# Serum tryptophan, tryptophan catabolites and brain-derived neurotrophic factor in subgroups of youngsters with autism spectrum disorders

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**Abstract**

**Background:** There is evidence that changes in neuro-immune responses coupled with dysfunctions in serotonin metabolism underpin the pathophysiology of autism spectrum disorders (ASD).

**Objective**: This study aimed to delineate whether ASD subgroups or characteristics show aberrations in tryptophan and brain-derived neurotrophic factor (BDNF) metabolism.

**Methods:** 65 individuals with ASD (diagnosed according to ICD criteria) and 30 healthy control patients were included. Measured were serum levels of tryptophan, kynurenine (KYN), kynurenic acid (KA), quinolinic acid (QA), BDNF and PRO-BDNF and total blood 5-HT and 5-OH-tryptophan (5-HTP).

**Results:** Elevated BDNF levels and lower tryptophan and KA levels were characteristics of both childhood autism and intellectual disability disorder, whilst elevated tryptophan and lower 5-HT synthesis were hallmarks of Asperger syndrome. A pathological MRI was associated with elevated tryptophan and lowered KA. Abnormal EEG results and dysmorphology were both associated with an elevated BDNF/ PRO-BDNF ratio. Any brain pathology and gastro-intestinal symptoms were accompanied by lowered KA.

**Conclusions:** Increased BDNF production and changes in the metabolism of tryptophan are associated with many ASD characteristics, showing particularly strong associations with childhood autism and Intellectual and Developmental Disabilities. Peripheral BDNF and tryptophan metabolism appear to take part in the pathophysiology of autism spectrum disorders and their phenotypes.

**Key words:** autism spectrum disorders, tryptophan catabolites, brain-derived neurotrophic factor, serotonin, intellectual disability disorder.

## Introduction

Autism spectrum disorder (ASD) refers to a group of complex neurodevelopment disorders characterized by repetitive and characteristic patterns of behavior, and early dysfunctions in social interactions and communication. Wing and Gould introduced the concept of this triad of impairments in autism, and argued for a broader autism phenotype (1). Autism represents different medical conditions rather than one single disease. The term ASD was introduced by Allen and included autistic disorder, Asperger's syndrome, and Pervasive developmental disorder not otherwise specified. Currently, in the DSM-5 diagnostic classifications system the sub-diagnosis have been replaced with ASD (2). ASD includes a spectrum of widespread phenotypic manifestations ranging from debilitating impairments to mild clinical symptoms. ASD is considered as one of the most common neurological developmental disorders, with a reported prevalence of at least 0.5 to 1.5 % (3-5).

Many ASD patients show a high prevalence of comorbid conditions, including language delay and epilepsy with prevalence rates of 50% and 15%, respectively (6, 7). Furthermore, numerous autistic patients suffer from an intellectual disability (ID). The proportion of children with ASD with ID (IQ <70) has decreased markedly from 1996 to 2000 from 61 to 40%, while no significant changes in prevalence were found from 2000 to 2010 (8). The same study (8) reported that ASD prevalence, both with and without ID, increased significantly from 2000 to 2010 with a 6.6% average annual increase for ASD with ID and 9.6% for ASD without ID.

Finally, it is important to note that many patients with ASD also have neuropsychiatric comorbid conditions, such as attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder, and tics (9). This means that ASD is a very complex condition including a broad range of phenotypes and representing a substantial quantity of specific neuro-

psychiatric conditions and syndromes. This complexity is reflected by the fact that the underlying etiological conditions and pathophysiological mechanisms are still unclear.

Case control (10-14) and cohort (15, 16) studies show that birth (obstetric) complications are associated with ASD. A review published in 2007 (17) examined prenatal and perinatal factors affecting risk of childhood autism and ASD. Based on seven epidemiological studies, the authors report that obstetric conditions emerge as significant trigger factors, including birth weight, duration of gestation and intrapartum hypoxia.

A recent meta-analysis (18) investigated whether children with ASD have altered peripheral blood levels of brain derived neurotrophic factor (BDNF). The review included 19 studies with 2896 ASD participants and demonstrated increased peripheral blood levels of BDNF as a manifestation of ASD in children. The authors state that their findings strengthen the clinical evidence of an abnormal neurotrophic factor profile in children with ASD. However, significant heterogeneity among studies was found in the meta-analysis. The authors discuss this and perform an investigation of the heterogeneity. They do not, however, discuss the possibility that the differences in findings among studies may be due to different subdiagnoses among the included ASD children. Previously, we have stressed the importance of such differentiation, as we found differences in several biomarkers in ASD subtypes (19, 20). Subgrouping of ASD patients showed increased BDNF levels in some ASD subgroups, including childhood autism (20).

Recent research on the pathophysiology of ASD is focused on the role of neuroimmune pathways and serotonin (5-HT) metabolism (21, 22). There is some evidence that ASDs are accompanied by a mild immune activation and an inflammatory response (23-25). Differentiation in ASD subgroups revealed significantly differences in cytokines, including IL-8 and IL-10, supporting the idea that an immune-inflammatory response is present in some but not all ASD subgroups, including childhood autism (19).

In addition to changes in neuro-immune responses, increasing evidence shows that dysfunction of the serotoninergic systems may play a role in the pathophysiology of ASD or childhood autism (26-28). The essential amino acid tryptophan is degraded into several neuroactive compounds, including kynurenic acid (KA), 3-hydroxykynurenine (3-OH-KYN) and quinolinic acid (QA), in an enzymatic cascade known as the tryptophan catabolite (TRYCAT) pathway. Elevated pro-inflammatory cytokines activate indoleamine 2,3-dioxygenase (IDO), which leads to an upregulated TRYCAT pathway, and thereby increased tryptophan degradation, both in the periphery and in the brain (23, 29, 30). Chronic activation of the TRYCAT pathway leads to production of several neuroactive, neuroprotective and neurotoxic TRYCATs. For example, QA acts as potent neurotoxin, which inhibits ATP production by mitochondria, activates nitro-oxidative stress pathways, disrupts neuron glial communication and blood brain barrier integrity, induces apoptosis of glial cells, and directly damages neurons and function through its agonistic activity at N-methyl D-aspartate (NMDA) receptors (NMDAr) (31). KA, on the other hand, functions as an antagonist of NMDA, amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors thereby acting to regulate levels of glutamate and dopamine, and binds to the alpha-7-nicotinamide acetylcholine receptor (31). An activated TRYCAT pathway is a feature of a number of medical conditions characterized by immune activation and chronic inflammation (32-34), as well as neuro-psychiatric diseases including Parkinson's disease (35-37), multiple sclerosis (38, 39), stroke (40-43), somatiform disorders (44), schizophrenia (45), psychosis (46) and Alzheimer's disease (47, 48). However, the role of tryptophan metabolism and the TRYCAT pathway in relation to BDNF production in the pathophysiology of ASD subgroups (including childhood autism, Asperger syndrome, ADHD and intellectual disability), possible trigger factors of ASD (including pregnancy and delivery complications) and ASD characteristics

(gastro-intestinal symptoms, dysmorphology, sleeping difficulties, brain imaging, EEG irregularities, pathological neuroimaging) has remained elusive.

Hence, the aim of the study was to delineate the involvement of tryptophan metabolism (measurements: serum tryptophan and whole blood 5-HT and 5-OH-tryptophan), the TRYCAT pathway (measurements: KYN, KA and QA) and BDNF metabolism (measurements: serum BDNF and PRO-BDNF) in ASD children subdivided into the above-mentioned subgroups and to examine the effects of possible trigger factors and ASD characteristics on these 5-HT/TRYCAT and neuro-immune pathways.

## Material and methods

### *Subjects*

The participants in this study were enrolled in an epidemiological study covering two counties in Norway, Oppland and Hedmark, aimed at identifying patients with ASD in that geographical region (49). The diagnostic assessment was conducted by certified personnel based on the Autism Diagnostic Interview–Revised, the Autism Diagnostic Observation Schedule, and the ICD-10 criteria for any ASD diagnosis. Seventy-nine patients who were diagnosed as ASD and agreed to participate in a further investigation underwent a medical examination in accordance with guidelines prepared by Southern and Eastern Norway Regional Health Authority (50). The medical investigation included medical and developmental history and a physical investigation (51). The medical and developmental history focused on birth, medical comorbid symptoms and family history, early development, age at and nature of symptom onset, sleep anamnesis, eating pattern and gastrointestinal symptoms, and existing psychiatric disorders.

Physical investigations included assessing somatic problems in each child, general status (height, weight, head circumference, vision, and organ status), and a neurological

examination (stigmata, skin phenomena, gross and fine motor skills, coordination, reflexes, and hearing function). Additional investigations included blood tests for chromosome investigation (using microarray-based comparative genomic hybridization) and the metabolic screening of urine. Electroencephalography (EEG), cerebral magnetic resonance imaging (MRI) and computer tomography (CT) were also applied when specific indications were present.

Of the 79 patients with ASD invited to participate in the research, blood samples were tested in 65 (52 boys) children with a mean age of 11.2 years. Almost half of the 65 patients (24 boys and 6 girls) were classified as childhood autism; the remaining patients were diagnosed as atypical autism (18.5%, 10 boys and 2 girls), Asperger's syndrome (24.6%, 15 boys and 1 girl), Rett syndrome (1 girl), and another ASD diagnosis (6 patients). 25 (39 %) had intellectual disability (IQ<70), 20 (31 %). Some of the ASD patients showed irregularities on EEG and of the 20 patients with pathological findings, 50 % had a clinical diagnosis of epilepsy according to ICD-10 criteria, and were treated with different kinds of anti-epileptic medication. 15 (23 %) patients had pathological neuroimaging (MRI or CT), the abnormalities found were unspecific and they did not indicate any kind of a specific medical disorder. 15 (23 %) patients had genetic abnormalities, shown as a broad spectre of chromosomal duplications, deletions, and translocations. 6 patients showed neurological abnormalities such as motor disturbances, nystagmus, cerebellar pathology and sensory dysfunction. Parents reported sleeping difficulties in 29 (45 %) of the patients. Birth complications such as emergency caesarean sections, low Apgar score, prematurity, were reported for 19 (29 %) of the ASD children. Pregnancy complications such as infections, diabetes, bleeding, preeclampsia together with perinatal asphyxia, resuscitation, perinatal infections, birth injuries, and congenital malformations were found in one third of the (32.9%) subjects. Medical investigation revealed that 14 (21%) of the patients had some sort of

dysmorphic traits such as macrocephaly, microcephaly, Café au lait spots, big or prominent ears and bilateral epicanthic folds.

The control individuals comprised 30 children (14 boys) with a mean age of 10.9 years who visited the Department of Pediatrics of the Innlandet Hospital Trust at Lillehammer with the following disorders: patients who underwent surgical procedures, patients with noninflammatory gastrointestinal disturbances, patients with noninflammatory cardiac or lung diseases, and healthy individuals.

***Blood collection and serum preparation***

Blood was collected in 7.5-ml serum gel tubes (S-Monovette, Cat. No. 1602, Sarstedt, Nümbrecht, Germany). The samples were left for at least 30 minutes (and a maximum of 120 minutes) at room temperature prior to being centrifuged at 1400×*g* for 12 minutes. The serum samples were kept for no longer than 5 hours before being aliquoted into volumes of 600–1000 μl in Eppendorf tubes, which were frozen instantly and then kept at –80ºC until being used in further experiments.

***Measurement of TRYCATs***

The following components in serum samples from 65 ASD patients and 30 healthy controls were measured using high-performance liquid chromatography (HPLC): KYN, KA and QA (collectively referred to as TRYCATs), as well as tryptophan, 5-HTP and 5-HT. The HPLC analyses were carried out at Laboratory of Medical Biochemistry, University of Antwerp, Belgium. Serum 5-HTP and 5-HT was determined by a modified version of the method of Anderson et al. (1987). For the assay of TRP, the sample was first diluted 50 times with 1% phosphoric acid and then incubated for 10 min at room temperature to liberate TRP from serum albumin. TRP was analysed as above except that the excitation and emission

wavelengths were 275 nm and 340 nm, respectively. QA was determined by HPLC with spectrophotometric detection at 272 nm after solid phase extraction on Oasis MAX 1 cc extraction catridges from Waters. In brief, 500 µl of working internal standard solution (6 nM 3,5-pyridinedicarboxylic acid in water) was added to 500 µl of working standard or serum; 100 µl of 6% phosphoric acid in water (v/v), was added to the serum sample tubes. The samples were loaded on solid-phase extraction tubes. Two washing steps were performed, the first with water and the second with 2% propionic acid in methanol/acetonitrile at 40:60. Finally, the extraction column was eluted with 5% trifluoroacetic acid in methanol/acetonitrile at 40:60. The solvent was evaporated till dryness, redissolved in solvent A, and then injected onto a Hypercarb 3u (100x3 mm) column from Thermo Scientific that was thermostated at 80$^{o}$C. Solvent A consisted of 2% perchloric acid in acetonitrile/water at 6:94, and solvent B was 2% perchloric acid in acetonitrile/water at 50:50. The gradient profile was as follows: starting at 5% solvent B, increasing to 30% at 11 min, than to 70% at 11 min, then to 70% at 11 min, and finally to 95% at 14 min. The flow rate was 1.2 ml/min. The within- and between-run coefficients of variations were 3.9% and 6.0% respectively.

***Measurement of BDNF and PRO-BDNF***

Plasma levels of BDNF and PRO-BDNF were assessed using Derived Neurotrophic Factor ELISA kit and Human Pro Brain-Derived Neurotrophic Factor ELISA kit obtained Nordic BioSite, Sweden.

***Statistical analysis***

Analysis of variance (ANOVAs) is employed to check differences in scale variables among diagnostic groups, whilst analysis of contingency tables ($X^2$-test) is employed to assess associations between nominal variables. Multivariate general linear model (GLM) analysis is

used to delineate the effects of explanatory variables (e.g. ASD subgroups, trigger factors or characteristics) on a set of dependent variables (the biomarkers), while adjusting for other background variables where needed (e.g. age, sex). When there is a significant effect in the multvariate GLM analysis, we carried out tests for between-subject effects to check univariate effects of the significant explanatory variables on the dependent variables. Estimated marginal means (SE) were computed and displayed (see table 3) for those contrasts that showed significant tests for between-subject effects. Parameter estimates were employed to check the impact and sign of scale independent variables on the dependent variables. We used binary regression analyses to delineate the most significant independent variables of dichotomous dependent variables, including childhood autism, Asperger's syndrome, dysmorphology, etc. We computed the odds ratio and 95% confidence intervals as well as Nagelkerke values and classification tables with sensitivity and specificity. We used Ln transformations of the scale variables to normalize the data distribution of the biomarkers (checked with the Kolmorov-Smirnov test), namely BDNF, PRO-BDNF, tryptophan, KYN, KA and QA. All biomarkers were z-transformed and the estimated marginal means (SE) of these z-values are shown in table 3. We computed z unit weighted composite scores (45) yielding two new variables based on the BDNF data, namely BDNFtot as z value BDNF (zBDNF) + zPRO-BDNF (reflecting total BDNF circulating in the plasma) and BDNF/PRO-BDNF ratio as zBDNF – zPRO-BDNF (reflecting the ratio between mature BDNF and PRO-BDNF levels). We additionally computed two new z unit weighted composite scores based on the tryptophan data, namely KYN/tryptophan ratio as zKYN – ztryptophan (reflecting IDO activity) and (5-HT+5-HTP)/tryptophan ratio as z5-HT + z5-HTP – ztryptophan (reflecting activation of the 5-HT synthesis pathway). All statistical analyses were performed using IBM SPSS windows version 22. Tests were 2-tailed and a p-value of 0.05 was used for statistical significance. Here we include some BDNF (20) and TRYCAT (namely tryptophan, KA, KYN and QA) (52) results,

which were published earlier, in order to examine their cumulative effects and the effects of new pathway-derived variables using multivariate statistical analyses performed on different ASD phenotypes.

## Results

### Socio-demographic data

The clinical data of the ASD population included in this study have been published previously (19). **Table 1** shows the demographic and biomarker data in subjects with ASD and normal controls. There were no significant differences in age between the two groups, while there were more males in the ASD study sample. BDNF and BDNFtot were significantly higher in ASD patients than in controls. KA was significantly lower in ASD subjects than in controls. There were no significant differences in the other variables among the study samples.

### Childhood autism and biomarkers

**Table 2** shows the results of 8 multivariate GLM analyses with the 4 BDNF biomarkers and 6 tryptophan biomarkers as dependent variables and ASD subgroups and characteristics as explanatory variables. Age (F=1.13, df=8/82, p=0.350) and sex (F=1.04, df=8/82, p=0.415) were not significant in this analyses and therefore we do not show these data in Table 2. The number of subjects who were included in the multivariate analysis was somewhat lower than the number of subjects shown in table 1 (namely 2 controls and 2 ASD) because there were 4 missing values in the multivariate data set, namely 1 5HT, 1 KYN and 2 QA). Regression #1 shows that there was a significant association between childhood autism and the biomarkers. Tests for between-subject effects showed significant effects of childhood autism on BDNF, BDNFtot, tryptophan and KA. **Table 3** shows the estimated marginal

means (SE) of the significant biomarkers indicating that childhood autism is accompanied by increased BDNF as compared with the other 2 study samples, increased BDNFtot as compared with controls, lowered tryptophan as compared with ASD subjects without childhood autism and lowered KA as compared with controls. **Electronic Supplementary File (ESF),** Figure 1 shows the mean values of the 10 biomarkers (in z transformations) in controls and ASD subjects with and without childhood autism.

**IDD and biomarkers**

Regression #2 shows that there were significant associations between IDD and the biomarkers. Tests for between-subjects effects and Table 3 (estimated marginal means) show that BDNF and BDNFtot were significantly higher in IDD patients as compared with non-IDD ASD patients and controls, tryptophan was significantly lower in IDD patients as compared with non-IDD ASD patients, and KA was lower in IDD as compared with controls. In order to examine whether IDD or childhood autism is associated with BDNF, BDNFtot, tryptophan and KA we performed a GLM analysis with those 4 biomarkers as dependent variables and IDD and childhood autism as explanatory variables. This analysis showed a significant effect of IDD ($F=3.23$, $df=4/89$, $p=0.016$; partial eta-squared=0.127), but not childhood autism ($F=1.26$, $df=4/89$, $p=0.291$; partial eta squatted=0.054). **Electronic Supplementary File (ESF),** Figure 2 shows the mean values of the 10 biomarkers (in z transformations) in controls and ASD subjects with and without IDD.

**Asperger's syndrome and biomarkers**

Table 2, regression #3 and Table 3 show significant associations of Asperger's syndrome with the biomarkers. While ASD without Asperger's syndrome was associated with increased BDNF and BDNFtot and lowered KA as compared with controls, those with Asperger's syndrome occupied an intermediate position and did not differ significantly from

either controls or ASD patients without Asperger syndrome. Moreover, tryptophan was higher in Asperger's syndrome as compared with controls and ASD without Asperger syndrome, while (5-HT+5-HTP)/ tryptophan ratio was lower in Asperger's syndrome versus ASD without Asperger syndrome. **Electronic Supplementary File (ESF),** Figure 3 displays the mean values of the 10 biomarkers (in z transformations) in controls and ASD subjects with and without Asperger's syndrome. There was no significant effect of ADHD. Although ADHD had a significant overall effect (F=1.96, df=16/164, p=0.019) post hoc analyses showed increased BDNF in both ASD patients with and without ADHD.

**ASD characteristics and biomarkers**

Table 2, regression #4 and Table 3 show that lowered KA was associated with ASD when combined with pathological MRI findings, whereas no such changes were found in ASD without pathological MRI findings or normal controls. Table 2, regression #5 and Table 3 show that BDNFtotal is higher and KA lower in ASD with gastro-intestinal symptoms as compared with controls. ASD with dysmorphology (regression #7) was characterized by increased BDNF/PRO-BDNF ratio as compared with normal controls, whereas ASD subjects without dysmorphology took up an intermediate position. Table 2, regression 7 shows that there was a trend toward a significant effect of birth complications on serum tryptophan levels (p=0.051). Table 3 shows a trend towards increased tryptophan levels in ASD subjects with birth complications as compared to the two other groups.

We have also examined the effects of other possible explanatory variables on the 10 biomarkers and found no significant effects of birth weight (F=0.69, df=8/46, p=0.697), age mother (F=0.83, df=8/45, p=0.583), age father (F=1.83, df=8/40, p=0.101), and pregnancy complications (F=0.82, df=8/46, p=0.591).

**Best predictions of ASD phenotypes and characteristics**

Table 4 shows the results of binary regression analyses with the ASD subgroups as dependent variables and the biomarkers as explanatory variables. Both ASD ($X^2$=9.17, df=1, p=0.002, Nagelkerke=0.135) and childhood autism ($X^2$=10.69, df=1, p=0.001, Nagelkerke=0.155, sensitivity=48.3% and specificity=75.8%) were predicted by increased BDNF values. IDD was predicted ($X^2$=20.79, df=2, p<0.001, Nagelkerke=0.295; sensitivity=52.0% and specificity=90.9%) by increased BDNF and lowered tryptophan values. Asperger's syndrome was best predicted ($X^2$=9.59, df=1, p=0.002, Nagelkerke=0.169) by increased tryptophan levels. Pathological MRI scans were significantly ($X^2$=15.62, df=2, p<0.001, Nagelkerke=0.304; sensitivity=50.0% and specificity=85.2%) associated with lowered KA but increased tryptophan levels. The subgroup with abnormal EEG measurements was characterized by increased BDNF/PRO-BDNF ratio ($X^2$=5.31, df=1, p=0.021, Nagelkerke=0.095). The subgroup with any brain pathology (that is an abnormality on EEG, MRI or brain nerve measurements) was characterized by lowered KA levels ($X^2$=5.31, df=1, p=0.021, Nagelkerke=0.095). Gastro-intestinal symptoms were best predicted by lowered KA levels ($X^2$=5.15, df=1, p=0.023, Nagelkerke=0.079), while dysmorphology was best predicted by increased BDNF/PRO-BDNF ratio ($X^2$=5.55, df=1, p=0.019, Nagelkerke=0.093).

**Discussion**

The first major finding of this study is that ASD children with an additional diagnosis of *IDD* had significantly higher levels of BDNF and total BDNF and significantly lower tryptophan levels as compared with non-IDD ASD patients and controls. Furthermore, KA levels were significantly lower in IDD patients as compared with controls, but not significantly different from non-IDD ASD patients. Here and in previous studies [15,17] we

reported comparable aberrations in these biomarkers in childhood autism. Croonenberghs and co-workers (53) found lower plasma tryptophan concentrations in 26 autistic subjects (aged 12-18 years) than in normal volunteers. Gevi et al. (54) showed differences in TRYCATs including KA, KYN, QA, 5-HT levels, in 30 ASD patients (aged 3-7 years) when compared to controls. There is a partial overlap between both childhood autism and IDD, namely in our study 16 of the 25 IDD patients showed childhood autism and the 9 others not. Importantly, we investigated whether the changes in the biomarkers were attributable to IDD or childhood autism and detected that IDD, and not childhood autism, was statistically significantly associated with changes in these biomarkers. To the best of our knowledge, no other studies have examined tryptophan or tryptophan catabolites in autistic children with IDD. Evidence from several studies with different methodological approaches suggests that both peripheral and CNS immune-inflammatory mechanisms may participate in the pathophysiology of both autism and IDD (55). This is supported by our previous findings that the level of interleukin-8 was significantly higher, while that of IL-10 was significantly lower in patients with childhood autism than in controls (19). Therefore, lowered tryptophan levels in IDD (and childhood autism) may be caused by immune-activation associated IDO-activation. On the other hand, we could not detect significant increases in the KYN/tryptophan ratio (computed as z KYN – z tryptophan), which would indicate increased IDO activity.

A second major finding is that tryptophan levels were higher in Asperger's syndrome than in controls and ASD patients without Asperger's syndrome (Bryn et al., 2017) and that the (5-HT+5-HTP)/ tryptophan 5-HTP/tryptophan ratio was lower in Asperger's syndrome versus ASD without Asperger syndrome. It has been suggested that central serotonergic responsivity is decreased in ASD (56), and that systemic alterations in serotonergic function may occur in autism (57). Additional evidence of central nervous system serotonergic abnormalities in autism was obtained in brain imaging studies (56). Elevated blood 5-HT

levels were in fact the first biomarker identified in ASD (26). Earlier studies also reported an elevated peripheral 5-HT synthesis in ASD. For example, Croonenberghs et al. [28] detected that administration of 5-HTP to autistic youngsters and controls increased 5-HT levels more in autistic youngsters than in controls, indicating an increased synthesis of 5-HT from 5-HTP. Our finding of elevated (5-HT+5-HTP)/ tryptophan ratio indicates that Asperger's syndrome is accompanied by lowered activity of the peripheral 5-HT synthesis pathway, which in turn could contribute to increased plasma tryptophan levels. Thus, it appears that the serotonergic pathophysiology may differ between Asperger's syndrome and IDD or childhood autism, which are accompanied by lowered tryptophan levels. Other differences between Asperger's syndrome and IDD/childhood autism are that the latter is accompanied by increased BDNF and lowered KA levels, whereas no such alterations are detected in Asperger's syndrome.

The third major finding of this study is that ASD characteristics are also accompanied by specific changes in BDNF production as well as the metabolism of tryptophan. Firstly, ASD subjects with a pathological MRI showed elevated tryptophan and lowered KA values, whilst ASD subjects with an abnormal EEG and dysmorphology showed elevated BDNF and PRO-BDNF levels. Furthermore, ASD subjects with any brain pathology and gastro-intestinal symptoms showed lowered KA levels. These findings show that the same pathways (tryptophan, TRYCAT and BDNF metabolism) which are associated with clinical diagnoses, are also associated with organic brain pathologies that underpin the same disorders. We will now discuss the possible involvement of these pathways in clinical ASD diagnoses and characteristics.

Firstly, lowered KA may be associated with the pathophysiology of IDD especially when abnormal MRI findings are present. KA acts as an endogenous antagonist of the glutamate-binding *N*-methyl-D-aspartate (NMDA) receptor, and, therefore may be neuroprotective by inhibiting excessive glutamate excitation and antagonizing the neurotoxic

effects of QA (58). In addition, KA has antioxidant and anti-inflammatory properties (59). Thus, lowered KA levels may lead to less neuroprotection and enhanced neuro-immune and neuro-oxidative pathways thereby increasing neurotoxic effects.

Secondly, our results indicate that elevated tryptophan levels are associated with a pathological MRI. Under physiological conditions, tryptophan and 5-HT have antioxidant and anti-inflammatory effects and therefore are neuroprotective (60-62). Nevertheless, a recent meta-analysis shows signs of increased oxidative stress in autism including lowered levels of many antioxidants [56]. Moreover, nitric oxide (NO) is significantly higher in children with autism as compared to healthy subjects (63), whilst dysregulated NO signaling is thought to play a role in neuropsychiatric disorders including ASD [57]. During immune activation tryptophan is a target of oxidative and nitrosative stress whereby tryptophan may become nitrosylated into nitric oxide (nitroso-)- tryptophan (NOW), as detected in patients with major depression (64). Reactive nitrogen species have the ability to modify a variety of amino acids on proteins, including nitration of aromatic amino acids, such as tryptophan, often resulting in modulation of the modified protein's function (65). S-nitrosylation, that is, the posttranslational modification of cysteine residues to regulate protein function, has been suggested to contribute to neurodevelopmental disabilities such as ASD (66). Moreover, severe nitrosylation has detrimental effects, including neurotoxic and neurodegenerative effects (67). Furthermore, the TRYCAT pathway is part of the 'compensatory (anti)inflammatory reflex system' (CIRS), which tends to downregulate an overzealous primary inflammatory response (68). Most TRYCATs (except QA) have anti-inflammatory effects and thus attenuate the primary inflammatory response through negative feedback mechanisms (30). On the other hand, increased levels of some TRYCATs may be detrimental, since they may exert cytotoxic, neurotoxic, excitotoxic, inflammatory and oxidative effects (31). Our findings may indicate that lowered KA levels and activity of the TRYCAT pathway,

as indicated by increased tryptophan, may further attenuate the negative immunoregulatory effects of TRYCATs.

Thirdly, our results indicate that increased BDNF and an increased BDNF/PRO-BDNF ratio are hallmarks of ASD, IDD, and pathological brain measurements including EEG and dysmorphology. While BDNF has neuroprotective, anti-inflammatory and anti-apoptotic properties, PRO-BDNF may induce neuronal apoptosis and death of cerebellar, motor and sympathetic neurons (69-71). Some reports also show that BDNF and PRO-BDNF may differently modulate synaptic plasticity (72). Thus, it is important to investigate both PRO-BDNF and BDNF, as well as the total BDNF levels, which reflect production. As already mentioned, a recent meta-analysis demonstrated increased peripheral blood levels of BDNF in ASD children (18). One theory is that increased BDNF may contribute to the pathophysiology of ASD through its ability to enhance synapse formation (18), a mechanism that would explain why subjects with ASD have increased numbers of synapses in the brain (73). It is interesting to note that under mitogenic stimulation, BDNF production was significantly increased in children with autism compared with typically developing subjects (74). These results suggest that immune cell-derived production of BDNF could be an important source of increased BDNF in autism. As such there are two possibilities: a) increased BDNF in ASD is a surrogate biomarker of immune activation without any causal relationship with the onset of ASD, or b) immune activation-related increases in BDNF play a role in the pathophysiology of autism and its phenotypes. These results are not affected by Val66Met genotypes as we were unable to find significant effects of this genotype on PRO-BDNF and BDNF levels (data now shown).

Fourthly, we detected that lowered KA levels were also associated with gastro-intestinal symptoms. This is an interesting finding given that some autistic patients show alterations in gut microbiome, gut permeability and maybe increased production of neurotoxic

bacterial catabolites (75, 76). In rodent models, colonic permeability is enhanced in mice that showed lower muscle kynurenine conversion into KA (77). This supports the theory that KA has some protective effects on gut permeability. There are, however, no studies in humans corroborating this theory.

Finally, our results show a trend toward a significant effect of birth complications on increased serum tryptophan levels (p=0.051). One study has reported that birth complications, especially at delivery where the fetus showed signs of physiological distress, predicted lower serotonin synthesis capacity (measured $^{11}$C-AMT trapping as a proxy), in the hippocampus and medial orbitofrontal cortex. These results suggest that limbic serotonin pathways may be particularly vulnerable to environmental challenges during the period when they undergo the most prominent neurodevelopmental changes. Higher levels of tryptophan, as found in our study, should enhance serotonin synthesis although it may be that more tryptophan has remained in the periphery thereby decreasing transport to the brain and consequently 5-HT synthesis.

A limitation of this study is that it has a cross-sectional design and therefore no causal modeling is possible. A strength is that we studied well-phenotyped ASD subjects and well-defined biomarkers using multivariate analyses, which gives the opportunity to study cumulative effects of pathway-derived biomarkers.

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## STATEMENT OF INTEREST

None to declare.

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**Figure 4** summarizes our results. In conclusion, this study reveals an association between increased BDNF production and changes in the metabolism of tryptophan and several characteristics of autism spectrum disorders in particular childhood autism and IDD. It is concluded that peripheral BDNF and tryptophan metabolism may take part in the pathophysiology of ASD and their phenotypes. Future research should examine the differences in the TRYCAT pathway, 5-HT synthesis and neurotrophic factors in relation to neuro-immune, neuro-oxidative and neuro-nitrosative pathways in the different ASD phenotypes.