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Kynurenine pathway and autism spectrum phenotypes: an investigation among adults with autism spectrum disorder and their first-degree relatives

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Abstract

Background. Increasing literature highlighted alterations of tryptophan (TRP) metabolism and kynurenine (KYN) pathway in children with autism spectrum disorder (ASD). However, no study specifically focused on adult samples. Meanwhile, several authors stressed the relevance of investigating neurobiological correlates of adult forms of ASD and of those subthreshold ASD manifestations frequently found in relatives of ASD probands, known as broad autism phenotype (BAP). This work aimed to evaluate circulating levels of TRP and metabolites of KYN pathway in a sample of ASD adults, their first-degree relatives and controls (CTLs), investigating also the correlations between biochemical variables' levels and ASD symptoms.

Methods. A sample of ASD adults, together with a group of first-degree relatives (BAP group) and unrelated CTLs were assessed by means of psychometric scales. Circulating levels of TRP, KYN, quinolinic acid (QA), and kynurenic acid (KYNA) were assessed in all subjects.

Results. ASD patients reported significantly higher total scores than the other groups on all psychometric scales. BAP subjects scored significantly higher than CTLs. ASD patients reported significantly lower TRP levels than BAP and CTL groups. Moreover, significantly lower levels of KYNA were reported in both ASD and BAP groups than in CTLs. Specific patterns of associations were found between autism symptoms and biochemical variables.

Conclusions. Our findings confirm in adult samples the presence of altered TRP metabolism through KYN pathway. The intermediate alterations reported among relatives of ASD patients further stress the presence of a continuum between subthreshold and full-threshold ASD phenotypes also from a biochemical perspective.

Introduction

Autism spectrum disorder (ASD) represents a highly heterogeneous neurodevelopmental condition characterized by an early onset of dysfunctions in social interactions and communication, stereotyped and rigid patterns of behavior, restricted interests, and impaired sensory integration processing, with or without intellectual impairment and language development alterations.[1] Although classically most of researches in the field of ASD were focused on children, recently there has been a growing interest in evaluating ASD presentations in adulthood. In particular, several authors stressed the importance of recognizing, among adult populations, milder forms of ASD, without intellectual impairment, which often remain underdiagnosed during childhood.[2-4] Adults patients with milder forms of ASD often come to clinical attention after the development of other psychiatric disorders, whose outcome is typically worsened by the comorbid ASD condition.[2-4] Moreover, the importance of evaluating the presence not only of milder ASD forms, but also of subthreshold autistic traits is recently being highlighted in the literature, due to the growing body of evidence that has associated these characteristics, even if below the threshold, with significant clinical correlates, including greater vulnerability toward the development of psychiatric disorders, limited adjustment to environmental stimuli/stressors, and suicidal ideation.[2-4] Subthreshold of full-threshold ASD features have been reported to be a vulnerability factor for developing trauma and stress-related symptoms also after milder life events, while the continuous use of strategies such as social camouflaging by ASD people in order to mask their symptoms and adjust to the environment may be itself a source of great distress, facilitating the development of anxiety and depressive symptoms.[3]

Subthreshold autistic traits were initially investigated among close relatives of ASD subjects not clinically affected by the disorder, who were often reported to show personality traits similar

to those of their probands, although less severe,[5,6] leading to the conceptualization of a "broad autism phenotype" (BAP).[2-5,7] This condition features many of the autism core symptoms, such as narrow interests, repetitive behaviors, deficits in social communications, and social skills even if of subclinical severity.[5,6,8] More recently, autistic traits were reported not to be limited to relatives of ASD probands, seeming also to be distributed in a continuum from the general to the clinical population,[9-13] further stressing the importance of investigating clinical and biological correlates of different phenotypes of the autism spectrum.

The underlying pathophysiological mechanisms of ASD remain mostly unknown. It is now clearly the key role of genetic heritability,[14-16] although it was also stressed the importance of the interaction between genetic vulnerability and environmental factors[17] such as mother's diabetes and obesity, oxidative stress, and maternal immune deregulation.[18,19] Some studies highlighted an increased immune and inflammatory response in ASD[20-22] even if the importance of immunological factors in ASD pathophysiology is still unclear.[7] Increased prooxidant species, together with decreased antioxidant ones, including glutathione depletion, were also reported in ASD children.[23] From a biochemical point of view, one of the most investigated factors in this field is the alteration of serotonin (5-HT) system.[24-26] About a third of ASD children seem to show hyperserotonemia, in particular when measured in platelets. However, this result appears to be less stable among adults and greatly vary depending on the specimen.[24,27,28] Considering BAP, even if an increasing body of evidences from neuroimaging studies corroborates the presence of significant neurostructural and neurofunctional alterations among first-degree relatives of ASD probands,[4,6,29] there is still a lack of biochemical research on this specific matter.[7,29] Biochemical studies including parents or siblings of ASD patients are very limited in number, and in some cases, this population has been used instead as a control group.[24] Recently, the possible role of tryptophan (TRP) metabolism gained interest in ASD studies, to the point that some authors hypothesized that altered TRP metabolism might be identified as "the unifying biochemical basis for ASD."[30] In mouse models, increased dietary TRP supplement was reported to reduce aggressiveness and to promote social behaviors, which are worsened by a TRP intake reduction.[31-33] In humans, acute TRP depletion has been linked to deficits in episodic memory consolidation for verbal information,[34], whereas in both adults and children with ASD, dietary deprivation of TRP seems to worsen ASD symptoms.[23,33,35]

TRP is as an essential amino acid that, through its catabolism, plays a significant role in neurogenesis and neurodegeneration.[36,37] Through the methoxyindole pathway, TRP is the precursor of peripherally and centrally produced serotonin (5-HT),[38] and acute depletion of TRP was reported indeed to inhibit 5-HT synthesis.[33,39] However, the relevance of TRP metabolism is not limited to the 5-HT pathway, but is also related to the regulation of excitotoxicity, oxidative stress, and immune and inflammatory responses, of which many TRP-derived metabolites act as key mediators.[33,40] In particular, most of the available TRP is metabolized through the kynurenine (KYN) pathway, or TRP catabolite (TRYCAT) pathway, which, through different branches, leads to the production of several crucial metabolites, such as kynurenic acid (KYNA), quinolinic acid (QA), picolinic acid (PA), nicotinamide adenine dinucleotide (NAD^+), and adenosine triphosphate.[41,42] The primary intermediate metabolite of the KYN pathway is the N-formyl-L-KYN, obtained through the action of the enzyme indole-2,3-dioxygenase (IDO) in the extrahepatic tissues[43] or, in a minor quote, by the TRP-2,3-dioxygenase (TDO) (mainly in the liver and kidneys).[44] This intermediate is then metabolized by the KYN formamidase into L-KYN, which opens the way to 2 different possible pathways: the so-called "neurotoxic branch" and the "neuroprotective branch." The "neuroprotective branch," via the enzyme KYN aminotransferase (KAT),[33,45] ends with the production of KYNA.[46] KYNA is known to exert a neuroprotective effect in various ways: first, it is an antagonist of N-methyl-D-aspartate (NMDA) receptors, being involved in the modulation of excitotoxicity linked to glutamate transmission. KYNA is also an antagonist of kainate and α-7 nicotinic acetyl choline receptors.[33,45] Moreover, KYNA can induce the amyloid degrading enzyme, scavenge free radicals, and act as an antioxidant; as an agonist of G-protein coupled receptor (GPR35), KYNA may promote the inhibition of the N-type Ca channels in the neural tissues and the regulation of the production of cyclic adenosine monophosphate (c-AMP).[33,45] Lastly, KYNA also seems to exert an agonistic activity on the aryl hydrocarbon receptor which interrupts the release of cytokines in macrophages and other kinds of cells.[33] Despite that, it should be noted that increased levels of KYNA have been hypothesized to exert a detrimental effect through an over-reduction of the cholinergic transmission.[33,45] Through the "neurotoxic branch," KYN is transformed into 3-hydroxykynurenine (3-HK), which is then metabolized in 3-hydroxyanthranilic acid (3-HAA): this latter leads to the production of QA or of PA. Finally, QA can be then further transformed, by QA phosphoribosyl transferase, into NAD^+. QA is an agonist of NMDA, kainate, and α-7 nicotinic acetyl choline receptors, and it is apparently capable of activating specific types of NMDA receptors in the striatum and the hippocampus: thus, its elevation may lead to altered glutamate transmission, which was reported in many inflammatory CNS diseases.[47] QA, acting as a glutamatergic excitotoxin during inflammation, may provide more energy to cells by increasing NAD+ levels.[33,48] Noticeably, NAD+ levels were reported to be decreased in ASD.[33] QA mediated excitotoxicity may be involved in ASD by altering the neuronal development, the formation of synapses, and neuronal connectivity since intrauterine life.[33] QA was also reported to increase prooxidant and reduce antioxidant power, promoting the decrease of glutathione levels and superoxide dismutase activity[33] and the elevation of intracellular calcium levels, which can lead to an impairment in cell homeostasis and mitochondrial functions, eventually inducing neuronal apoptosis.[33,49,50] Other metabolites of the KYN neurotoxic branch may show further detrimental effects, as in the case of the induction of oxidative stress by 3-HK and 3-HAA and of the induction of apoptosis by 3-HK. On the other hand, 3-HAA may have a neuroprotective activity by suppressing the immune and inflammatory activity.[45,51] As a matter of fact, both KYNA and QA are considered relevant modulatory compounds of neuronal circuits, acting in different ways in specific brain areas during the development of CNS,[52] as highlighted by many studies that stressed their function in synaptogenesis.[53-55] Noticeably, it was reported that, while KYN pathway may modulate the inflammatory response, inflammatory processes through several cytokines may induce, in a vicious circle, the TRP metabolism through the KYN pathway enhancing IDO and TDO activities. Adrenal cortisol hormones, secreted in response to distress, can also induce the KYN pathway, specifically shifting it toward the production of QA.[56] The role of gut microbiota in TRP metabolism should also be considered. In particular, gut microbiota may be responsible of the shifting of TRP metabolism through the indole route by gut bacteria, increasing indolyl 3-acetic acid and indolyl

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lactate.[57] These data are of particular interest when considering the frequent report of gut microbiota alterations among ASD children.[7]

In the field of ASD, TRP levels were mainly investigated in child samples, typically reporting reduced circulating levels of TRP (and/or increased TRP concentrations in the urine) when compared with controls,[32,58-61] although these results have not always been confirmed.[62,63] Results on post-puberal patients were limited: Minderaa et al[64] did not find differences between ASD subjects and controls with respect to TRP levels, whereas Croonenberghs et al[65] reported reduced levels of TRP in a sample of ASD individuals with an intelligence quotient of >55. Noticeably, a more recent study highlighted increased levels of TRP in the urine of subjects with infantile autism than in those of subjects with Asperger's syndrome.[40] Studies on the KYN pathway in ASD are even more limited. The first studies in this field did not find significant differences between ASD children and controls with respect to QA levels in cerebrospinal fluid[66] or plasma KYN levels.[67] More recently, Lim et al[23] investigated 15 Omani families with ASD children, using their age-matched healthy siblings as a control group. They reported an increased KYN/TRP ratio and QA concentrations together with reduced PA among ASD patients. The authors hypothesized that an increased KYN/TRP ratio may reflect the IDO activity, which may be modulated by several cytokines, eventually implying an involvement of neuroimmune mechanisms in ASD pathogenesis.[23] Bryn et al[51] reported lower circulating levels of KYNA and higher KYN/KYNA in ASD children than in controls, without significant differences for TRP, QA, and 3-HK levels. They also reported lower TRP levels in the Childhood autism subgroup with respect with the Asperger's syndrome subgroup. The increased KYN/KYNA ratio led the authors to hypothesize an increased neurotoxic potential, eventually underlain by a reduced KAT activity, as previously highlighted for Parkinson's or Alzheimer's disease.[51] A further study in ASD children and controls[68] reported lower serum TRP and KYNA levels among subjects with Childhood autism and intellectual disability disorder (IDD), whereas among subjects with Asperger's syndrome, TRP levels were instead increased, showing also a lower 5-HT synthesis. The authors suggested that reduced TRP levels may be specifically linked to IDD.[51] No difference was found for KYN. A more recent study led among younger children highlighted instead reduced 3-HAA and increased 3-HK and KYNA levels in serum of ASD patients than of controls, without differences in TRP and KYN levels.[45] Globally, studies on KYN pathway in ASD are still limited, showing a specific lack of investigations on adult samples and on the issue of BAP.

In this framework, our study aimed to evaluate circulating levels of TRP and its metabolites of the KYN pathway (specifically KYN, KYNA, and QA) in a sample of adult ASD patients and of their first-degree relatives, focusing also on investigating eventual correlations between the levels of biochemical parameters and specific autism spectrum symptoms, as measured by the psychometric scales.

Methods

Recruitment procedures

A group of ASD subjects (ASD group) with no or only milder intellectual impairment and/or language development alteration was recruited among adult out- or in-patients treated at the Psychiatric University Clinic of the Azienda Ospedaliera Universitaria Pisana. A group of ASD patients' first-degree relatives was also recruited, as a population with increased presence of BAP features according to previous literature.[4,23,24] In order to recruit the relatives' group, which was labeled "BAP group," for each recruited patient, the participation in the study of one relative (a parent or a sibling) was requested. Major exclusion criteria for both groups were: being unable to fill out the assessments due to language or intellectual impairment; a diagnosis of Schizophrenia, substance use disorder, neurodegenerative diseases, or other relevant medical/neurological disorders. Due to the reported high prevalence of comorbid anxiety or mood disorders in adult ASD patients,[2-4] the presence of other mental disorders (with the exception of the above-reported ones) was assessed but not considered in the exclusion criteria. In addition, for the ASD group, subjects were excluded if aged below 18 or over 65 years, whereas for the BAP group, they were excluded only if aged below 18 years. A further exclusion criterion for the BAP group was the presence of a diagnosis of ASD or of another neurodevelopmental disorder according to DSM-5. The CTL group was recruited on a voluntary basis. Exclusion criteria for this group were the same used for the ASD group, with the exception that subjects in the CTL group were also excluded if they received a diagnosis of a psychiatric disorder according to DSM-5 criteria. All subjects were assessed by means of a structured clinical interview and psychometric scales. In order to perform the biochemical evaluation, a blood sample was collected from each participant. All subjects received clear information about the study and had the opportunity to ask questions before providing a written informed consent. The study was conducted in accordance with the declaration of Helsinki, and all procedures were approved by the local ethical committee. All data were treated according to Italian and European Privacy laws and rules.

Psychometric instruments

Psychometric evaluation included a preliminary structured interview with mental health professionals by means of the Structured Clinical Interview for DSM-5 Disorders (SCID-5), which is the gold standard instrument for the assessment of major mental disorders according to DSM-5 criteria.[69] In addition, patients were assessed with the Adult Autism Subthreshold Spectrum (AdAS Spectrum), the Ritvo Autism and Asperger Diagnostic Scale, 14-item version (RAADS-14), the Ruminative Response Scale (RRS), and the Work and Social Adjustment Scale (WSAS).

The Adult Autism Subthreshold Spectrum

The AdAS Spectrum is an instrument tailored for the assessment of the wide range of subthreshold and full-threshold autism spectrum manifestations in adults without intellectual or language impairment.[70] It is composed of 160 dichotomous items, grouped in 7 domains: *Childhood/adolescence*, *Verbal communication*, *Nonverbal communication*, *Empathy*, *Inflexibility and adherence to routine*, *Restricted interests and rumination*, and *Hyper-hypo reactivity to sensory input*. In the validation study, the instrument demonstrated excellent reliability and strong correlations with other scales frequently used in this field, such as the RAADS-14 or the AQ (Pearson's r correlation = 0.77).[70]

The Ritvo Autism and Asperger Diagnostic Scale

The RAADS-14 is a questionnaire developed as a brief version of the RAADS-Revised, aiming to evaluate the presence of autism spectrum symptoms. It is composed of 14 items, with answers organized in a 4-point Likert scale. The RAADS-14 features

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3 domains: *Mentalizing deficits, Social anxiety*, and *Sensory reactivity*. The instrument reported excellent internal consistency in the validation study.[71]

The Ruminative Response Scale

The RRS is a questionnaire designed for assessing the tendency toward ruminative thinking, which showed excellent internal consistency in the validation study.[72] The version used in this work is composed of 22 items, grouped in 3 dimensions: *Brooding*, *Reflection*, and *Depression*. Answers are rated on a 4-point Likert scale. [73]

The Work and Social Adjustment Scale

The WSAS is an instrument widely used in the literature for evaluating the impact of the investigated symptoms on work and social adjustment. It is composed by 5 items (*Work*, *Home management*, *Social leisure activities*, *Private leisure activities*, and *Ability to form and maintain close relationships*). For each item, subjects are requested to rate in a 9-point Likert scale how much their symptoms affect their "ability to carry out the activity."[74]

Biochemical assessment

Collection of biological samples

A sample of peripheral venous blood (15 mL) was collected from all the subjects. All blood withdrawals were performed between 9 and 10 a.m. from subjects fasting for at least 12 hours. Blood samples were collected in vacutainer tubes containing K_3EDTA as anticoagulant or a clot activator for serum separation. The K_3EDTA vacutainer tubes were centrifuged at 150 g for 15 minutes in order to separate the platelet rich plasma (PRP) from the other cellular elements.[75] Then the PRP samples were transferred in Falcon tubes and centrifuged again together with clot activator tubes at 1500 g for 15 minutes in order to obtain platelet poor plasma (PPP) and platelet pellet. The clot activator tubes were instead centrifuged only one time, at 1500 g for 15 minutes, in order to obtain serum aliquots. All centrifugations were conducted at room temperature. All the samples were aliquoted in high-quality, low-binding protein Eppendorf Safe-Lock test tubes, properly marked and then stored at −80°C until the day of the assays.

Determination of biochemical parameters

Determinations of TRP, KYN, KYNA, and QA circulating levels were conducted by means of enzyme-linked immunosorbent assays (ELISAs), previously validated and commercially available. In particular, indirect competitive ELISA kits produced by ImmuSmol (Bordeaux, France) were used. According to the kit instructions, TRP, KYN, and QA were measured in PPP, KYNA in serum. The kits provided two 96-well microtiter plates: one for the analyte derivatization step and another for the actual ELISA assay. This latter plate contained a pre-adhered aliquot of the analyte object of investigation in the bottom of each well. The competitive reaction of these ELISA kits featured the addition in the wells of the derivatized analyte, which would compete with the pre-adhered analyte for a specific antibody also added into the wells. After incubation and washing steps, the detection reaction was performed, through the addition of a secondary antibody associated with the horseradish peroxidase (HRP) enzyme. This latter antibody was bound depending on the quantity of the first antibody linked to the analyte pre-adhered on the bottom of the wells. Subsequently, the HRP substrate 3,3′,5,5′-tetramethylbenzidine was added to the wells. After proper incubation procedures, the reaction was stopped through the addition of a stop solution which changed the color produced by the enzymatic reaction from blue to yellow. Considering the competitive nature of the assay, the intensity of the color decreased with the increase of the analyte concentration. The final absorbance was measured by a plate reader spectrophotometer at $\lambda = 450$ nm within 10 minutes from the stop reaction. For the quantitative analysis of each analyte, standard curves were calculated by performing a 4-parameter logistic regression, on the basis of the absorbance values of the standards. Finally, the calibration curve allowed the interpolation of concentrations of the analytes in the samples.

Statistical analysis

In this study, nonparametric analyses were used because the data did not respect the normality tests and variance homoscedasticity. Categorical sociodemographic variables were compared among groups by means of chi-square tests. Kruskal-Wallis one-way analysis of variance was used for the comparison of continuous sociodemographic variables, psychometric instrument scores, and biochemical parameter concentrations among groups. *Post hoc* comparisons were performed with Dunn's test. Within ASD and BAP groups, the Mann-Whitney *U*-test was used for the comparison of biochemical parameter concentrations between subjects with or without specific diagnoses or pharmacological treatments. Spearman's correlation coefficient (r) was performed in order to assess eventual correlations among different biochemical variables and between biochemical variables and scores reported on psychometric instruments. All analyses were performed by means of SPSS, version 24 (IBM Corp., Armonk, NY, USA). Calculation of calibration curves and regression analyses during the biochemical assays were performed by means of GraphPad Prism (Version 7.0; San Diego, CA, USA).

Results

Sociodemographic and clinical features

A total of 72 participants was recruited: 24 subjects with ASD (ASD group), 24 unaffected relatives of ASD individuals (BAP group), and 24 unrelated controls (CTL group). Significant differences were found for sex composition and mean age: in particular, the BAP group reported a significantly higher age than the other 2 groups, whereas CTLs also reported a higher age than ASD individuals. Moreover, the ASD group was composed in a higher proportion of males with respect to the other groups, whereas BAP subjects were in higher proportion females also with respect to the CTL group. (see Table 1). Most of ASD subjects reported other mental disorders in comorbidity (91.67%), whereas about 45.83% of subjects in the BAP group reported to have at least 1 psychiatric diagnosis. The patient sample was composed by adults subjects followed at a psychiatric department; thus, almost all the members were not drug-free. About a quarter of BAP subjects (25%) were also in treatment with psychopharmacological therapies (see Table 2).

Comparison of psychometric instruments scores among groups

Considering the AdAS Spectrum, the ASD group scored significantly higher than BAP and CTL groups on the total and on all domains with the exception of *Empathy* for which no significant difference was found between BAP and ASD individuals. Moreover, BAP subjects reported significantly higher total and domain

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Table 1. Sociodemographic Features of the Sample

		ASD (n = 24) (mean ± SD, mean rank)	BAP (n = 24) (mean ± SD, mean rank)	CTL (n = 24) (mean ± SD, mean rank)	*H*	*P*
Age (years)		27.75 ± 6.97, 20.77	55.42 ± 10.25, 57.71	33.29 ± 8.05, 31.02	39.92	<.001[a]
Mean BMI (Kg/m^2)		26.32 ± 7.14, 41.69	25.34 ± 6.32, 37.31	23.02 ± 3.92, 30.50	3.484	.175
					Chi-square	*P*
Sex (n[%])	M	17(70.8%)	3(12.5%)	9(37.5%)	17.09	<.001[b]
	F	7(29.2%)	21(87.5%)	15(62.5%)		

Notes: Significant post hoc comparisons: [a]BAP > CTL > ASD, P < .05; [b]M: ASD > CTL > BAP, P < .05.
Abbreviations: ASD, autism spectrum disorder; BAP, broad autism phenotype.

Table 2. Psychiatric Disorders and Pharmacological Treatments Among Groups

	ASD (n = 24) n (%)	BAP (n = 24) n (%)	CTL (n = 24) n (%)
Psychiatric disorders			
Anxiety disorders	7(29.17%)	9(37.50%)	–
Obsessive-compulsive disorder	3(12.50%)	0(0%)	–
Major depressive disorder	0(0%)	3(12.50%)	–
Bipolar I disorder	10(41.67%)	0(0%)	–
Bipolar II disorder	7(29.17%)	1(4.16%)	–
Feeding and eating disorders	3(12.50%)	1(4.16%)	–
Post-Traumatic Stress Disorder (PTSD)	1(4.16%)	0(0%)	–
At least one psychiatric disorder (other than ASD)	22(91.67%)	11(45.83%)	
Pharmacological treatment			
Antidepressants	10(41.67%)	4(16.67%)	–
Anxiolytics	12(50%)	2(8.33%)	–
Lithium	12(50%)	0(0%)	–
Other mood stabilizers (antiepileptics)	18(75%)	2(8.33%)	–
Antipsychotics	22(91.67%)	0(0%)	–
At least 1 pharmacological treatment	23(95.83%)	6(25%)	

Abbreviations: ASD, autism spectrum disorder; BAP, broad autism phenotype.

scores than CTLs with the exception of the *Childhood/adolescence* domain. Considering RAADS-14, subjects with ASD reported significantly higher total and domain scores than the other 2 groups, whereas the BAP group reported, in turn, significantly higher total and domain scores than CTLs, with the exception of *Social anxiety* domain score. A similar pattern was reported for the RRS: significantly higher total and domain scores were highlighted in the ASD group than in the other ones, although the BAP group also showed significantly higher scores than CTLs. When considering the WSAS, ASD individuals reported higher total scores (indicating a poorer adjustment) than the other groups, whereas BAP subjects reported significantly higher scores than CTLs. The ASD group reported higher scores also on single WSAS items, with the exception of *Close relationships*, for which no significant difference was found between ASD and BAP individuals. Finally, the BAP group scored higher than CTLs on all WSAS items with the exception of *Work* and *Home management* (see Table 3).

Comparison of biochemical variables among groups and correlations among biochemical variables

Before performing the comparison of biochemical variables among groups, in order to evaluate the possible impact on psychiatric comorbidities or pharmacological treatment, we compared in both ASD and BAP groups the levels of biochemical parameters depending on the presence of the most frequently reported comorbid disorders for each group and on the presence of specific pharmacological therapy. In the ASD group, no significant difference was found for any biochemical variable between subjects with or without bipolar I, bipolar II, or anxiety disorders, w in the BAP group, no significant difference was found for biochemical variables between subjects with or without major depressive disorder or anxiety disorders in the BAP group. No significant difference in levels of biochemical variables was found also when comparing in the ASD group subjects in treatment or not with antidepressants, anxiolytics, antipsychotics, or mood

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Table 3. Comparison of Psychometric Instrument Scores Among Groups

	ASD (n = 24) (mean ± SD, mean rank)	BAP (n = 24) (mean ± SD, mean rank)	CTL (n = 24) (mean ± SD, mean rank)	*H*	*P*
AdAS Spectrum					
Childhood/adolescence	10.46 ± 3.69, 51.52	5.57 ± 3.37, 31.33	3.26 ± 2.14, 19.63	31.58	<.001[b]
Verbal communication	10.13 ± 4.88, 53.77	4.19 ± 2.34, 32.45	1.57 ± 1.67, 16.26	42.95	<.001[a]
Nonverbal communication	12.50 ± 4.41, 52.53	6.95 ± 3.60, 32.88	3.57 ± 2.25, 17.48	36.67	<.001[a]
Empathy	5.25 ± 2.79, 49.29	3.14 ± 2.48, 36.55	0.82 ± 1.03, 17.20	31.99	<.001[c]
Inflexibility and adherence to routine	21.25 ± 6.77, 52.60	12.24 ± 5.97, 34.67	5.39 ± 3.46, 15.46	41.59	<.001[a]
Restricted interests and rumination	12.75 ± 4.30, 51.90	7.24 ± 4.12, 34.69	3.09 ± 1.90, 16.17	38.54	<.001[a]
Hyper-hypo reactivity to sensory input	6.46 ± 3.44, 50.81	3.10 ± 2.34, 34.50	0.87 ± 1.22, 17.48	34.11	<.001[a]
Total score	78.79 ± 21.94, 55.04	42.43 ± 16.85, 33.79	20.83 ± 13.41, 16.02	45.54	<.001[a]
RAADS-14					
Mentalizing deficits	11.43 ± 4.85, 51.86	4.00 ± 5.00, 31.25	0.36 ± 0.95, 16.75	40.26	<.001[a]
Social anxiety	6.81 ± 3.08, 51.40	2.23 ± 2.69, 30.39	0.09 ± 0.29, 18.05	38.76	<.001[b]
Sensory reactivity	10.46 ± 3.69, 48.05	4.45 ± 3.13, 32.95	3.45 ± 2.63, 18.68	28.97	<.001[a]
Total score	22.81 ± 8.36, 52.36	8.27 ± 8.53, 32.70	0.77 ± 1.44, 14.82	43.67	<.001[a]
RRS					
Reflection	12.09 ± 3.06, 47.29	8.90 ± 2.61, 32.36	6.41 ± 1.94, 16.36	30.60	<.001[a]
Brooding	13.14 ± 3.04, 48.93	9.68 ± 2.71, 33.32	6.71 ± 1.49, 15.21	34.81	<.001[a]
Depression	32.71 ± 6.65, 49.57	23.19 ± 4.62, 31.24	17.62 ± 3.84, 15.19	37.09	<.001[a]
Total score	57.95 ± 10.74, 49.38	41.05 ± 8.86, 30.20	30.76 ± 6.53, 14.86	38.66	<.001[a]
WSAS					
Work	5.25 ± 2.02, 50.35	2.00 ± 2.29, 30.23	0.41 ± 0.73, 18.55	33.56	<.001[b]
Home management	4.70 ± 2.76, 47.60	1.82 ± 2.15, 31.36	0.32 ± 0.65, 19.91	26.11	<.001[b]
Social leisure activities	4.75 ± 2.63, 47.50	2.27 ± 2.64, 33.52	0.23 ± 0.53, 17.84	29.29	<.001[a]
Private leisure activities	5.40 ± 2.66, 47.80	2.95 ± 2.98, 34.34	0.14 ± 0.35, 16.75	32.32	<.001[a]
Close relationships	3.65 ± 3.03, 44.15	1.91 ± 2.02, 34.59	0.09 ± 0.29, 19.82	21.94	<.001[c]
Total score	23.75 ± 9.77, 49.98	10.95 ± 10.20, 33.02	1.18 ± 2.20, 16.09	36.22	<.001[a]

Notes: Significant post hoc comparisons: [a]ASD > BAP > CTL, P < .05; [b]ASD > BAP, CTL, P < .05;[c]ASD, BAP > CTL, P < .05.
Abbreviations: AdAS Spectrum, Adult Autism Subthreshold Spectrum; ASD, autism spectrum disorder; BAP, broad autism phenotype; RAADS-14, Ritvo Autism and Asperger Diagnostic Scale, 14-item version; RRS, Ruminative Response Scale; WSAS, Work and Social Adjustment Scale.

stabilizers (lithium or antiepileptics) or when comparing in the BAP group subjects in treatment with antidepressants, anxiolytics, or antiepileptics.

According to the results from the comparison of biochemical variables among groups, circulating levels of TRP were significantly lower in both the ASD and BAP groups with respect to CTLs. KYNA levels were instead significantly lower in the ASD group than in the other 2 groups. No significant difference was found for QA and KYN levels. Considering the ratio among biochemical variables, KYNA/KYN ratio was reported to be significantly lower in ASD individuals than in CTL ones, whereas KYNA/TRP and KYNA/QA were revealed to be significantly lower in the ASD group than in the BAP group, without significant differences with respect to CTLs. QA/KYN, QA/TRP, and KYN/TRP did not show significant differences among groups (see Table 4 and Figures 1 and 2). Significant correlations were found between levels of KYN, KYNA, and QA (see Table 5).

Correlations between biochemical variables and scores reported on psychometric scales

Both AdAS Spectrum and RAADS-14 total scores reported significant negative correlations with TRP and KYNA levels. Moreover, all AdAS Spectrum domain scores also showed negative correlations with TRP levels (with the exception of the *Childhood/adolescence* domain) and KYNA levels (with the exception of the *Empathy* domain; see Table 6). All RAADS-14 domains reported significant negative correlations with KYNA levels, whereas TRP was significantly and negatively correlated only with the *Sensory reactivity* domain (see Table 7). RRS total and domain scores were also all significantly and negatively correlated with both TRP and KYNA levels (see Table 8). Finally, for the WSAS, only 1 significant and negative correlation was found, between the item exploring *Close relationships* and TRP levels (see Table 9).

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Table 4. Comparison of Biochemical Parameters Among Groups

	ASD (n = 24) (mean ± SD, mean rank)	BAP (n = 24) (mean ± SD, mean rank)	CTL (n = 24) (mean ± SD, mean rank)	*H*	*P*
TRP (μM)	49.778 ± 20.957, 29.30	49.895 ± 17.566, 29.68	67.170 ± 25.698, 45.33	9.77	.008[a]
KYN (ng/mL)	535.729 ± 275.166, 32.70	653.960 ± 347.114, 38.18	563.579 ± 304.097, 34.29	0.89	.642
KYNA (ng/mL)	10.395 ± 6.031, 23.28	16.525 ± 7.486, 40.05	17.099 ± 8.129, 41.60	11.84	.003[b]
QA (ng/mL)	49.429 ± 30.435, 33.74	51.154 ± 40.542, 32.55	53.282 ± 26.704, 38.46	1.13	.567
QA/KYN	0.101 ± 0.058, 35.72	0.085 ± 0.041, 30.55	0.11 ± 0.71, 38.40	1.80	.406
QA/TRP	0.006 ± 0.004, 37.11	0.006 ± 0.004, 36.93	0.005 ± 0.004, 31.21	1.32	.518
KYN/TRP	0.057 ± 0.026, 37.83	0.079 ± 0.059, 39.52	0.051 ± 0.046, 28.15	4.38	.112
KYNA/TRP	0.001 ± 0.001, 27.85	0.002 ± 0.001, 43.68	0.002 ± 0.001, 33.90	7.15	.028[c]
KYNA/KYN	0.023 ± 0.017, 25.76	0.035 ± 0.026, 36.73	0.033 ± 0.013, 42.27	8.19	.017[d]
KYNA/QA	0.264 ± 0.164, 26.43	0.436 ± 0.289, 40.39	0.371 ± 0.186, 38.27	6.42	.040[c]

Notes: Significant post hoc comparisons: [a]ASD, BAP < CTL, P < .05; [b]ASD < BAP, CTL, P < .05; [c]ASD < BAP, P < .01; [d]ASD < CTL, P < .05.
Abbreviations: ASD, autism spectrum disorder; BAP, broad autism phenotype; KYN, kynurenine; KYNA, kynurenic acid; QA, quinolinic acid; TRP, tryptophan.

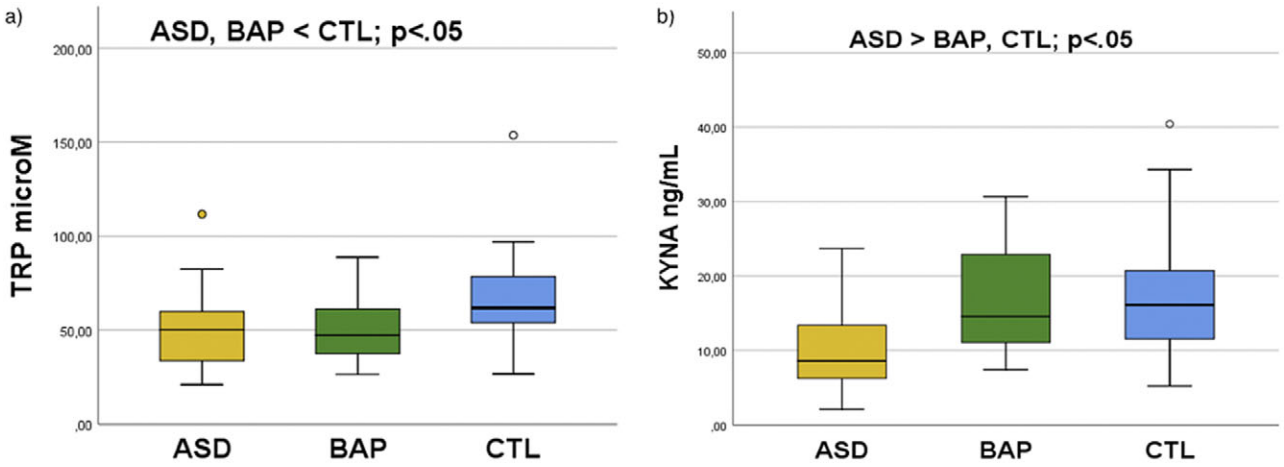

Figure 1. Comparison of biochemical parameters with significant differences among groups. (a) Tryptophan. (b) Kynurenic acid.

Discussion

Comparison of psychometric scales among groups

Results from the comparison of psychometric scale scores among groups showed the presence of intermediate scores in the BAP group, with a significant difference with respect to the other 2 groups, globally confirming the BAP theoretical model and the correct identification of the relatives' group as a group of subjects with increased autistic traits.[4,6,15,76-79] Although previous studies reported that the impairment in social and relational skills seemed to be the more frequent and severe autistic feature in relatives of ASD subjects,[80] our results also show a higher impairment in social skills and empathy in the BAP group, which did not differ from the patients for the scores reported on these domains. A worse global adjustment, and in particular in leisure and relational areas, was also found, confirming results from previous literature and stressing the impact that autistic traits may exert on quality of life also when subthreshold.[4]

Comparison of biochemical variables among groups and correlations among biochemical variables

To the best of our knowledge, this is the first study among adult ASD patients specifically focused on the KYN pathway, and including also relatives of ASD probands. In line with most of the available literature, most of which regarding ASD children,[32] our results highlighted lower levels of TRP in both ASD and BAP groups, supporting also in adult samples the hypothesis of a link between TRP metabolism alterations and ASD, which could be present also in subthreshold autism spectrum conditions. TRP is an essential precursor for the biosynthesis of 5-HT and melatonin, while reduced TRP could lead to the alteration of the 5-HT pathway, which may eventually be implied in the pathophysiology of neurodevelopmental alterations.[24] Noticeably, depletion of dietary TRP is also known to exacerbate autism symptoms.[35] On the other hand, our findings are in contrast with previous studies which reported in ASD children lower TRP levels only among subjects with IDD and instead increased TRP levels among subjects with Asperger's Syndrome.[51,68]

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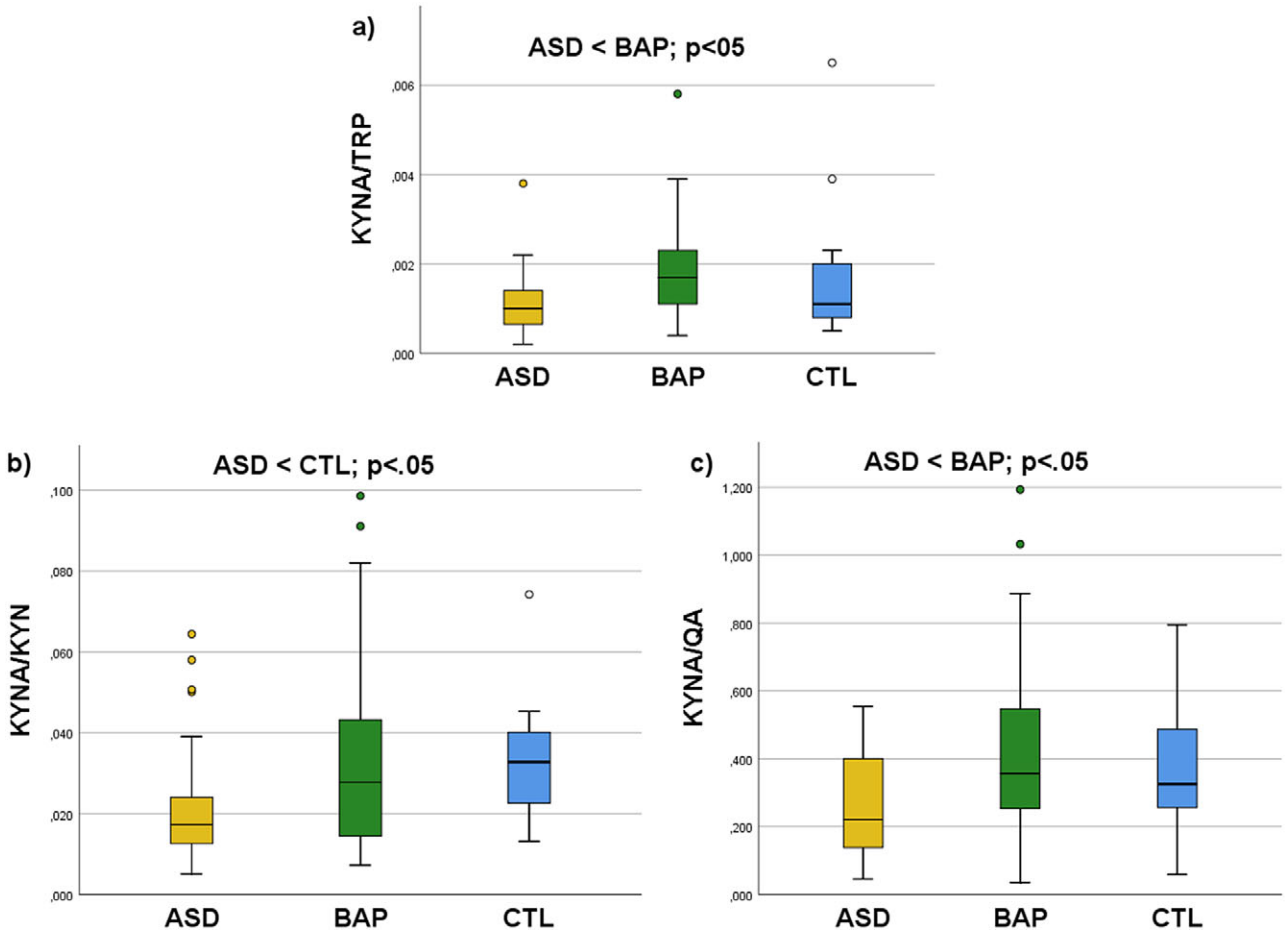

Figure 2. Comparison of biochemical parameters' ratios with significant differences among groups. (a) Kynurenic acid (KYNA)/tryptophan. (b) KYNA/kynurenine. (c) KYNA/quniolinic acid.

Table 5. Correlations (Spearman *r*) Between Biochemical Parameters in the Whole Sample

	QA (ng/mL)	KYNA (ng/mL)	KYN (ng/mL)	TRP (μM)
TRP (μM)	−.029	.039	−.034	–
KYN (ng/mL)	.564**	.337**	–	−.034
KYNA (ng/mL)	.267*	–	.337**	.039
QA (ng/mL)	–	.267*	.564**	−.029

Abbreviations: KYN, kynurenine; KYNA, kynurenic acid; QA, quinolinic acid; TRP, tryptophan.
*$P < .05$.
**$P < .01$.

Moreover, we did not find significant differences for KYN and QA levels, which were instead reported in some of the previous works on children samples.[23,51] However, results in adult and children sample may not be directly comparable, due to the reported age dependency of KYN metabolism.[81] Noticeably, TRP and KYN were reported to be altered also among depressed adults: in particular, increased KYN and reduced TRP levels were highlighted in these patients, while a link was revealed between suicidal behaviors, increased QA, and decreased KYNA levels. However, a recent study did not confirm these findings, reporting increased TRP and reduced KYN levels in hospitalized patients with major depressive disorder or bipolar

Table 6. Correlations (Spearman *r*) Between Biochemical Parameters and Adult Autism Subthreshold Spectrum (AdAS Spectrum) Scores in the Whole Sample

AdAS Spectrum	Childhood/ adolescence	Verbal communication	Nonverbal communication	Empathy	Inflexibility and adherence to routine	Restricted interests and rumination	Hyper-hypo reactivity to sensory input	AdAS total score
TRP (μM)	−.174	−.316*	−.259*	−.290*	−.259*	−.252*	−.350**	−.300*
KYN (ng/mL)	−.085	−.147	.005	−.013	−.093	−.113	−.006	−.059
KYNA (ng/mL)	−.314*	−.366*	−.344**	−.166	−.362**	−.444**	−.322**	−.362**
QA (ng/mL)	−.160	−.077	−.009	−.024	−.053	−.021	−.026	−.067

Abbreviations: KYN, kynurenine; KYNA, kynurenic acid; QA, quinolinic acid; TRP, tryptophan.
*$P < .05$.
**$P < .01$.

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Table 7. Correlations (Spearman *r*) Between Biochemical Parameters and RAADS-14 in the Whole Sample

	Mentalizing deficit	Social anxiety	Sensory reactivity	RAADS-14 total score
TRP (μM)	−.228	−.197	−.281*	−.275*
KYN (ng/mL)	.053	−.033	−.065	−.005
KYNA (ng/mL)	−.344**	−.336**	−.266*	−.331**
QA (ng/mL)	−.040	−.093	−.100	−.079

Abbreviations: KYN, kynurenine; KYNA, kynurenic acid; QA, quinolinic acid; RAADS-14, Ritvo Autism and Asperger Diagnostic Scale, 14-item version; TRP, tryptophan.
*$P < .05$.
**$P < .01$.

Table 8. Correlations (Spearman *r*) Between Biochemical Parameters and RRS Scores in the Whole Sample

	Reflection	Brooding	Depression	RRS total score
TRP (μM)	−.398**	−.351**	−.394**	−.407**
KYN (ng/mL)	.056	.013	−.035	−.027
KYNA (ng/mL)	−.262*	−.253*	−.351**	−.336**
QA (ng/mL)	−.081	−.091	−.034	−.057

Abbreviations: KYN, kynurenine; KYNA, kynurenic acid; QA, quinolinic acid; RRS, Ruminative Response Scale; TRP, tryptophan.
*$P < .05$.
**$P < .01$.

disorder, while no correlation was found between TRYCAT alterations and suicidality.[82]

When considering the possible factors linked to reduced TRP levels in ASD and BAP groups, besides dietary and endocrine factors,[40] possible metabolic alterations should also be evaluated. Although no difference was found for QA levels in this study, TRP may be preferentially transformed in other metabolites of the KYN pathway, such as xanthurenic acid.[57] The role of microbiota should also be taken into account, considering the ability of gut bacteria of affecting TRP metabolism.[57,83] Moreover, growing evidence is pointing out the presence of microbiota alterations and gut dysbiosis in ASD.[7] As in previous studies in children,[51] our results revealed lower levels of KYNA in the ASD group than in CTL and BAP subjects. Interestingly, the KYN/TRP ratio was not different among groups despite the lower levels of TRP in ASD and BAP subjects, whereas higher KYNA/TRP and KYNA/QA were reported among BAP when compared with the ASD group, eventually reflecting lower levels of excitotoxicity among BAP subjects.[51] These findings suggest that the metabolism of the BAP group may succeed, eventually via compensatory enzymatic activities, in producing levels of KYNA similar to those of the CTLs despite reporting reduced TRP levels, resulting in a lower impact on biochemical functioning. Globally, results from the comparison of the biochemical variables among groups, featuring reduced levels of TRP and KYNA but QA levels similar to CTLs in ASD patients, may support the hypothesis of an impairment of the neuroprotective branch of the KYN pathway in this population, together with a conservation of the neurotoxic pathway, which may not be properly counterbalanced as a result. Moreover, our results seemed also to stress the presence of a continuity between ASD and BAP subjects, which may show similar but milder alterations, with the eventual involvement of compensatory processes. Finally, it is worth mentioning that we did not find significant differences when comparing levels of biochemical parameters depending on the presence of other psychiatric comorbidities or of a current psychopharmacological treatment. On the other hand, in previous studies, mood/anxiety disorders and/or pharmacological treatments were reported to influence KYN metabolism[33]: these results may suggest that the impact of autism spectrum condition on biochemical parameters' concentrations may had overcome the influence of the presence of other conditions or pharmacological therapies.

Correlations between biochemical variables and scores reported on psychometric scales

In line with the results from the comparison analysis, both TRP and KYNA reported significant negative correlations with the total scores of the scales measuring the presence of ASD-related symptoms, the AdAS Spectrum, and the RAADS-14. Moreover, TRP levels were significantly and positively correlated with almost all the AdAS Spectrum domains with the exception of the *Childhood/adolescence* domain, showing the highest correlations with the domains measuring the altered reactivity to sensory input and the verbal communication impairment, whereas for the RAADS-14, a significant correlation was found only with the *Sensory reactivity* domain. KYNA was instead significantly correlated with all the domains of AdAS Spectrum and RAADS-14 (highest correlations with the rumination and verbal impairment dimension), with the only exception of the AdAS Spectrum *Empathy* domain. The association between TRP and altered sensory reactivity may be in agreement with the altered processing of different sensory stimuli highlighted in TRP depletion models by previous studies.[84,85] The link of both KYNA and TRP with impaired verbal communication could be instead in line with previous studies, which reported that TRP depletion seemed to worsen the communication skills in rats,[31,32] and to be associated with a reduction of episodic memory for verbal information in humans.[33,34] Episodic memory for verbal information is, in turn, linked to communication abilities.[86] TRP supplementation was also reported to reduce aggressive behaviors, further confirming its involvement in the social brain.[33] Only scant literature is available on the association

Table 9. Correlations (Spearman *r*) Biochemical Parameters and WSAS Scores in the Whole Sample

	Work	Home management	Social leisure activities	Private leisure activities	Close relationships	WSAS total score
TRP (μM)	−.166	−.148	−.183	−.149	−.271*	−.167
KYN (ng/mL)	−.066	−.055	−.012	−.051	−.082	−.063
KYNA (ng/mL)	−.228	−.191	−.150	−.207	−.203	−.242
QA (ng/mL)	.007	−.067	−.100	−.060	−.123	−.063

Abbreviations: KYN, kynurenine; KYNA, kynurenic acid; QA, quinolinic acid; TRP, tryptophan; WSAS, Work and Social Adjustment Scale.
*$P < .05$.

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between KYNA and specific clusters of symptoms, in particular in the field of ASD, and results are still controversial. Some studies in schizophrenia stressed an association of cognitive inflexibility with increased KYNA levels,[45,87-89] whereas others hypothesized an anxiolytic role for KYNA, eventually related to its antagonistic activity on NMDA receptors.[90] On the other hand, somewhat in line with our results, an association was found in rats between social isolation rearing and decreased KYNA.[91] However, both TRP and KYNA reported their highest correlations with the specific dimension of ruminative thinking as measured by the RRS, and, in the case of KYNA, by the AdAS Spectrum. Ruminative thinking is defined as a maladaptive, repetitive kind of thinking which does not typically lead to active problem solving but to an over-fixation on the problems and on the related negative feelings. Rumination is considered a core feature of the autism spectrum, but it is also a trans-nosographic dimension, which may be underlain by the presence of subthreshold autistic traits, associated with worse psychopathological outcome, increased vulnerability toward stressful events, and suicidality risk.[11,92-95] Although the specific association between ruminative thinking and TRYCAT alterations was not previously investigated, a role of glutamatergic transmission in ruminative thinking pathophysiology may be supported by studies that reported a reduction of rumination symptoms in mental disorders with the use of Ketamine, also among SSRI-resistant subjects. Ketamine, as KYNA, is an antagonist of NMDA receptor, thus influencing glutamatergic transmission.[96-98] No significant correlations were found for the WSAS, with the exception of a negative correlation between TRP levels and scores reported on the *Close relationships* item. This result, while further supporting the possible impact on TRP levels on the relational area, may suggest that KYNA and TRP levels, despite being associated with the presence of autistic symptoms, would not vary depending on the impact of symptoms on subjects' global functioning. In conclusion, the reported correlations between psychometric scales and biochemical variables further highlighted the possible key involvement of KYN pathway in ASD pathophysiology, eventually with specific associations with different clusters of symptoms.[7,33,51,99,100]

Limitations of the study

Several limitations should be considered with respect to this study. First, the investigation was led with a cross-sectional design, and it was not possible to make inferences about eventual temporal relationships between the variables. Second, the sample size was small, and our groups showed significant differences in sex and age distribution, eventually implying biases and limiting the extensibility of the results. In particular, groups were significantly different with respect to mean age: the BAP group, being mainly composed of parents of the patients' group, reported the oldest mean age. Moreover, in line with the higher frequency of ASD in male gender, the ASD group was composed in a higher proportion of males.[1] Females were more represented in the BAP group, because the mother was often the only relative who agreed in participating in the study. These data may be considered in light of the reported presence among mothers of a higher burden related to caregivers, with a frequent lack of father involvement in parental care.[101]

Third, the high rate of psychiatric comorbidities, particularly in the ASD group, is another factor that may have influenced our findings, even though dedicated analyses were performed for evaluating the possible impact of comorbidities and pharmacological treatment on levels of biochemical variables. It should be noted that high rates of psychiatric comorbidities in ASD patients and, to a lesser extent, in the group of the relatives are in agreement with the literature that stressed the high comorbidity between ASD and other psychopathological conditions, as well as the increased presence of mental disorders among unaffected relatives of ASD patients.[2,4,11,102] In addition, ASD adults without intellectual impairment often come to clinical attention only after the development of other comorbid psychiatric disorders, in particular if ASD symptoms remained under-recognized during early life: thus, in the specific population investigated here, psychiatric comorbidities may be overrepresented.[2,11] Moreover, considering that the ASD group was recruited among patients followed at a psychiatric clinic, most of the subjects were under pharmacological treatment. In addition, no information was available regarding participants' nutritional state, which may exert a significant influence on TRP levels and metabolism. Finally, most of the psychometric instruments used in the study were self-reported, eventually allowing biases in overstimation or underestimation of symptoms by the subjects.

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