



# The Gut-Immune-Brain Axis in Autism Spectrum Disorders; A Focus on Amino Acids

Joris H. J. van Sadelhoff<sup>1,2</sup>, Paula Perez Pardo<sup>1</sup>, Jiangbo Wu<sup>3</sup>, Johan Garssen<sup>1,2</sup>, Jeroen van Bergenhenegouwen<sup>1,2</sup>, Astrid Hogenkamp<sup>1</sup>, Anita Hartog<sup>1,2</sup> and Aletta D. Kraneveld<sup>1,4\*</sup>

<sup>1</sup> Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, Netherlands, <sup>2</sup> Danone Nutricia Research, Utrecht, Netherlands, <sup>3</sup> Laboratory of Neuroimmunology, Department of Symptom Research, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, <sup>4</sup> Veterinary Pharmacology, Institute for Risk Assessment Studies, Faculty of Veterinary Sciences, Utrecht University, Utrecht, Netherlands

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### \*Correspondence:

Aletta D. Kraneveld  
a.d.kraneveld@uu.nl

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Autism spectrum disorder (ASD) is a range of neurodevelopmental conditions that affect communication and social behavior. Besides social deficits, systemic inflammation, gastrointestinal immune-related problems, and changes in the gut microbiota composition are characteristic for people with ASD. Animal models showed that these characteristics can induce ASD-associated behavior, suggesting an intimate relationship between the microbiota, gut, immune system and the brain in ASD. Multiple factors can contribute to the development of ASD, but mutations leading to enhanced activation of the mammalian target of rapamycin (mTOR) are reported frequently. Hyperactivation of mTOR leads to deficits in the communication between neurons in the brain and to immune impairments. Hence, mTOR might be a critical factor linking the gut-brain-immune axis in ASD. Pharmacological inhibition of mTOR is shown to improve ASD-associated behavior and immune functions, however, the clinical use is limited due to severe side reactions. Interestingly, studies have shown that mTOR activation can also be modified by nutritional stimuli, in particular by amino acids. Moreover, specific amino acids are demonstrated to inhibit inflammation, improve gut barrier function and to modify the microbiota composition. In this review we will discuss the gut-brain-immune axis in ASD and explore the potential of amino acids as a treatment option for ASD, either via modification of mTOR activity, the immune system or the gut microbiota composition.

**Keywords:** autism spectrum disorder, amino acids, mammalian target of rapamycin, nutritional intervention, gut-immune-brain axis

## THE GUT-BRAIN AXIS IN AUTISM SPECTRUM DISORDER

Autism spectrum disorder (ASD) is a spectrum of neurological disorders that become apparent in early childhood and last throughout a person's life. This spectrum of disorders is characterized by varying levels of deficits in social behavior and communication, as well as stereotypical behaviors (1). Estimates of the prevalence of ASD vary widely among different studies, largely attributable to differences in diagnostic criteria, the ages and gender of the children screened, as well as the geographical location (2). Meta-analyses comprising studies in different geographical locations indicate a global incidence of ASD between 0.6 and 0.7% (3, 4). Over the past decades, the

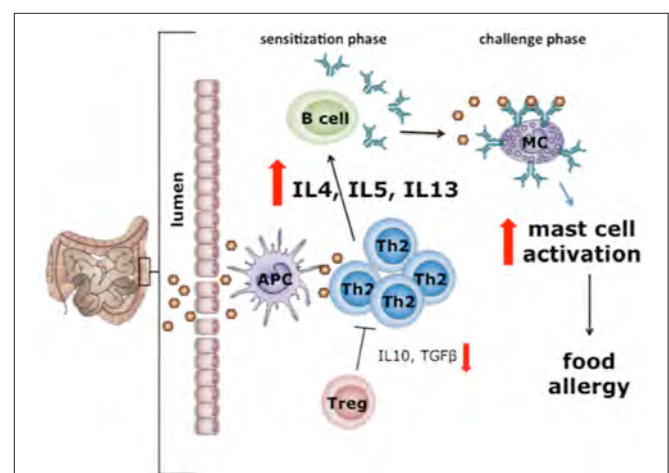
reported global prevalence has increased markedly (4, 5). Studies suggest that part of this increase is attributable to changes in diagnostic criteria, an increased awareness and the ability to diagnose at a younger age (3, 6). However, these changes alone are not sufficient to explain the rise in ASD prevalence. Other factors, including prenatal, natal and postnatal environmental factors like diet, intestinal microbiota, and immunological triggers are likely to also contribute to this increase (7–9).

In addition to behavioral deficits, autistic individuals often present with mild systemic inflammation and gastrointestinal disorders (10, 11). Although current epidemiological data is limited, a recent meta-analysis confirms that children with ASD experience four times more gastrointestinal symptoms than control groups and that gastrointestinal symptoms may identify a unique subgroup of children with ASD (12). Moreover, numerous studies showed a link between immune disturbances and ASD (11, 13, 14). Although research into the relation between immune disturbances and ASD is still in its early phases, studies suggest that many different aspects of the immune system are involved (15–17). For instance, ASD has been associated with a deregulated activation of microglia and astroglia (the “immune-like” cells in the brain) (18, 19), autoimmunity (20), increased T cell activation (21), deregulated innate immune functioning (11) and mutations in genes controlling the functioning of immune cells (11, 22, 23). The deregulated immune functions in people with ASD are reflected by abnormal cytokine levels in their body fluids. More specifically, levels of pro-inflammatory and allergy-associated type 2 helper T (Th2) cell-derived cytokines (e.g., TNF $\alpha$ , IL-4, IL-5, IL-6, IL-8) are shown to be upregulated in either cerebrospinal fluid, plasma or serum of people with ASD (21), whereas levels of regulatory cytokines IL-10 and TGF- $\beta$  are downregulated (24–26). Multiple studies investigated the relation between the aberrant cytokine levels and autism. One example is a study performed by Hashim et al. (27). Here, it was shown that serum levels of TGF- $\beta$  negatively correlated with the severity of autistic symptoms. A similar negative correlation was found between the severity of autism and the frequency of regulatory T (Treg) cells in blood, which appears to be lower in autistic individuals (28). As TGF- $\beta$  plays a critical role in Treg cell development (29), there might be a causal relation between lower TGF- $\beta$  levels, lower Treg levels and autism severity. Besides its role in the development of Treg cells, TGF- $\beta$  is also indicated to play an important role in brain development (30, 31). More specifically, TGF- $\beta$  regulates survival and differentiation of neurons and directs the growth of synapses, as observed in various invertebrate models (30, 31). Hence, a lack of TGF- $\beta$  could contribute to dysregulated brain functioning, representing another mechanism by which aberrant TGF- $\beta$  levels could be involved in the development of autistic phenotypes observed in autistic patients. The presence of a relation between abnormal cytokine levels in autistic individuals and autism severity is further corroborated by two large studies in children with ASD (32, 33). In these studies it was demonstrated that the increase of a broad range of cytokines including pro-inflammatory, Th2 as well as type 1 helper T (Th1) cell-derived cytokines and chemokines correlates with aberrant (autistic) behavior and impaired development (32, 33).

The exact mechanisms underlying the increased production of pro-inflammatory cytokines and allergy-associated Th2 cytokines in autistic individuals are yet to be elucidated. However, a potential explanation of these findings might be sought in the observation that allergy, especially food allergy, and eosinophilic esophagitis are often reported in ASD patients (34, 35). A food-induced allergic disease is an unfavorable immune reaction that can occur on exposure to specific food components, particularly proteins (36). Classically, food allergy is regarded as a type 1 hypersensitivity, i.e., an IgE-mediated immune response to harmless food proteins (Figure 1).

## THE IMMUNE-BRAIN AXIS IN ASD WITH A FOCUS ON FOOD ALLERGY

It has been shown from parental reports that food allergies are observed more often in autistic individuals than in the general population (39–41). This differential occurrence of food allergies might be even more evident than reported, since impaired communication by autistic individuals could lead to underdiagnoses (39). Multiple studies investigated the relation of food allergies with ASD. For instance, it has been reported that autistic children have higher serum levels of IgA, IgG, and IgM specific for cow's milk proteins compared to healthy controls (42, 43). Interestingly, Lucarelli et al. showed that autistic children on a cow's milk free diet showed improved behavioral symptoms (43). Vice versa, challenging autistic individuals with cow's milk led to an increase in hallmark behaviors associated with ASD,



**FIGURE 1 |** In the first phase of allergy or type 1 hypersensitivity reactions sensitization to the food allergen takes place (sensitization phase). During this phase, allergens cross the intestinal mucosal barrier and are presented via MHC-II to naïve T cells that develop into type 2 helper T (Th2) cells. Th2 cells induce the plasma cells to produce IgE antibodies (37), after which IgE binds to the FcεR1 receptor on mast cells. In the challenge phase, upon a second exposure to the same allergen, cross-linking of mast cell-bound IgE occurs. In turn, this leads to mast cell degranulation, a process resulting in the release of several mediators including histamine, cytokines and tryptases. The release of these mediators is the direct cause of most of the allergic intestinal symptoms, including nausea, vomiting, cramps, and diarrhea (38). With permission of Dr J. Wu.

providing evidence for a relation between food allergic reactions and behavior (43). Preclinical studies provide further evidence for a relation between food allergies and ASD, as cow's milk allergic (CMA) mice show autistic-like behavior such as reduced social interaction, increased repetitive behavior, disturbed spatial memory and reduced exploration behavior (44). The changes in behavior of CMA mice were associated with increased neuronal activation in the prefrontal cortex (PFC). Similarly, patients with ASD show enhanced neuronal activation in the PFC in response to tasks involving facial recognition (45) and attention (46). Furthermore, the activation of the dopaminergic system in the PFC of CMA mice was reduced as compared to non-allergic mice (44). Interestingly, in mouse models for ASD as well as in patients with ASD, dampening of the dopaminergic system in the PFC is observed as well (47–49). Taken together, it can be hypothesized that intestinal allergic responses may affect brain circuits involved in social behavior in ASD patients with food allergies.

A variety of studies examined the underlying mechanism of the apparent relation between the induction of food allergy and autistic behavior. As described earlier, individuals with ASD are associated with a pro-inflammatory cytokine profile in various body fluids and a disbalance in Th2 and Treg cells, which are common features for allergic individuals as well (21, 27, 32). Besides differences involving T cells, ASD is also associated with deviations involving the activation of mast cells, which are cells that, upon activation, play a central role in driving allergic reactions. For instance, mast cell activation is shown to be increased in ASD patients, resulting in elevated release of inflammatory and neurotoxic mediators, which in turn could contribute to the development of autistic phenotypes (14, 50, 51). The influence of mast cells on behavior is not limited to the effects of mediator-release, as mast cells can also influence behavior via direct mast cell-neuron interactions, which take place in the brain (52) and the gut (53, 54). The potential of mast cell abnormalities to drive ASD-associated behavior in human is supported by a study performed by Theoharides et al. (55). In this study, the occurrence of mastocytosis, as characterized by the presence of proliferating and hyperactivated mast cells in several organs, is investigated in children with ASD (55). This study indicated that the incidence of mastocytosis is ten times higher in ASD patients than in the general population (55). In addition, 15% of the people who suffered from mastocytosis showed aberrant behaviors, such as concentration problems, decreased attention span, memory impairment, irritation, and distraction (55). These abnormal behaviors are similar to those encountered in ASD, suggesting that hyperactivation of mast cells, as occurs during allergic reactions, might be one of the mechanisms underlying the relation between food allergies and ASD-associated behavior. The potential involvement of mast cell hyperactivation in inducing ASD-associated behavior is further supported by studies showing that mast cell hyperactivation is associated with increased intestinal permeability (55). Accordingly, it is frequently reported that people with ASD have an increased intestinal permeability (56, 57). This disruption of the intestinal barrier might be involved in inducing ASD-like behavior, as preclinical research demonstrated that animals with ASD who suffer from defects in the intestinal barrier show increased levels of bacterial

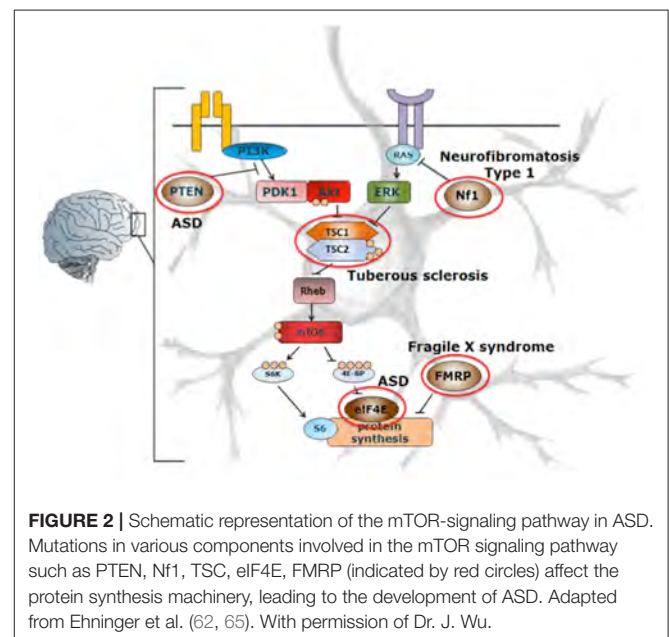
toxins in the bloodstream, which in turn could influence brain function (58).

## THE MAMMALIAN TARGET OF RAPAMYCIN IN ASD AND ITS CONNECTION WITH THE IMMUNE SYSTEM

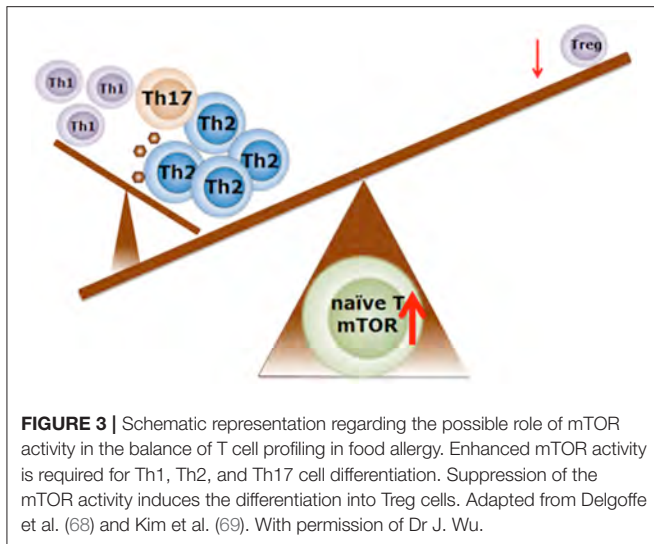
ASD is thought to be a neurodevelopmental disorder caused by multiple genetic and environmental factors. The genes involved in ASD are variable and diverse, but mutations in genes related to the mammalian target of rapamycin (mTOR) pathway, including *NF1* (59), *PTEN* (60), *TSC1* (61), *TSC2* (62), *eIF4E* (63), *FMRP* (64), are among those most widely associated with ASD (Figure 2).

NF1, PTEN, and TSC1/TSC2 act as negative regulators of mTOR complex 1 (mTORC1), of which single gene mutations lead to enhanced mTOR activity in the brain in various mouse models (60, 62, 65). Enhanced activation of mTOR leads to an increase in the phosphorylation of proteins downstream of mTORC1, such as S6 kinase (S6K) and eIF4E-binding proteins (4E-BPs), thereby promoting cap-dependent protein translation (66, 67). In this way, enhanced mTOR activation leads to an increase in the translation of neuroligins (67), which are proteins that are involved in the formation and maintenance of synapses between neurons. In turn, this results in an increased synaptic excitation/inhibition ratio, which may contribute to the development of ASD phenotypes (67). Interestingly, apart from its important role in neurological disorders, the mTOR-signaling pathway is also involved in directing immune responses (Figure 3).

Several studies suggest that Th1 and T helper 17 (Th17) cell differentiation are specifically regulated by mTORC1 signaling,







whereas Th2 cell differentiation depends on mTOR complex 2 (mTORC2) signaling (68, 70, 71). This is supported by studies that show that T cells in which mTORC2 activity is eliminated fail to differentiate into Th2 cells *in vitro* and *in vivo*, but are able to differentiate into Th1 and Th17 cells (72). When mTORC1 and mTORC2 are both inhibited using rapamycin, T cells differentiate into Treg cells (72). Indeed, rapamycin-induced mTOR inhibition resulted in elevated numbers of Treg cells in tissue culture of nasal polyps obtained from patients suffering from chronic allergic rhinitis (73). Further evidence of the ability of mTOR to direct immune responses is provided by a study by Kim et al. showing that mTORC1 activation in mast cells is associated with survival, differentiation, migration, and cytokine production (69). Finally, increased mTOR activity is shown to attenuate autophagy (74) which, in the intestine, is an important process to limit chronic intestinal inflammation (75). Thus, hyperactivity of mTOR in the intestinal tract might lead to a loss of immune regulation and barrier integrity, leading to an inflammatory profile possibly associated with allergy. Taken together, these findings reveal that in ASD the mTOR signaling pathways could be disturbed at the level of the immune system, the gastro-intestinal tract as well as the brain. As such, the mTOR signaling pathways could be considered as a central point in the gut-immune-brain axis. Manipulation of the mTOR signaling pathway may therefore serve as a therapeutic strategy for inflammation-associated neurodevelopmental disorders including ASD.

In order to more specifically investigate the role of mTOR in ASD, a variety of mTOR-inhibitors have been used. The mTOR inhibitor that is most commonly used for this purpose is rapamycin. Rapamycin interacts with the intracellular receptor FK506 binding protein (FKBP12). The formed FKBP12-rapamycin complex binds to mTOR and directly inhibits mTORC1, while mTORC2 is inhibited indirectly in a manner not fully understood (76, 77). As described earlier, mTORC1 drives translation of proteins, like neuroligins, that increase autistic-like phenotypes (77). Therefore, inhibition of mTORC1

by rapamycin might represent a therapeutic strategy for ASD patients. Indeed, rapamycin treatment improved ASD-associated behavior in various animal models for ASD, including the widely used BTBRT(+)/lpr3(tf)/J mouse model (78–81). Moreover, rapamycin treatment was shown to inhibit the social behavioral deficits and normalize the repetitive self-grooming behavior of CMA mice (82). In these mice, the humoral immune responses and mucosal mast cell activation in the intestines were also inhibited (82). At the molecular level, several changes were observed in the intestines of rapamycin-treated CMA mice that might underlie the observed improvement in behavioral outcomes. For instance, it has been shown that rapamycin treatment inhibited the enhanced phosphorylation and activation of the downstream target of mTOR p70 ribosomal protein S6 kinase (p70S6K) in the small intestines (82). Moreover, rapamycin significantly increased the mRNA expression of forkhead box P3 (Foxp3) both in the ileum and the Peyer's Patches of CMA mice, indicating an increase in Treg cell development (82). Finally, rapamycin treatment increased the expression of Treg cell-associated cytokines, including IL-10 and TGF- $\beta$  in the small intestine of CMA mice (82). Till this day, however, the effects of targeting mTOR on possible immune dysregulations found in the rodent ASD models mentioned above remain understudied.

Collectively, these data support the relevance to further investigate the therapeutic potential of mTOR inhibiting strategies in both genetic and idiopathic forms of ASD. However, the occurrence of serious side effects of chronic administration of pharmacological mTOR inhibitors like rapamycin, such as severe immunosuppression, is reason for concern and limits their potential as a viable treatment option for ASD (83, 84). Hence, safer alternative strategies to normalize mTOR activity are required.

## TARGETING ASD-ASSOCIATED ABNORMAL MTOR ACTIVITY WITH AMINO ACIDS

Besides pharmacological compounds, nutritional components are also able to modulate mTOR activity. Often described as being the most potent nutrition-derived modulators of mTOR are amino acids (AAs) (85, 86). Although it is not yet fully understood how AAs regulate mTOR activity, AA transporters seem to play a crucial role. A variety of mechanisms involving AA transporters are proposed by which AAs can regulate mTOR activity. First, AA transporters can form a passageway for AAs through the plasma membrane. Once inside the cell, the AAs activate cytoplasmic AA sensors by interacting with a multi-protein complex involving RAG GTPases, Ragulator, and the v-ATPase on the lysosomal surface (87–89). This interaction recruits mTORC1 to lysosomes, where it can be activated. One example of a cell-surface AA transporter is the well-studied heterodimeric CD98, which transports preferably branched chain AAs (BCAAs). In combination with a glutamine transporter (SLC1A5), CD98 exchanges leucine for glutamine to increase the intracellular leucine concentration which, in turn,

activates mTORC1 through different signaling molecules (90). Another mechanism by which AAs can modulate mTORC1 activation involves a specific subset of AA transporters, known as AA transceptors, which have a dual transporter-receptor function. The binding or translocation of AAs by transceptors initiates an intracellular signaling cascade, ultimately leading to the modulation of mTORC1 activation (89, 91). Thus, AA transceptors can modulate mTORC1 activation by sensing the extracellular AA concentration. Finally, AAs can also modulate mTORC1 activity by mechanisms that do not involve the translocation of AAs across the plasma membrane (89, 91). Studies have demonstrated that specific AAs can bind and activate G-protein-coupled receptors, which in turn induce lysosomal localization and activation of mTORC1 (89). One example is the G protein-coupled receptor family C group 6 member A (GPCR6A), which is activated by basic, aliphatic and polar AAs such as serine, arginine and lysine.

As AAs can modulate mTORC1 activity via multiple mechanisms, which involve receptors and transceptors that specifically recognize different types of AAs, it is likely that the modulation of mTORC1 activity is different for each type of AA. Consistently, a previous study using mammary epithelial cells demonstrated that the BCAAs leucine (Leu), isoleucine (Ile), and valine (Val) enhanced mTORC1 activity, whereas histidine (His), lysine (Lys), and threonine (Thr) suppressed mTORC1 activity (92). Similar results were found in an *in vitro* allergic mast cell model (93). Inhibition of mTOR signaling by a combination of the AAs His, Lys and Thr in this mast cell model was accompanied by an inhibition of acute mast cell degranulation and an attenuated allergy-associated cytokine production following antigen-IgE mediated activation (93). Based on the outcomes of these *in vitro* studies, a diet containing relatively higher amounts of His, Lys and Thr and relatively lower amounts of Leu, Ile, and Val was developed for preclinical ASD studies (93). It was hypothesized that mTOR signaling could either be normalized or reduced in ASD animals using this AA diet. Consistent with this hypothesis, the AA diet restored the social interaction and normalized the repetitive behavior of CMA mice (93). In addition, dietary intervention with the AAs inhibited the enhanced mTOR signaling pathway in the PFC and amygdala of CMA mice. Since the mTOR signaling pathway is centrally involved in directing immune responses including differentiation of T cells (94), T cell responses were measured as well. Indeed, the CMA-induced Th2 and Th17 cell immune responses were downregulated by the specific AA diet (93). These effects of dietary AAs on effector T cells was accompanied by an upregulation of Treg cells in the small intestines of CMA mice. In another set of experiments using the same AA diet, the diet normalized the repetitive behavior of BTBRT(+)/lpr3(tf)/J mice, a frequently used murine model for ASD (93). This outcome was associated with an inhibition of the enhanced mTOR signaling pathway in the PFC of these mice, suggesting beneficial effects of the AA diet in both genetic and idiopathic forms of ASD (93).

The studies described above further establish mTOR activity to be a crucial factor in the gut-immune-brain axis playing a key role in the regulation of ASD-associated behavior. It has become clear that not only the effects of mTOR on the

brain are important, but also the effects of mTOR concerning the regulation of immune responses and intestinal health. This warrants further investigation into the activity status of mTOR in peripheral blood mononuclear cells and intestinal biopsies of autistic patients. These data are needed to further substantiate mTOR as a therapeutic target or as biomarker in patients with ASD. Finally, based on the positive outcomes of an AA-based nutritional intervention on autistic behavior in mouse models for ASD, a proof of principle clinical study in ASD patients using this specific diet is warranted.

## THE EFFECTS OF AMINO ACIDS ON THE GUT-IMMUNE-BRAIN AXIS IN ASD BEYOND MTOR

As described, specific AAs can modulate the activity of mTOR in a manner that might be beneficial for people with ASD. However, the potential of AAs to be beneficial for ASD is not limited to mTOR modulation. Numerous studies have shown that AAs can also exert beneficial effects on other components in the gut-immune-brain axis. Below, the interactions of AAs with the immune system and the gut microbiota (composition) will be discussed in relation to ASD.

## THE POTENTIAL EFFECTS OF AMINO ACIDS ON THE DERAILED IMMUNE SYSTEM CHARACTERISTIC FOR ASD

Beyond targeting the ASD-enhanced mTOR signaling, AAs have been demonstrated to exhibit anti-inflammatory capabilities. As previously described, intestinal and systemic inflammation, as evaluated by a.o. higher levels of pro-inflammatory cytokines (e.g., IL-6, TNF $\alpha$ , and IL1 $\beta$ ) are characteristic for people with ASD. In fact, inflammation is shown to induce ASD-associated behavior. Therefore, inhibition of systemic and/or intestinal inflammation might be beneficial for people diagnosed with ASD. Below, the immunomodulatory effects, mostly anti-inflammatory effects, of several AAs are described in relation to ASD.

Numerous studies investigated the immune modulatory functions of AAs in *in vitro* studies. Among these are studies that demonstrate anti-inflammatory effects of AAs on immune cells that might be affected in people with ASD. For instance, application of the BCAAs Leu, Ile and Val individually reduced the production of NO as well as mRNA and protein levels of the pro-inflammatory cytokine IL-6 in LPS-stimulated macrophages *in vitro* (95). Moreover, application of Leu, Ile, and Val individually or in combinations inhibited the acute degranulation and IL-6 production in antigen-IgE-activated mast cells, as previously mentioned (96). Whereas, increasing the availability of BCAAs has anti-inflammatory effects on specified immune cells, a decreased availability of the BCAAs leads to immune cell impairments. For instance, human lymphocytes cultured in medium containing Leu, Ile and Val at levels lower than the typical concentration in adult human plasma showed decreased ability to proliferate (97, 98). Not only *in vitro*, but also *in*

*vivo* experiments demonstrate the importance of an adequate availability of BCAAs for normal immune cell functioning. For example, reducing the levels of Leu, Ile, and Val by 50% in an otherwise normal mice diet increased the susceptibility of mice to salmonella infections, potentially due to an impaired antibody production (99). These mice also showed a lowered number of spleen cells, which might be indicative of a decreased lymphocyte proliferation *in vivo* (99). Interestingly, Novarino et al. identified inactivating mutations in the gene branched chain keto-acid dehydrogenase kinase (BCKDK) in several ASD patients (100). Loss of BCKDK function resulted in hypercatabolism of Leu, Ile and Val, leading to abnormally low levels of these BCAAs in serum and in the brain (100). Knowing the importance of adequate BCAA availability for normal immune cell functioning, these mutations might contribute to the immune impairments observed in a subgroup of individuals with ASD.

In addition to the observed anti-inflammatory effects of the BCAAs *in vitro* and in mouse models, this group of AAs also has demonstrated anti-inflammatory effects in immune compromised humans. For example, in cirrhotic patients, daily supplementation of Leu, Ile and Val improved innate immune cell functions including the phagocytic function of neutrophils and natural killer activity of lymphocytes, which are processes that are also impaired in people with ASD (17, 101, 102). Also, in patients with rectal cancer, infusing a solution enriched in Leu, Ile and Val led to an increase in the number of CD4+ (Th) cells in blood, and led to an increased ratio of CD4+/CD8+ (cytotoxic T) cells in the blood (103). Studies have demonstrated that autistic individuals have fewer CD4+ cells and a lower CD4+/CD8+ cell ratio in blood, indicating immune impairments (104–106). Interestingly, Abd El-Aziz et al. showed a negative correlation between the CD4+/CD8+ cell ratio in blood of individuals with ASD and the severity of autistic behavior. Thus, factors like Leu, Ile and Val that increase the ratio of CD4+/CD8+ cells might provide therapeutic benefits for ASD patients (104).

Besides their modulatory capacities on peripheral immune cells, the BCAAs also affect microglial cells, the immune cells of the brain. Culturing rat microglial cells in a medium enriched with these BCAAs induced a more anti-inflammatory state of the microglial cells after LPS stimulation (107). This was supported by a decreased microglial production of IL-1 $\beta$ , TNF $\alpha$ , and inducible nitric-oxide synthase (iNOS), as well as an increased production of IL-10, both at the level of mRNA and at the protein level (107). Moreover, microglial phagocytic capacity, which is critical for normal brain functioning and is impaired in individuals with ASD (108), was stimulated by the presence of high levels of the BCAAs (107). However, neuroprotective functions of microglial cells were impaired in the presence of high levels of Leu, Ile and Val, as represented by a decreased production of the neuroprotective factor Insulin-like growth factor 1 (IGF-1) (107). The latter observation is in line with the possible detrimental effects of mTOR-hyperactivation.

Alongside the BCAAs, the essential AAs Thr, Lys, and His also have immunomodulating capacities, potentially via modulation of mTOR signaling (93). Of these AAs, Thr has been most widely

associated with immunomodulation *in vivo*. Animal studies have demonstrated that Thr is critical for intestinal barrier and immune function and can improve intestinal epithelial integrity in inflammatory conditions where the gut barrier is impaired, as is the case in ASD (109). For instance, in various rat models of colitis, a Thr-enriched diet was shown to restore mucus synthesis and normalize the gut microbiota, thereby improving intestinal function (110, 111). Studies in animals and humans also suggest an increased requirement of Thr by the intestines in inflammatory conditions in order to maintain intestinal barrier function (112, 113). Although less widely studied, the AA His also interacts with the immune system. Apart from its use for the generation of histamine, a key molecule involved in immune responses, His exhibits anti-inflammatory effects on a variety of immune cells. For instance, *in vitro* studies show that supplementation of His suppressed IL-8 secretion in TNF $\alpha$ -activated human epithelial cells, PBMCs and monocytes (114, 115). The immunomodulating ability of His is also demonstrated *in vivo*, as dietary His improved murine colitis by inhibiting the production of pro-inflammatory cytokines by macrophages (116). Studies examining the immunomodulatory effects of Lys are limited. However, dietary deficiency of Lys has been shown to limit lymphocyte proliferation and induces a pro-inflammatory state, represented by increased levels of pro-inflammatory cytokines and NO in the kidney, liver and spleen of piglets (117). Taken together, data from both *in vitro* and *in vivo* experiments suggest that there is a potential for the AAs Thr, Lys, and His to ameliorate the compromised gut-immune-brain axis in ASD.

In addition to the AAs that are demonstrated to interact with mTOR, several other AAs also have anti-inflammatory capabilities, in particular glycine (Gly), glutamine (Gln) and glutamate (Glu). *In vitro* studies demonstrated that supplementation of Gly protects intestinal epithelial cells against inflammatory oxidative stress (118), and reduces pro-inflammatory cytokine production while enhancing IL-10 expression of LPS-stimulated monocytes (119). The capacity of Gly to exert anti-inflammatory effects is further supported by *in vivo* studies using a variety of animal immune models. For instance, oral intake of Gly reduced TNF $\alpha$  production and improved the survival rate of LPS-injected rats (120), reduced inflammation and oxidative stress in a mouse model of cancer cachexia (121) and inhibited the onset of CMA in mice (122). As the pro-inflammatory, allergic-like status of the intestines in people with ASD might play a role in the induction of autistic-like behavior, it can be speculated that dietary Gly supplementation might have therapeutic potential for individuals with ASD.

The AAs Gln and Glu are related to each other, both in terms of structure and their immunomodulatory capabilities. Both Gln and Glu are critical for intestinal growth and functions and are shown to reduce intestinal hyperpermeability both *in vitro* and *in vivo* (123–125). Remarkably, plasma levels of Gln are reported to be lower in patients with ASD as compared to healthy individuals (126). The reduced bioavailability of Gln might be involved in the mucosal barrier impairments observed in individuals with ASD. This is supported by studies demonstrating that low levels of serum Gln correlate with intestinal barrier disruption and



inflammation in children (127, 128). Besides its involvement in intestinal barrier function, Gln is also widely known as an anti-inflammatory agent, likely via inhibition of the activation of nuclear factor kappa B (NF- $\kappa$ B) and signal transducers and activators of transcription (STAT) (129). In turn, this inhibition leads to suppression of the production of inflammatory cytokines such as IL-6, TNF $\alpha$  and IL-8 as shown for various immune cells *in vitro*, in animal immune models and in immune-compromised human (129–131). As such, Gln is proposed as candidate for treating inflammatory disorders. Anti-inflammatory effects of Glu include the inhibition of Th2 cells, the stimulation of the production of regulatory cytokine IL-10 by Treg cells and the stimulation of Treg cell development via modulation of the cytokine expression by dendritic cells (132, 133).

Besides their involvement in inflammatory immune responses, Gly and Glu are also neuroactive AAs. Glu is the primary excitatory neurotransmitter of the brain, which is stored in the form of Gln until it is transferred to presynaptic terminals and converted back to Glu (134). Normally, Glu has a protective effect on cognitive function and neural plasticity. However, excessive levels of Glu may be neurotoxic and may play a role in neuroinflammation events in the pathogenesis of ASD (135). Accordingly, multiple studies report higher levels of Glu in serum, plasma and the brain of people with ASD as compared to healthy controls (136–138). In contrast, levels of Gly, a major inhibitory neurotransmitter, are found to be unchanged in patients with ASD (126). The imbalance between Gly and Gln might drive an imbalance in inhibitory and excitatory neurotransmitters, which is indicated to lead to excessive neuroinflammation (137).

As discussed, specific AAs can modify the functions of the immune system in a way that might be beneficial for individuals with ASD. Conversely, the derailed immune system observed in ASD can also modulate the bioavailability of specific AAs, and thereby drive the pathogenesis of ASD. This has been demonstrated most clearly for tryptophan (Trp), of which plasma levels are consistently shown to be lower in ASD patients (139–141). Apart from being a precursor for the neurotransmitter serotonin, Trp is extensively metabolized in the kynurenine pathway (142). This pathway produces several immunomodulatory and neuroactive compounds, examples of which include kynurenine (142) and quinolinic acid (143), respectively. In pro-inflammatory conditions, like ASD, the activation of the kynurenine pathway is enhanced (144), leading to abnormal production of metabolites with neurotoxic effects (144, 145). Furthermore, the kynurenine pathway produces metabolites that induce apoptosis in Th1 cells but not in Th2 cells, suggesting that an increased activation of this pathway favors Th2 polarization, which is characteristic for ASD (146). Finally, aberrant activation of the kynurenine pathway in ASD also diverts Trp from the serotonin synthesis route (144, 147, 148). Psychological complications characteristic for a.o. ASD patients may originate from serotonin deficiency mediated by the depletion of Trp following kynurenine pathway activation (139, 148), which is also reported in a murine model for ASD (149, 150). Whereas, aberrant Trp metabolism might

induce neurotoxicity and a Th2-type immune status, Trp can also act as an anti-inflammatory agent. For instance, dietary supplementation of Trp in a mouse and piglet model for colitis reduced inflammation (151, 152), whereas mice fed a low-Trp diet became increasingly susceptible to chemically induced inflammation (153). Promising effects of a multi-nutrient diet containing high Trp on disturbed behavior and reduced intestinal and CNS serotonin levels are reported in a murine model for ASD (96). Whether dietary supplementation of Trp has therapeutic potential for ASD patients remains open to speculation: it might further drive abnormal activation of the kynurenine pathway, however, it could also provide benefits by reducing inflammation and normalizing serotonin synthesis.

## THE INTERACTION BETWEEN AMINO ACIDS AND THE GUT MICROBIOME IN RELATION TO ASD

Accumulating evidence has demonstrated a bidirectional communication between the brain and the gut, with the intestinal microbiota as a key factor in both health and disease (154, 155). The microbiota can affect cognition and behavior, as has been demonstrated in an increasing number of studies (156–158). Modification of the microbiota composition or absence of the microbiota impacts the hypothalamic-pituitary-adrenal (HPA) axis, the immune system and behavior (158). The importance of gut microbiota in early life and brain development is demonstrated by animal studies showing that maternal separation of neonates does not only lead to a dysbiotic state of the microbiota that persists into adulthood, but also results in long-term cognitive and behavioral deficits (159). Nevertheless, the mechanistic link between the gut microbiota, gut immune homeostasis, and brain functioning remains poorly understood. A variety of mechanisms including immune, hormonal and neural pathways are likely to be involved and may depend on the nature of the microbiota dysbiosis. Also for ASD, the microbiome-gut-immune-brain axis is suggested to be important as profound changes in the microbial composition and activity have been reported (160). Below, we describe the possible interaction between AAs and the gut microbiota in perspective of ASD.

Over the last decades extensive studies have revealed that the gut microbiota composition plays an important role in the metabolism and recycling of nitrogen-containing compounds (161, 162). One of the major nitrogenous compounds utilized by gut bacteria are AAs. AAs derived from either the host or from food enter the gut and are metabolized by gut bacteria into a wide variety of products (163). Hence, it can be speculated that the gut microbiota regulates the AA pool and composition that is available to the host. This is supported by a study demonstrating that gut bacteria alter the AA distribution in the gastrointestinal tract (164). Alterations in AA availability may lead to changes in signaling pathways that are sensitive to AAs, including the mTOR signaling and inflammatory pathways (77, 165). For instance, studies demonstrated that people with ASD

TABLE 1 | Overview of the complex roles of amino acids in the microbiome-gut-immune-brain axis in ASD.

| AA  | Body fluid levels in ASD       | Effects on the microbiota             | Effects on the intestines            | Effects on the immune system                                                                             | Effects on mTOR                                                                                        | Effects in the brain                                                        |
|-----|--------------------------------|---------------------------------------|--------------------------------------|----------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| Leu | ↓ Blood, urine, CSF (126)      | ↓ Proteobacteria (194)<br>↓ F/B (194) |                                      | ↓ MC activation (93)<br>Inflammatory response mφ (95)<br>Essential for lymphocyte proliferation (97, 98) | Essential for Ig production (100)<br>↑ MC and EC (92)                                                  | ↑ Microglia phagocytosis (107)<br>↓ Neuro-protective factor microglia (107) |
| Ile | ↓ Blood, urine, CSF (126)      | ↓ Proteobacteria (194)                |                                      | ↓ MC activation (93)<br>Inflammatory response mφ (95)<br>Essential for lymphocyte proliferation (97, 98) | Essential for Ig production (100)<br>↑ MC and EC (92)                                                  | ↑ Microglia phagocytosis (107)<br>↓ Neuro-protective factor microglia (107) |
| Val | ↓ Blood, urine, CSF (126)      | ↓ Proteobacteria (194)                |                                      | ↓ MC activation (93)<br>Inflammatory response mφ (95)<br>Essential for lymphocyte proliferation (97, 98) | Essential for Ig production (100)<br>↑ MC and EC (92)                                                  | ↑ Microglia phagocytosis (107)<br>↓ Neuro-protective factor microglia (107) |
| His | ↑ Blood (126)<br>↓ Urine (126) |                                       | ↓ Inflammatory response EC (115)     | ↓ MC activation (93)<br>Th2 and Th17 cells (93)<br>↑ Treg cells (93)                                     | ↓ Inflammatory response mφ (116) and PBMC (114, 115)<br>↓ Brain, MC and EC (92)                        |                                                                             |
| Thr | ↓ Blood (126)                  | Fermented into SCFA (163, 174)        | ↑ Epithelial barrier (109)           | ↓ MC activation (93)<br>Th2 and Th17 cells (93)<br>↑ Treg cells (93)                                     | ↓ Brain, MC and EC (92)                                                                                |                                                                             |
| Lys | ↑ Blood, Urine (126)           |                                       |                                      | ↓ MC activation (93)<br>Th2 and Th17 cells (93)<br>↑ Treg cells (93)                                     | Essential for downregulating inflammatory cytokines and NO production (117)<br>↓ Brain, MC and EC (92) |                                                                             |
| Gly | - (126)                        |                                       | Protects EC (118)                    | ↓ Inflammatory response mφ (119)                                                                         |                                                                                                        |                                                                             |
| Gln | ↓ Blood (126)                  | ↓ F/B (191)                           | ↑ Epithelial barrier (123)           | ↓ MC activation (131)<br>Inflammatory cytokine and iNOS production in various immune models (129–131)    |                                                                                                        |                                                                             |
| Glu | ↑ Blood and brain (126)        |                                       | ↑ Epithelial barrier (124, 125)      | ↓ Th2 cells (132, 133)<br>↑ Treg cells and regulatory cytokine IL-10 (132, 133)                          |                                                                                                        | Neurotoxic at high levels (135)                                             |
| Arg | - (126)                        | ↓ F/B (190)                           |                                      |                                                                                                          |                                                                                                        |                                                                             |
| Trp | ↓ Blood (126)                  |                                       | ↓ Intestinal inflammation (151, 152) | Essential for serotonin synthesis (149, 150)                                                             |                                                                                                        | Essential for serotonin synthesis (149, 150)                                |

A selection of the complex effects of AAs on (from left to right) the microbiota composition, intestinal inflammation and intestinal barrier function, the immune system, mTOR activity and on cells in the brain. This table is limited to the effects that are relevant for ASD. The ↓ indicates a downregulation of the corresponding target by the specific amino acid, whereas the ↑ indicates an upregulation. Leu, leucine; Ile, isoleucine; Val, valine; His, histidine; Thr, threonine; Lys, lysine; Gly, glycine; Gln, glutamine; Glu, glutamate; Arg, arginine; Trp, tryptophan; MC, mast cell; EC, epithelial cell; CSF, cerebrospinal fluid; SCFA, short chain fatty acids; F/B, Firmicutes/Bacteroidetes ratio; PBMC, peripheral blood mononuclear cells; Ig, immunoglobulin; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; mφ, macrophages; Th2, T helper 2 cells; Th17, T helper 17 cells; Treg, regulatory T cells.

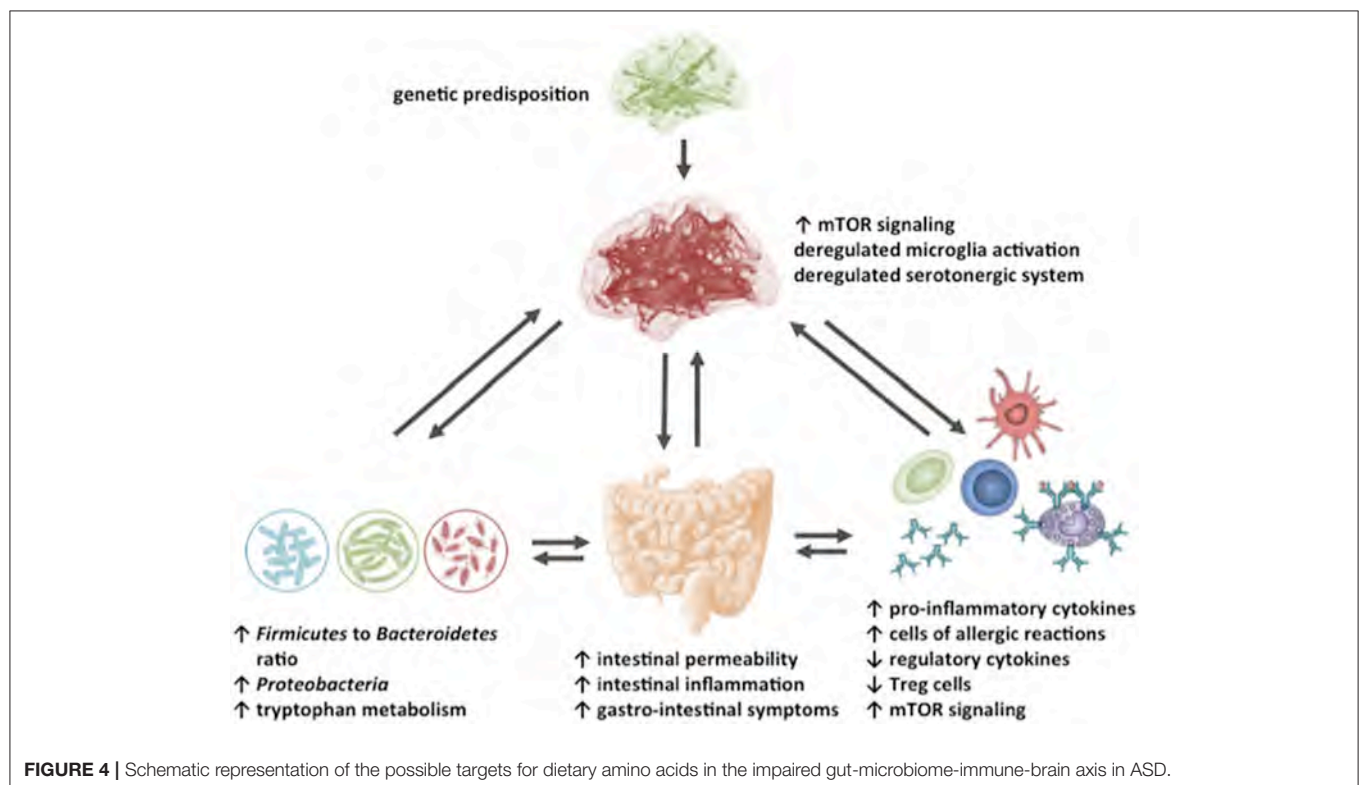


have lower BCAA levels in their plasma, urine and cerebrospinal fluid (141, 166, 167). A deficiency of any of the BCAAs is shown to cause immune cell impairments in various animal models, leading to a more pro-inflammatory immune status (168), which is also observed in people with ASD. A possible explanation for the abnormal levels of BCAAs might be an increased conversion of these AAs by gut *Proteobacteria*, as these are among the most extensive BCAA-fermenting bacteria (169) and are shown to be more abundant in people with ASD (170–172).

Gut bacteria do not only alter AA availability, but they also play a role in the availability of AA-derived metabolites. For instance, levels of Thr are shown to be decreased in patients with ASD (141, 167, 173). Gut bacteria use Thr for a.o. the production of short-chain fatty acids (SCFAs), mainly propionate (163, 174). Studies have revealed an increased abundance of propionate in people with ASD (175, 176). In fact, elevated propionate production is postulated to be involved in the pathogenesis of ASD, as this SCFA is shown to cross the blood-brain barrier and is able to induce ASD-like behavior in adult rodents, possibly via activation of microglia (177–179). The gut microbiome also has a role in the regulation of Trp metabolism (180, 181). Deviations in either endogenous or bacterial Trp metabolism have been associated with a variety of immune-related disorders, including ASD (182, 183). For instance, a recent study demonstrated a significant reduction of serotonin bioavailability in the intestines of a mouse models for ASD (96, 149). This reduction was accompanied by a down-regulation of the gene *Tph1*, indicating a lower

production of serotonin from dietary Trp (149). Interestingly, serotonin production from Trp was positively associated with the abundance of the *Blautia* bacteria present in the gut. In both mouse models of ASD and in humans with ASD, the abundance of *Blautia* bacteria is reported to be lower compared to healthy controls (149, 184). This change in the microbiota might underlie the abnormal Trp-metabolism and serotonin bioavailability that is frequently observed in ASD patients as well as in *in vivo* models for ASD and thus may drive ASD pathogenesis.

As discussed, the gut microbiota influences the bioavailability of AAs and their metabolites. Vice versa, the availability of AAs can also influence the microbiota composition. For instance, several studies have reported that the gut microbiota of people with ASD as well as in murine ASD models is characterized by a significantly higher ratio of *Firmicutes* to *Bacteroidetes*, mainly due to a decrease in *Bacteroidetes* (171, 185, 186). The ratio of *Firmicutes* to *Bacteroidetes*, which are the two most abundant bacterial phyla in human, is considered of significant relevance in human health (187). An increased *Firmicutes/Bacteroidetes* ratio might be involved in inducing inflammation, as this is observed in several inflammatory conditions and as this ratio is shown to be positively associated with dysregulated humoral and cellular immunity (188, 189). Interestingly, dietary supplementation of arginine (Arg) in healthy mice decreased the *Firmicutes* to *Bacteroidetes* ratio in the small intestine, mainly due to an increase in *Bacteroidetes* (190). Similarly, dietary supplementation of Gln decreased the *Firmicutes/Bacteroidetes* ratio in sows (191).



Whether modulation of Gln and Arg intake can restore the aberrant *Firmicutes/Bacteroidetes* ratio observed in ASD patients, and subsequently improve their immune status remains to be examined.

Furthermore, several studies have reported an increase in the abundance of *Proteobacteria* in people with ASD (170–172). *Proteobacteria* are often found to be increased in inflammatory conditions such as colitis and inflammatory bowel disease and have been identified as a marker for microbiota instability and gut inflammation (192, 193). Supplementation of BCAAs was shown to largely reduce the abundance of *Proteobacteria* in mice, accompanied by lower concentrations of inflammatory marker LPS-binding protein (LBP) in serum (194), which has been shown to be elevated in ASD patients (195). In addition, BCAA supplementation also reduced the *Firmicutes/Bacteroidetes* ratio in middle-aged mice (194). However, whether BCAA supplementation is able to restore the abnormal gut microbiota in ASD, and which underlying mechanisms are involved remains to be further investigated.

## CONCLUSION

In **Table 1**, an overview is presented of the complex roles of AAs described in this review regarding the microbiome-gut-immune-brain axis in ASD. Besides behavioral deficits, people with ASD are characterized by systemic inflammation, gastrointestinal immune-related disturbances and changes in the gut microbiota composition. Moreover, differences in levels of specific AAs in

various body compartments, including the intestinal tract, blood, urine and brain have been reported in patients with ASD, as well as in rodent models for ASD. This review described that specific AAs can modulate the intestinal epithelial immune barrier and are able to tune the mucosal immune system, possibly through influencing the mTOR pathway in immune cells. Moreover, specific AAs can influence neuroinflammatory processes and can target the aberrant mTOR signaling in the brain, thereby influencing neuronal activity and disturbed behavior associated with ASD. Finally, though limited reports are available, dietary AAs can influence the changed intestinal microbiota composition and activity of people with ASD. **Figure 4** shows a graphical overview of the different targets for dietary AAs in ASD. Taken together, this review creates more insight in the complex pathogenic relevance of the microbiome-gut-immune-brain axis in ASD and warrants additional research into the opportunities for AA-based nutritional interventions as treatment for ASD in the system medicine approach.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**Conflict of Interest Statement:** JvS, JG, JvB, AH are current employees of Danone Nutricia Research.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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