 2015; Iwasa et al., 2015; Fischer et al., 1974; Campollo et al., 1992; Kinny-Koster et al., 2016).

AA CLINICAL TRIALS: INFORMATION MINED FROM PAST INTERVENTIONS PROPELS FUTURE INSIGHTS

In the past decade, interest has surged in AAs as potential interventions to address disease or support health. To understand the breadth of foundational clinical information on EMMs, we conducted a systematic analysis of AAs and related metabolites and precursors in studies registered on clinicaltrials.gov between 2009 and 2019 (Figure 2). We identified 1,171 trials that studied EMMs as potential interventions, with an average of 104 trials initiated per year, which varied in length, number, and scope (Figure 2A). During this 10-year span, trials evaluating EMMs referenced a total of 687 unique Medical Subject Headings (MeSH) terms (Figure 2A), which are controlled vocabulary terms established by the National Library of Medicine and recommended as keywords to describe trials registered on clinicaltrials.gov. As the MeSH terms represent standardized vocabulary, there is to be an expected decrease in novel MeSH terms over time. However, there were a number of novel MeSH terms introduced each year, with 34 new MeSH terms referenced in 2019, demonstrating a growing number of potential applications for the study of EMMs (Figure 2B). Of the 25 most commonly referenced MeSH terms in these clinical trials, most were related to nervous system (31%) or metabolic (14%) disease-related terms, whereas 15% were related to general categories like "syndrome" or "disease" (Figure 2C). The number of EMMs studied over time

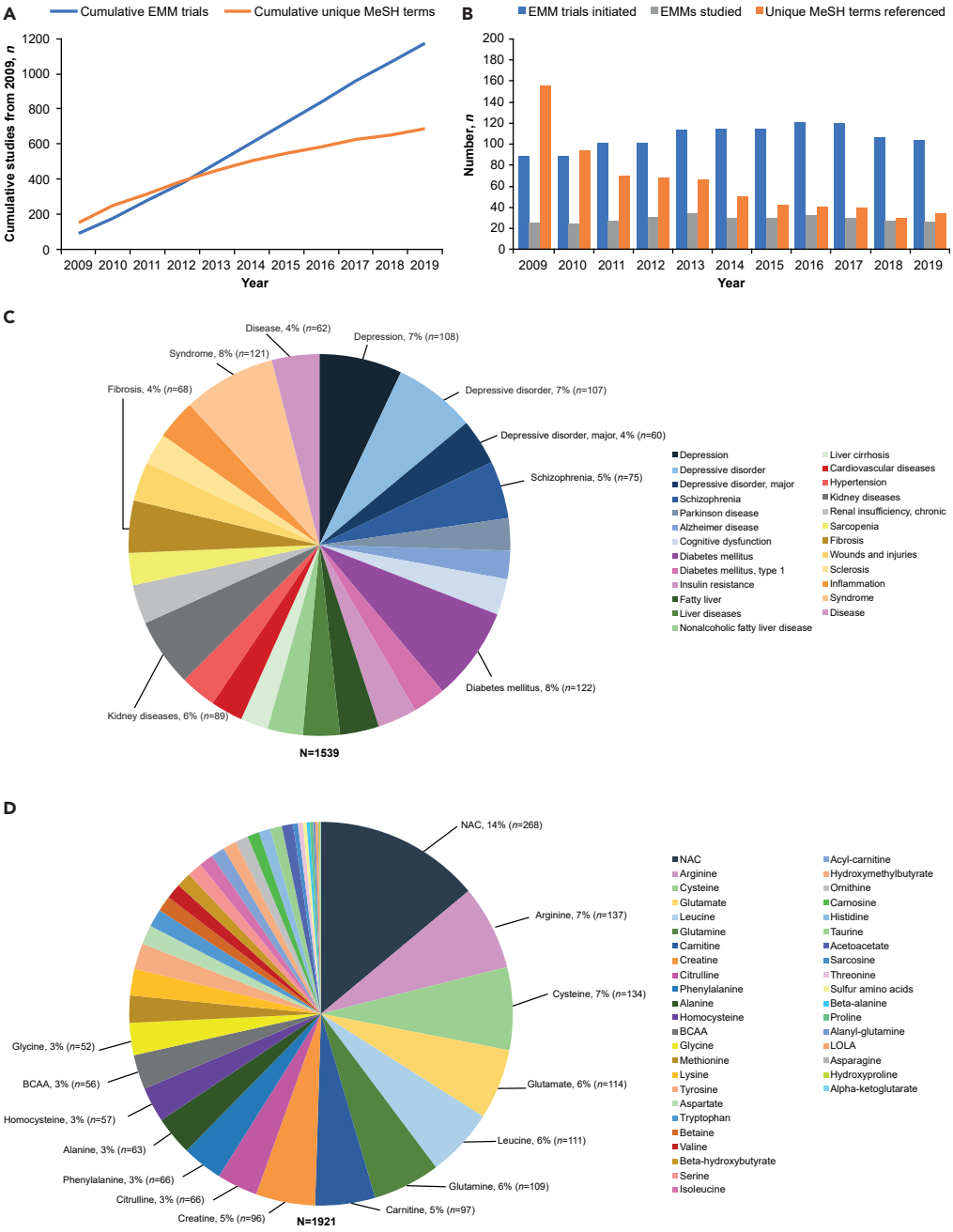

Figure 2. Clinical Trials of AAs and Related Molecules Registered in Clinicaltrials.gov Between 2009 and 2019

We used natural-language processing to create a rank-scoring algorithm for clinicaltrials.gov, identifying relevant trials based on AAs and related metabolites and precursors of interest. Scores are calculated for each trial based on the terms of interest and their frequency. We used a hierarchical dictionary of inclusion and exclusion terms to rank order all trials and used a scoring threshold to identify high-scoring relevant trials. Inclusion criteria terms included specific EMM terms like alanine and glutamate. Exclusion criteria included terms that may have produced false-positive results, such as carbamylglutamate or norleucine.

(A) Cumulative data for unique EMM trials and referenced MeSH terms each year.

(B) The number of EMM trials initiated, EMMs studied, and unique MeSH terms referenced in studies from 2009 to 2019.

(C) The 25 most commonly referenced MeSH terms related to the primary disease or condition being studied between 2009 and 2019. The legend is organized so that the MeSH terms are grouped by organ system or disease type, with nervous system-related terms shown first and metabolic-related terms shown second. The most common categories (≥4%) are labeled.

Figure 2. *Continued*
(D) EMMs reported in clinical trials between 2009 and 2019. The legend is organized in order of frequency, and EMMs with ≥3% frequency are labeled.
BCAA, branched-chain amino acid; EMM, endogenous metabolic modulator; HIV/AIDS, human immunodeficiency virus/acquired immunodeficiency syndrome; LOLA, L-ornithine and L-aspartate; NAC, N-acetyl cysteine; NAFLD, nonalcoholic fatty liver disease.

remained consistent each year, with an overall average of 28 (Figure 2B). In all, 41 EMMs were evaluated as potential therapeutic interventions, with NAC (268 trials) and arginine (137 trials) being the most frequently studied EMMs (Figure 2D).

The studies from clinicaltrials.gov alone contain a wealth of information that may inform the design of EMM compositions. For example, many of these trials measure the effects of EMMs on therapeutically relevant biomarkers (i.e., liver enzymes, plasma ammonia concentration) and structural measures (i.e., liver steatosis, muscle mass) related to chronic liver disease and associated complications. In addition, the range of doses tested for each EMM across clinical studies can help establish the precedent of safe and tolerable use across a number of populations and diseases. Below, we summarize recent examples of clinical studies on single and combination AA interventions in multifactorial diseases.

Single AA Interventions

AAs have been most commonly evaluated with single AA interventions in clinical trials. In NASH, there are several examples of translational research that have established a range of EMM-specific biological activities in disease pathogenesis and management (Chakravarthy et al., 2020b). Clinically, serine administration (two weeks) improved liver function markers and resulted in a decrease in hepatic steatosis ranging from 1% to 23% in patients with NAFLD (n = 6) (NCT02599038) (Mardinoglu et al., 2017). In a phase 2b placebo-controlled trial in children with NAFLD (n = 88), cysteamine, a cysteine metabolism-related molecule, significantly reduced serum aminotransferase and lobular inflammation (NCT01529268) (Schwimmer et al., 2016). L-carnitine supplementation for 24 weeks had several downstream effects, including reduced liver enzymes, inflammatory markers, and blood glucose and improved lipid profiles, insulin sensitivity, and hepatic tissue histology versus placebo in patients with NASH (n = 36) (Malaguarnera et al., 2010). L-carnitine administration for more than six months significantly attenuated skeletal muscle loss in patients with cirrhosis (n = 35), when compared with propensity score-matched controls (Ohara et al., 2018). Arginine supplementation for 21 days showed downstream improvements on glucose homeostasis, blood pressure, lipid parameters, endothelial function, and waist circumference when compared with placebo in patients who were obese and insulin resistant with type 2 diabetes (n = 16) (Lucotti et al., 2006). However, the effects of arginine on specific liver-related parameters are unknown in patients with NAFLD/NASH.

Additional notable studies demonstrating positive results with individual AAs in multifactorial diseases include trials of glutamine (NCT01179217) or arginine (NCT01796678) in sickle cell disease (SCD) (Niihara et al., 2018; Morris et al., 2013). L-glutamine, which is now an approved therapeutic for SCD, administered for 48 weeks reduced sickle cell-related pain and led to significantly fewer acute chest syndrome episodes compared with placebo in children and adults with SCD (n = 152) (Niihara et al., 2018). NAC has been explored for the symptoms of mild traumatic brain injury (NCT00822263) (Hoffer et al., 2013). Single AA clinical studies provide a basis for future translational investigation of AAs as multitarget treatment strategies in patients with multifactorial diseases.

AA Combinations Targeted to a Single Biology

AAs have been used in combination to target a single pathogenic process, most commonly through BCAAs, other essential AAs (EAAs), and urea cycle AAs. BCAAs have been tested extensively with both preclinical and clinical approaches and have shown potential benefit in liver conditions (Holecek, 2018; Muto et al., 2005; Marchesini et al., 2003; Kitajima et al., 2018; Miyake et al., 2012; Honda et al., 2016; Li et al., 2013). In a 12-month oral BCAA supplementation multicenter trial, BCAA administration led to reductions in both hospital admissions and mortality, as well as improvements in Child-Pugh scores in patients with cirrhosis (n = 59) compared with controls (Marchesini et al., 2003). Similarly, a 2-year multicenter trial demonstrated improvement in the number of hepatic failure events and survival rate in patients with decompensated cirrhosis after BCAA supplementation (n = 314) versus diet therapy (Muto et al., 2005). In a small subgroup of Japanese patients with cirrhosis (n = 11), improvements in hypoalbuminemia with

BCAA supplementation over 48 weeks were accompanied by changes associated with targeted BCAA biology, such as significant reduction from baseline in visceral fat mass and skeletal muscle fat accumulation, glucose tolerance improvement, reduction in Child-Pugh score, and maintenance of skeletal muscle mass (Kitajima et al., 2018). Experience with BCAA in NASH specifically is limited; preliminary observations of two patients with NASH-related cirrhosis showed that BCAA supplementation improved glucose tolerance (Miyake et al., 2012), and these observations are supported by rodent models in which BCAAs led to improved steatosis and plasma lipid levels (Miyake et al., 2012; Honda et al., 2016; Li et al., 2013).

The urea cycle AAs, L-ornithine and L-aspartate, are often administered in combination in a stable salt known as LOLA (Kircheis and Luth, 2019). LOLA promotes urea synthesis and reduces blood ammonia, a key toxin in HE pathogenesis (Gebhardt et al., 1997; Kircheis and Luth, 2019). LOLA has potential as a treatment for overt and minimal HE (Alvares-Da-Silva et al., 2014; Kircheis et al., 1997; Sharma et al., 2014; Stauch et al., 1998). In a double-blind randomized trial, LOLA (six months) versus placebo significantly improved secondary HE prophylaxis among patients with cirrhosis (n = 73) (Varakanahalli et al., 2018).

Various combinations of EAAs have been used to stimulate and support protein production in other multifactorial diseases. For example, EAA combinations have been studied in muscle-related conditions, such as sarcopenia in the elderly (Dillon et al., 2009; Kim et al., 2012). In a double-blind, placebo-controlled randomized trial of EAAs in older patients undergoing total knee arthroplasty (n = 16), EAA supplementation (twice daily for 1 week before and 2 weeks after total knee arthroplasty) reduced muscle atrophy and improved muscle function compared with placebo (NCT00760383) (Dreyer et al., 2013). In a follow-up study in older patients undergoing total knee arthroplasty (n = 19), EAA supplementation (twice daily for 1 week preoperatively and 6 weeks postoperatively) significantly reduced muscle atrophy compared with placebo, although it had no effect on muscle function (NCT02145949) (Dreyer et al., 2018). Previous studies have evaluated single or combination AAs in disease modification; however, it is clear that additional studies are needed to elucidate the roles of AAs in disease pathology and as potential treatment strategies.

ADVANCEMENTS IN NETWORK BIOLOGY AND COMBINATORIAL THERAPEUTIC DESIGN PRINCIPLES

The interconnected disease nodes that underlie multifactorial diseases can be uncovered using a network biology approach, which includes the use of genomics, proteomics, transcriptomics, and metabolomics (Barabasi et al., 2011; Hasin et al., 2017; Yan et al., 2018; Zhu et al., 2012). The network biology systems-level approach has been proposed in therapeutic development for multifactorial diseases like NAFLD and NASH (Mardinoglu et al., 2018). Using network-based drug combination modeling, product candidates can be designed to provide simultaneous targeting of disease pathways, while minimizing toxic effects (Cheng et al., 2019). Computer-modeled combinatorial studies are paired with cellular or animal studies to build an empirical understanding of drug-pathway interactions (Flobak et al., 2019; Menden et al., 2019). However, cellular studies may be misinterpreted owing to major limitations in these systems, such as the masking of metabolic regulation by traditional nutrient-rich media (Cantor, 2019). Likewise, nonclinical studies may not account for the metabolic differences between humans and cellular or animal model systems (Cantor et al., 2017; Cantor, 2019). Our *in vitro* studies evaluate EMM combinations with human primary cellular models, which are compelling systems because of their potential fidelity with human metabolism (Marukian et al., 2019; Comb et al., 2020).

Network-based combination design strategy can be applied to the development of EMM compositions. EMM compositions can be designed based on the known cellular interactions of AAs and related metabolites and precursors, clinical safety and efficacy of these molecules, and metabolic profiling for health and/or disease paired with combination design principles (Figure 3). In sum, preclinical, clinical, and network-based research influence EMM composition design that can be used to target biologies related to health and/or multifactorial disease treatment (Figure 3). The subset of EMMs that we have focused on in this article, specifically AAs, related metabolites, and precursors, have well-understood safety profiles. In addition, these EMMs have a history of evaluation in single or combination studies, reducing the risk for off-target toxicities potentially introduced by chemical entities that have not yet been thoroughly assessed for safety.

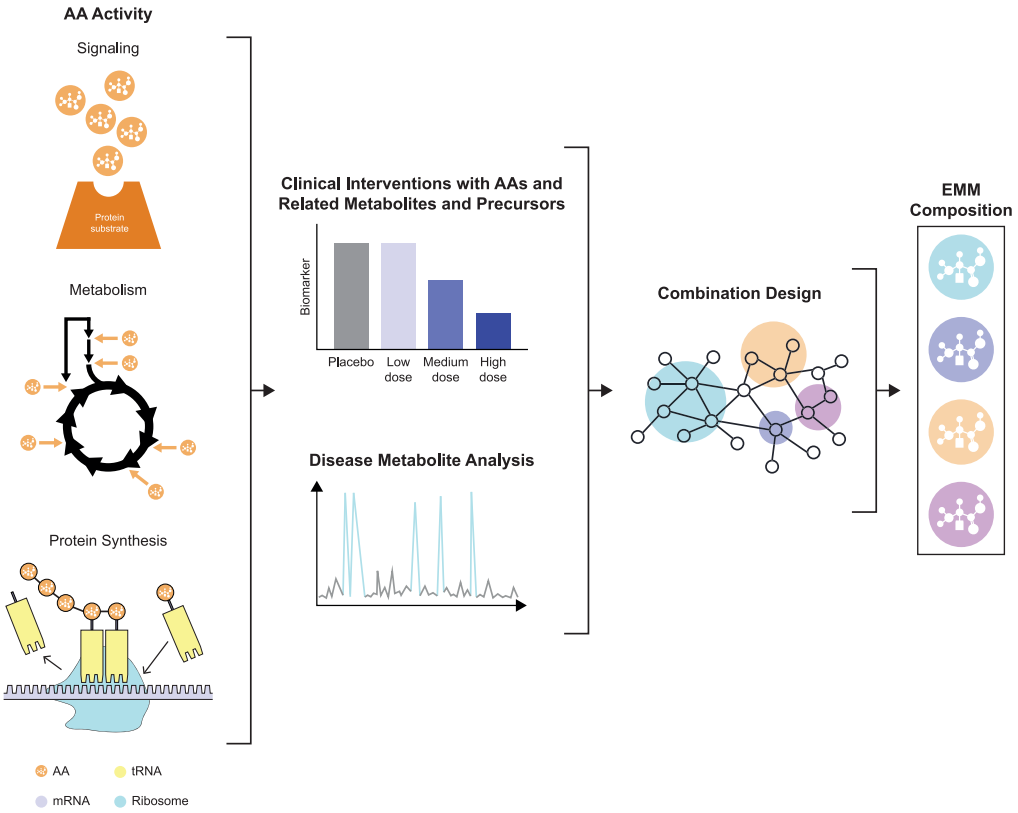

Figure 3. Rationally Designed EMM Compositions in the Treatment of Multifactorial Diseases

A comprehensive understanding of AA activity combined with clinical insights on AAs and related metabolites and precursors and disease metabolite analysis enables combinatorial design strategies for selecting EMM compositions as multitargeted therapeutics.

AA, amino acids; EMM, endogenous metabolic modulator; mRNA, messenger RNA; tRNA, tRNA.

EMM COMPOSITIONS FOR MULTIFACTORIAL DISEASES

Emerging research on health and disease metabolite analysis and pharmacodynamic effects after EMM combination administration hints at the potential for rational EMM composition design. In a hepatocyte genome-scale metabolic model, plasma concentrations of glycine, serine, and other metabolites were negatively correlated with hepatic steatosis (Mardinoglu et al., 2017). Based on these metabolic models, a combination of NAC, nicotinamide riboside, and serine was administered in mice and shown to prevent hepatic steatosis (Mardinoglu et al., 2017). This composition was further studied in healthy male participants (n = 9); the combination resulted in reductions relative to baseline in plasma levels of biomarkers associated with liver fat (i.e., kynurenine, kynurenate, pyruvate, and ornithine) and insulin resistance (i.e., BCAAs) (NCT03838822) (Zhang et al., 2020). In a separate study in the clinic, patients with type 2 diabetes and hypertension (n = 12) received a combination of arginine and NAC. The combination of arginine and NAC improved endothelial function through increased nitric oxide bioavailability via simultaneous oxidative stress reduction and nitric oxide production (Martina et al., 2008).

We have established a foundation of translational research that supports the specific design of EMM compositions for multifactorial diseases. For example, metabolic profiling and clinical research studies on EMMs contributed to the design of AXA2678, an EMM composition targeted for a number of potential disuse-induced sequelae associated with muscle atrophy. AXA2678 contains BCAAs and other select EAAs (lysine, histidine, phenylalanine, and threonine) designed to stimulate muscle protein synthesis, as well as nonessential AAs that can impact nitric oxide (arginine), TCA cycle (glutamine), and oxidative stress reduction (NAC) pathways. In our study of muscle structure during and after disuse in healthy young men (n = 10), AXA2678 was safe and well tolerated and was shown to attenuate muscle atrophy and fat accumulation (Holloway et al., 2019).

Additional EMM compositions in development are being studied for potential multifactorial effects in liver disease. AXA1665 comprises eight AAs being evaluated to assess their potential impact on AA dysregulation, ammonia handling, and muscle structure and function and could have a potential to impact patients with cirrhosis (Chakravarthy et al., 2020a). In a single-center, two-part clinical study, AXA1665 restored AA dysregulation and reduced ammoniagenesis in subjects with mild to moderate hepatic insufficiency ($n = 9$) (NCT04147936) (Chakravarthy et al., 2020a). AXA1125, a combination of five AAs and an AA precursor (NAC), produced multifactorial effects on metabolism, inflammation, and fibrosis in NASH cell and mouse models (Lee et al., 2017; Marukian et al., 2019). In a multicenter, open-label clinical study, AXA1125 (three times a day over 12 weeks) improved markers of metabolism, inflammation, and fibrogenesis, as well as relevant clinical measures in subjects with NAFLD and type 2 diabetes ($n = 23$) (Chakravarthy, 2018; Chakravarthy et al., 2019; Harrison et al., 2020). Our clinical data support the potential for EMMs to target distinct pathways in multifactorial diseases.

CONSIDERATIONS FOR EMM COMPOSITION DESIGN WITH AAS AND RELATED METABOLITES AND PRECURSORS

Thorough understanding of biology and disease pathogenesis is required to identify the optimal combination and ratio of EMMs with the potential to elicit disease-modifying effects. A significant consideration with EMM composition design in the context of AAs is the achievement of a fine-tuned balance of pharmacokinetic profiles of all AAs. It is necessary to achieve appropriate target exposures of AAs to mediate the desired pharmacodynamic processes, while leaving nontargeted physiological mechanisms intact and minimizing the risk for safety/tolerability issues. Administration of AAs in combination is known to result in altered pharmacokinetics compared with single AA administration, partially because of AA interactions at the level of intestinal and target tissue transport (Broer and Fairweather, 2018). One clinical study evaluated the plasma pharmacokinetic profiles of leucine, valine, and isoleucine both individually and in combination following oral administration in five healthy male subjects. After its administration individually, leucine significantly reduced plasma concentrations of isoleucine, valine, and phenylalanine (Matsumoto et al., 2014). When administered as a combination of isoleucine, valine, and leucine, the plasma concentrations of isoleucine and valine peaked at later time points and declined more rapidly than after single administration of leucine, isoleucine, or valine (Matsumoto et al., 2014). Likewise, administration/ingestion of AXA1665 led not only to alterations in the concentrations of AAs included within AXA1665, but also altered concentrations of non-dosed AAs (Chakravarthy et al., 2020a). Thus, combinations of AAs can lead to pharmacokinetic alterations and result in targeted pharmacodynamic effects.

Another consideration when designing EMM compositions is that some AAs, particularly at high concentrations, have been shown to contribute to compromised safety or negative outcomes. Certain AAs should be selectively avoided at particular doses and in certain populations. For example, in patients with advanced liver disease ($n = 3$), a high-protein diet or mixed AA infusion of EAAs and nonessential AAs led to the development of HE, which improved with reduced-protein diet or slowing of infusion (Fischer et al., 1974). Specifically, glutamine degradation to ammonia in the gut and kidneys may exacerbate the encephalopathic phenotype in patients with cirrhosis (Holecek, 2014). In patients with compromised renal function, AA intake may initially improve renal function (i.e., higher glomerular filtration rate) (Woods, 1993); however, over time, AAs place excessive strain on the kidneys and accelerate renal decline (Brenner et al., 1982). Additionally, patients with chronic kidney disease are recommended to adhere to a low-protein diet to avoid acceleration of renal function decline (Kidney Disease: Improving Global Outcomes (Kdigo) Ckd Work Group, 2013). An understanding of multifactorial disease pathophysiology underlies EMM composition design, such that certain EMMs should possibly be selectively avoided owing to potential detrimental effects in specific disease contexts.

Finally, when considering the total body of clinical and preclinical work on AAs and related metabolites and precursors, there are mixed results with AAs and conflicting reports on sufficient protein intake recommendations for patients with chronic liver or kidney disease (Brenner et al., 1982; Fischer et al., 1974; Holecek, 2014; Woods, 1993). Differing results with AAs may correspond to variations in experimental design related to population or model organisms, doses or formulations of AAs administered, or analytical methods. BCAAs were associated with improved glycemic control in preliminary observations of patients with NASH-related liver cirrhosis, and conversely, with increased insulin resistance in one study of patients with obesity (Badoud et al., 2014; Miyake et al., 2012). Although BCAA supplementation improved survival in patients with cirrhosis, studies in mice demonstrate inconsistent effects of BCAA supplementation: steatosis reduction in an obesity/diabetic model and steatosis induction in a NASH model (Honda et al., 2017;

Zhang et al., 2016; Yoshida et al., 1989). The differing effects of BCAAs are potentially due to the varying impact of BCAA levels observed within specific disease metabolic profiles (Iwasa et al., 2015; Lake et al., 2015). Regarding EAAs, original findings of improved muscle function after EAA supplementation versus placebo in older patients undergoing total knee arthroplasty were not replicated in a follow-up study, possibly due to EAA formulation differences (Dreyer et al., 2013, 2018). The researchers reported using inconsistent EAA formulation practices, which led to inadvertently low leucine administration in the follow-up study (Dreyer et al., 2013, 2018). The rational design of EMM compositions may provide a solution to achieve the appropriate balance of EMMs and target only positive health outcomes for specific disease metabolic profiles.

CONCLUDING REMARKS

There is an opportunity to utilize multitargeted treatments with favorable safety profiles that more wholly address underlying multifactorial disease pathology. The convergence of advances in AA biology, clinical pharmacology, and metabolomics has revealed a broad relationship between AA metabolism and disease. The intersection of AAs and disease modification presents a case for using AAs and related metabolites and precursors in combination as EMM compositions in the treatment of multifactorial diseases. We propose that rationally designed EMM compositions have the potential to regulate biological processes that promote overall health and, importantly, have significant potential as safe, multitargeted therapeutics given the long clinical history and expansive roles of EMMs in biology.

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AUTHOR CONTRIBUTIONS

M.J.H. conceptualized the project and contributed to visualization of the figures. M.J.H., M.V.C., and R.A. wrote the original draft. M.J.H., M.V.C., R.A., and T.T. provided review and editing in writing support of the manuscript.

DECLARATION OF INTERESTS

M.J.H., M.V.C., and T.T. are employees and R.A. is a former employee of Axcella Health Inc.; all authors own stock options in the company.

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