Metabolic Brain Disease
https://doi.org/10.1007/s11011-018-0186-6

ORIGINAL ARTICLE

Relationship between absolute and relative ratios of glutamate, glutamine and GABA and severity of autism spectrum disorder

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Received: 14 October 2017 / Accepted: 8 January 2018

Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental pathology characterized by an impairment in social interaction, communication difficulties, and repetitive behaviors. Glutamate signaling abnormalities are thought to be considered as major etiological mechanisms leading to ASD. The search for amino-acidic catabolytes related to glutamate in patients with different levels of ASD might help current research to clarify the mechanisms underlying glutamate signaling and its disorders, particularly in relation to ASD. In the present study, plasma levels of the amino acids and their derivatives glutamate, glutamine, and γ-aminobutyric acid (GABA), associated with their relative ratios, were evaluated using an enzyme-linked immunosorbent assay (ELISA) technique in 40 male children with ASD and in 38 age- and gender-matched neurotypical health controls. The Social Responsiveness Scale (SRS) was used to evaluate social cognition, and the Childhood Autism Rating Scale (CARS) was used to assess subjects' behaviors. Children with ASD exhibited a significant elevation of plasma GABA and glutamate/glutamine ratio, as well as significantly lower levels of plasma glutamine and glutamate/GABA ratios compared to controls. No significant correlation was found between glutamate levels and the severity of autism, measured by CARS and SRS. In receiver operating characteristic (ROC) curve analysis, the area under the curve for GABA compared to other parameters was close to one, indicating its potential use as a biomarker. Glutamine appeared as the best predictive prognostic markers in the present study. The results of the present study indicate a disturbed balance between GABAergic and glutamatergic neurotransmission in ASD. The study also indicates that an increased plasma level of GABA can be potentially used as an early diagnostic biomarker for ASD.

Keywords autism · neurotransmitter · glutamate excitotoxicity · gamma-aminobutyric acid · glutamine · childhood autism rating scale

Introduction

Autism spectrum disorder (ASD) is a heterogeneous and complex neurodevelopmental disorder characterized by impairment in social interaction, communicative disturbance, and repetitive patterns of behaviors (APA 2013; Christensen et al. 2016). Research indicates that ASD has a complex and multifactorial etiology, involving interactions between

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Published online: 03 February 2018

different genetic, neurological, immunological, nutritional, and environmental factors (Kwong et al. 2000; Boddaert et al. 2009; Mitchell et al. 2011; Bjørklund and Chartrand 2016; Bjørklund et al. 2016; Endreffy et al. 2016; Matelski and Van de Water 2016; El-Ansary et al. 2017; Meguid et al. 2017). The prevalence of ASD is remarkably increased during the last decades (Elsabbagh et al. 2012; Lavelle et al. 2014). Recently in the US, a prevalence of 1 on 68 children (1.47%) has been reported to suffer from ASD (Zablotsky et al. 2015; Christensen et al. 2016), and this trend is 4.5 times more common in boys than in girls (Christensen et al. 2016). However, no statistics are yet available in Saudi Arabia concerning the prevalence of ASD (Zeina et al. 2014).

Recent reports showed that an imbalance in the excitatory and inhibitory mechanisms in the GABA and glutamate neurophysiology was observed in ASD individuals (Rojas et al. 2014). In the human brain, glutamate is one of the major excitatory neurotransmitters (Pittenger et al. 2011; Naaijen et al. 2017). Glutamate is responsible for many neurological functions, including cognition, memory, behavior, movement, and sensation. It also plays significant roles in the brain development, including synapse induction and their relationship with astrocytes, cell migration, synaptic spatial organization in the cerebellum, cell differentiation, and death (Moriyama et al. 2000; Balakrishnan et al. 2014; Kim et al. 2017). Because of these critical and essential functions, glutamate dysregulation has been associated with some neurodevelopmental and neurodegenerative disorders such as schizophrenia, ASD, and epilepsy (Javitt 2004; Santoro et al. 2012). Regulation of the synaptic level of glutamate is essential to prevent accumulation of glutamate in the synaptic cleft, which would result in overstimulation of glutamate receptors leading to neuronal excitotoxicity and damage (Mark et al. 2001; Choudhury et al. 2012). On the other side, γ-aminobutyric acid (GABA) in the brain is responsible for synaptic inhibition (Bjørklund 2013). Neurons cannot make their amino acid neurotransmitters glutamate and GABA without glutamine, as a precursor released from astrocytes into glutamatergic or GABAergic neurons (Reubi et al. 1978; McKenna et al. 2011). Glutamate is taken up from the synaptic space into astrocytes, where is converted into glutamine, transported to neurons and reused. The 'glutamate-glutamine cycle' is essential to avoid excitotoxicity (Shimmura et al. 2013) and the equilibrium between excitatory and inhibitory neurotransmission is essential for the proper brain development (Choudhury et al. 2012; Wu and Sun 2015). It has been reported that this 'glutamate-glutamine cycle' is impaired in the brains of ASD patients (Shimmura et al. 2013), and an imbalance between excitation and inhibition in glutamate signaling could be a possible cause of ASD (Fatemi 2008).

A recent multi-regression study in Saudi Arabia reported that differences in the glutamate and glutamine were a significant predictive variable of ASD, using a multi-regression stepwise test, with glutamate or glu/gln ratio as a dependent variable in the ASD group (El-Ansary 2016). The multiple regression analysis also revealed a marked association between reduced GABA, glutamate excitotoxicity (affecting glutamine plasma levels) and neuroinflammation in autistic patients (El-Ansary and Al-Ayadhi 2014). This evidence would suggest that these parameters were associated with severity of ASD, as assessed by standardized questionnaires on ASD severity. Levels of glutamine in ASD were recently examined, and it was found that both plasma glutamate and glutamine serve as possible biomarkers of the typical IQ found in ASD (Shimmura et al. 2011). Very few associations of these biomarkers with moderate or even severe autism have been conducted, yet the impaired levels of glutamate and glutamine, which should biochemically reflect glutamate pathway and GABA, have been observed in the attention-deficit hyperactivity disorder (ADHD), which usually co-occur with severe autism (Maltezos et al. 2014; Zablotsky et al. 2017)). In Saudi Arabia, no studies have been ever conducted to evaluate the potential relationship between glutamate (GABA) and glutamine in ASD. The present study aims to analyze serum levels of glutamate, glutamine, and GABA, and their relative ratios related to glutamate excitotoxicity in plasma of Saudi children with ASD, and also to compare these values with the serum concentrations found in a neurotypical control group of age and sex-matched children.

Materials and methods

Compliance with ethical standards

All protocols used in the present study followed the ethical guidelines of the Declaration of Helsinki (WMA 2013) and were approved by the King Khalid Hospital Ethical Committee, Riyadh, Saudi Arabia (Protocol 15/0367/IRB). Written consent was obtained from parents, tutors or caregivers of both the ASD children and the neurotypical controls, who participated in the study.

Participants

In the present study, 40 male children with ASD were enrolled from the Autism Research and Treatment Center at King Khalid University Hospital in Riyadh. At the beginning of the study, we conducted a sample size and a population study to select the suitable subjects, no covariates regarding ages and sexes biased our recruitment protocol. The sample size was assessed using Minitab® Software v18, according to previous reports (Rudzki et al. 2017; Kadam and Bhalerao 2010) based on an available hospitalized population. The sample size was calculated based on a control group expected to present a conversion rate of less than 17% and a minimum detectable effect of 95%, on

statistical significance (positive) at 95%. Size sample fulfills the minimum size for a t-test analysis.

The mean age of the ASD children was 6.8 ± 5.2 years (Mean ± SD). In all subjects, the diagnosis of ASD was confirmed using the Developmental Diagnostic Dimensional Interview (3Di) (Skuse et al. 2004), the Autism Diagnostic Observation Schedule (ADOS) (Rutter et al. 2005, 2012), as well as the Autism Diagnostic Interview-Revised (ADI-R). None of the ASD children underwent special supplements, alternative treatments or undergoing any pharmacological therapy during the study. No ASD patient was ever positively diagnosed with OCD, ODD, ADHD or other mood/psychiatric disorders. The control group consisted of 38 age- and gender-matched healthy children with a mean age of 8 ± 3.2 years and enrolled from the pediatric clinic at King Saud Medical City in Riyadh. All participants were screened via anamnestic interviews with their parents for current or past physical illness, current pharmacotherapy, and vaccination. Subjects who had dysmorphic features, fragile X syndrome, severe neurological (e.g., seizures), psychiatric (e.g., bipolar disorder) or other known medical conditions, including endocrine, pulmonary, kidney, liver, and cardiovascular disease were excluded from the study.

Behavioral assessment

The behavioral assessment was performed using the Childhood Autism Rating Scale (CARS), a standardized questionnaire. It was developed for children over the age of 2 years, with the purpose to differentiate children with ASD from the ones with other developmental disabilities (Reber 2012; Breidbord and Croudace 2013). CARS consists of 15 items assessing behaviors associated with autism, including general impressions (Chlebowski et al. 2010) and is usually completed by a physician involved in the study, based on clinical and behavioral manifestations of the patients and anamnestic interviews. Each item is scored on a scale ranging from one to four. Total scores can range from a score of 15 to 60. Scores below 30 indicate that the individual is in the non-autistic range. Scores between 30 and 36.5 indicate mild to moderate autism, and scores from 37 to 60 indicate severe autism (Chlebowski et al. 2010).

The Social Responsiveness Scale (SRS) is an instrument to evaluate the social aspects of ASD. It was developed for children between 4 and 18 years to identify ASD in the pediatric population and to screen and support clinical diagnosis. Usually, patients or patient's parents completed this questionnaire, also with the assistance of a physician or practitioner. The domains of the 65 items questionnaire are social awareness, social cognition, social communication, social motivation, restricted interests, and repetitive behavior associated with ASD (Salley et al. 2013). The answers are rated on a scale of 1 ("not true"); 2 ("sometimes true); 3 (often true); and 4 ("almost always true"). A score of ≥76 is considered severe, i.e., strongly related to autistic disorder. A score of 60–75 indicates mild to moderate deficiencies in reciprocal social behavior, and scores of 59 and below are considered normal (Reber 2012).

Blood collection procedure

After an overnight fast, blood samples were collected in 7 ml tubes containing K_2-EDTA. Samples were centrifuged at 3000 rpm (1450 *g*) for 15 min at 4°C. The plasma was decanted and aliquoted to prevent multiple freeze-thawing cycles. The blood samples were stored at −80°C until analysis.

Biochemical assays

The quantitative determination of glutamate, GABA, and glutamine was measured in blood plasma from the ASD children and neurotypical controls using enzyme-linked immunosorbent assay (ELISA) technique. The applied assays were based on the method of competitive binding enzyme immunoassay technique. Descriptions of kits-assays are described below.

Assay of glutamate

Glutamate was analyzed using an ELISA diagnostic kit from MyBioSource (San Diego, CA, USA). The procedure requires three steps, and the first one is an extraction step. Plasma samples and the standards were added together with the diluent in the extraction plate followed by a derivatization step in which the extracted plasma samples and the standards were added together with sodium hydroxide, the equalizing reagent, and D reagent in a reaction plate, covered, and shaking for 2 h, followed by addition of the Q Buffer into all wells. In the last step, the extracted plasma samples and standard compete with glutamate antiserum for a specific number of binding sites on the glutamate microtiter strips, a mechanism lasting for 15–20 h at 2–8°C. Free antigen and free antigen-antiserum complexes are then removed by three times wash. After that, the anti-rabbit IgG-peroxidase conjugate is added, incubated for 30 min on a shaker, and aspirated by washing. Tetramethylbenzidine (TMB), as the enzyme substrate, was then added to detect the antibody bound to the solid phase. The absorbance of the solution in the wells is read with the use of a microplate reader at 450 nm, and the concentration of unknown titers was calculated using the standard curve.

Assay of GABA

Gamma-aminobutyric acid was analyzed using an ELISA kit from MyBioSource (San Diego, CA, USA). The kit has a microtiter plate that is pre-coated with an antibody specific to GABA. During the assay, the GABA in the standard or

sample competes with a fixed quantity of biotin-labeled GABA for sites on the pre-coated monoclonal antibody. After washing the excess conjugate and unbound standard or sample from the plate, avidin conjugated to horseradish peroxidase (HRP) was added. TMB as enzyme substrate was used to detect the antibody bound to the solid phase, the developed color was read at 450 nm, and the concentration of GABA was measured using the standard curve.

Assay of glutamine

For the measurement of glutamine quantity in human plasma, an ELISA kit from MyBioSource (San Diego, CA, USA) was employed. This kit, based on competitive enzyme immunoassay technique, uses a GLN-HRP conjugate and a monoclonal anti-glutamine. On the pre-coated plate, the assay sample and the buffer are incubated 1 h together with GLN-HRP conjugate, then decanted, and then five times washed. Next, the wells are incubated with TMB as a HRP enzyme substrate. The intensity of the yellow color that appeared after adding stop reagent was spectrophotometrically measured at 450 nm in a microplate reader.

Statistical analyses

Statistical Program for Social Sciences (SPSS, IBM-SPSS, Inc., Chicago, IL, US) was used for all analyses. A Wilcoxon-Mann-Whitney test was used for comparisons between mild to moderate and severe autism groups from CARS on the levels of glutamate, glutamine, and GABA, taking into account the results of the Shapiro-Wilk test for normality. Ratios were also used to evaluate the biochemical and functional relationship between the biochemical markers altogether, as statistical comparisons in this context were not entirely foreseen by just evaluating absolute plasma levels of every single biomarker, without their ratios. Also, data were expressed as mean ± standard deviation (SD). A multivariate analysis was also accomplished by using both a one-way MANOVA and a multi-regression test. To assay variance distribution in the present study, a Levene test was also performed. Potential correlations between CARS and biochemical analytes were evaluated by Spearman rank correlation test. Positive or negative correlations $r > 0.80$ were considered significant. A significant difference between neurotypical controls and ASD patients or between different severity levels (i.e., mild to moderate and severe) within the CARS, and SRS scales with biochemical analytes were considered significant at the H_0 hypothesis with a P value <0.05.

Furthermore, biomarkers were evaluated by receiver operating characteristics (ROC) curve using the same software (SPSS). In a ROC analysis, the area under the curve (AUC) provides a useful measure for comparing different biomarkers. It can be used for the comparison of differences for a parameter between ASD and control subjects or between different autism severity levels (i.e., mild to moderate and severe) within the CARS and SRS scales. AUC close to one indicates an excellent diagnostic and predictive biomarker. Moreover, predictiveness curves for absolute and relative values of the measured parameters in the ASD patients were drawn using SPSS software, to assess the performance of these biomarkers in the Saudi population. Moreover, it displays crucial risk information that does not appear by the ROC curve.

Results

GABA and glutamine levels, as well as glutamate/GABA, and glutamate/glutamine ratios, were significantly different between the ASD group and controls (Table 1). No significant differences were observed when this comparison was performed between mild and moderate CARS ASD severity and severe ASD (Table 1). The ASD group showed an increase in GABA and glutamate/glutamine of 80.65% ($p = 0.001$) and 56.98% ($p = 0.027$), respectively, compared to controls. There was a significant decrease in the levels of glutamine, and glutamate/GABA of 24.33% ($p < 0$ 0.001) and 37.41% ($p < 0.001$), respectively, in the ASD group compared with the control group. Furthermore, a decrease in glutamate levels in the ASD group was observed, although non-significant ($p > 0.05$) (Table 1).

When the autism severity, as assessed by CARS and SRS, was compared with glutamate and glutamate/GABA ratio, an increase in the level of glutamate with the severity occurred, while this level decreased compared to GABA in both scales. The glutamine level increased with higher autism severity on the CARS scale, whereas it decreased with increased severity on the SRS scale. In the glutamate/glutamine ratio, the levels tended to decrease with severe CARS scale, whereas it increased with increasing SRS scale. These results were, however, not significant at the $p < 0.05$ level and so multivariate analysis was latterly used to assess this issue.

Except for the absolute value of glutamate, all other parameters, and their relative ratios, were correlated with ASD severity, measured as CARS (Fig. 1). Table 2 and Fig. 2 illustrates the ROC analysis data as the AUC, cutoff values, specificity, and sensitivity of the measured parameters. The AUC was found to be close to one in GABA for ASD and different severity of both scales (CARS and SRS). The ROC analysis demonstrated 82.5% sensitivity (82.5% of the patients with ASD had elevated GABA values compared to the neurotypical controls) and 86.8% specificity (only 13.2% of neurotypical control individual had elevated GABA) (Table 2 and Fig. 2). Predictiveness curves, calculated according to Pepe et al. (2008) showed that at the best Youden cutoff the predictive risk to retrieve false positive or false negative is the

Table 1 Nonparametric test (Wilcoxon-Mann-Whitney test) between 40 male Saudi children with autism spectrum disorder (ASD) and 38 age- and gender-matched neurotypical controls

Parameter		Groups	N	Mean ± S.D.	Percent change	*P* value
Glutamate (Glu) (ng/ml)	Groups	Controls	38	45,643.82 ± 18,801.39	100.00%	*0.726*
		ASD children	40	45,344.88 ± 17,252.72	99.35%	
	CARS	Mild to Moderate	21	41,429.52 ± 15,979.10	90.77%	*0.081*
		Severe	19	49,672.37 ± 17,987.99	108.83%	
	SRS	Mild to Moderate	10	38,982.00 ± 21,153.42	85.40%	*0.396*
		Severe	14	45,067.86 ± 18,291.53	98.74%	
γ-Aminobutyric acid (GABA) (ng/ml)	Groups	Controls	38	9.13 ± 6.69	100.00%	*0.001**
		ASD children	40	16.50 ± 14.95	180.65%	
	CARS	Mild to Moderate	21	18.17 ± 20.16	198.92%	*0.524*
		Severe	19	14.66 ± 5.03	160.47%	
	SRS	Mild to Moderate	9	25.18 ± 30.00	275.69%	`
		Severe	14	14.64 ± 4.64	160.31%	
Glutamine (Gln) (ng/ml)	Groups	Controls	38	7.13 ± 2.05	100.00%	*0.001**
		ASD children	39	5.39 ± 2.40	75.67%	
	CARS	Mild to Moderate	20	4.97 ± 2.38	69.78%	*0.183*
		Severe	18	6.00 ± 2.37	84.16%	
	SRS	Mild to Moderate	9	5.43 ± 1.82	76.17%	*0.619*
		Severe	12	4.66 ± 2.76	65.36%	
Glu/GABA ratio	Groups	Controls	38	5718.22 ± 2669.91	100.00%	*0.001**
		ASD children	38	3579.13 ± 2022.28	62.59%	
	CARS	Mild to Moderate	20	3418.83 ± 2047.60	59.79%	*0.539*
		Severe	18	3757.24 ± 2037.45	65.71%	
	SRS	Mild to Moderate	9	2916.96 ± 1954.37	51.01%	*0.616*
		Severe	13	3299.72 ± 1860.95	57.71%	
Glu/Gln ratio	Groups	Controls	36	7143.80 ± 4226.72	100.00%	*0.027**
		ASD children	36	11,214.60 ± 8741.36	156.98%	
	CARS	Mild to Moderate	19	12,539.64 ± 10,183.83	175.53%	*0.623*
		Severe	17	9733.68 ± 6786.79	136.25%	
	SRS	Mild to Moderate	9	8525.06 ± 5646.99	119.34%	*0.394*
		Severe	12	14,821.15 ± 12,081.12	207.47%	

Results are presented as Mean ± S.D. *P* values demonstrate the significant differences either between total ASD participants relative to control or between mild-moderate relative to severe ASD patients using the Childhood Autism Rating Scale (CARS) and the Social Responsiveness Scale (SRS) as autism severity measures

*: *P* value <0.05 shows significant alteration

highest for glutamate alone, while it reaches the lowest value for its association with glutamine in the Glu/Gln ratio (Fig. 3).

When considering biochemical markers statistics, the Shapiro-Wilk test assessed a parametric distribution. The one-way MANOVA assessed that each biochemical marker changed with CARS or SRS (Table 3), but this effect was due to the strong association of biomarkers with the scoring evaluations, as these markers were retrieved only from ASD children (Table 3). The Levene's test for homogeneity of variances showed that variances were homogeneously dispersed in the ASD and control populations, aside for the comparison SRS/glutamate (Table 3). Therefore, a much stronger statistical test, evaluating multiple regression between CARS/SRS and biochemical markers was performed, showing that only glutamine levels explained the variability of ASD severity (CARS/SRS) ($p = 0.03956$, Table 3). Finally, when the Spearman correlation coefficients were calculated, the results showed that CARS was correlated with SRS and glutamine ($P < 0.009$ and $P < 0.038$, respectively), while GABA was negatively associated with glutamine ($P < 0.001$). The glutamate/GABA ratio was positively associated with glutamate/glutamine ($P < 0.001$) (Table 4).

Discussion

Although many recent studies are concerned with the screening for biomarkers of early diagnosis of ASD, no specific biomarker to date has been found to accurately mirror the etiological mechanism of this complex disorder directly. Neuroinflammation, oxidative stress, together with glutamate

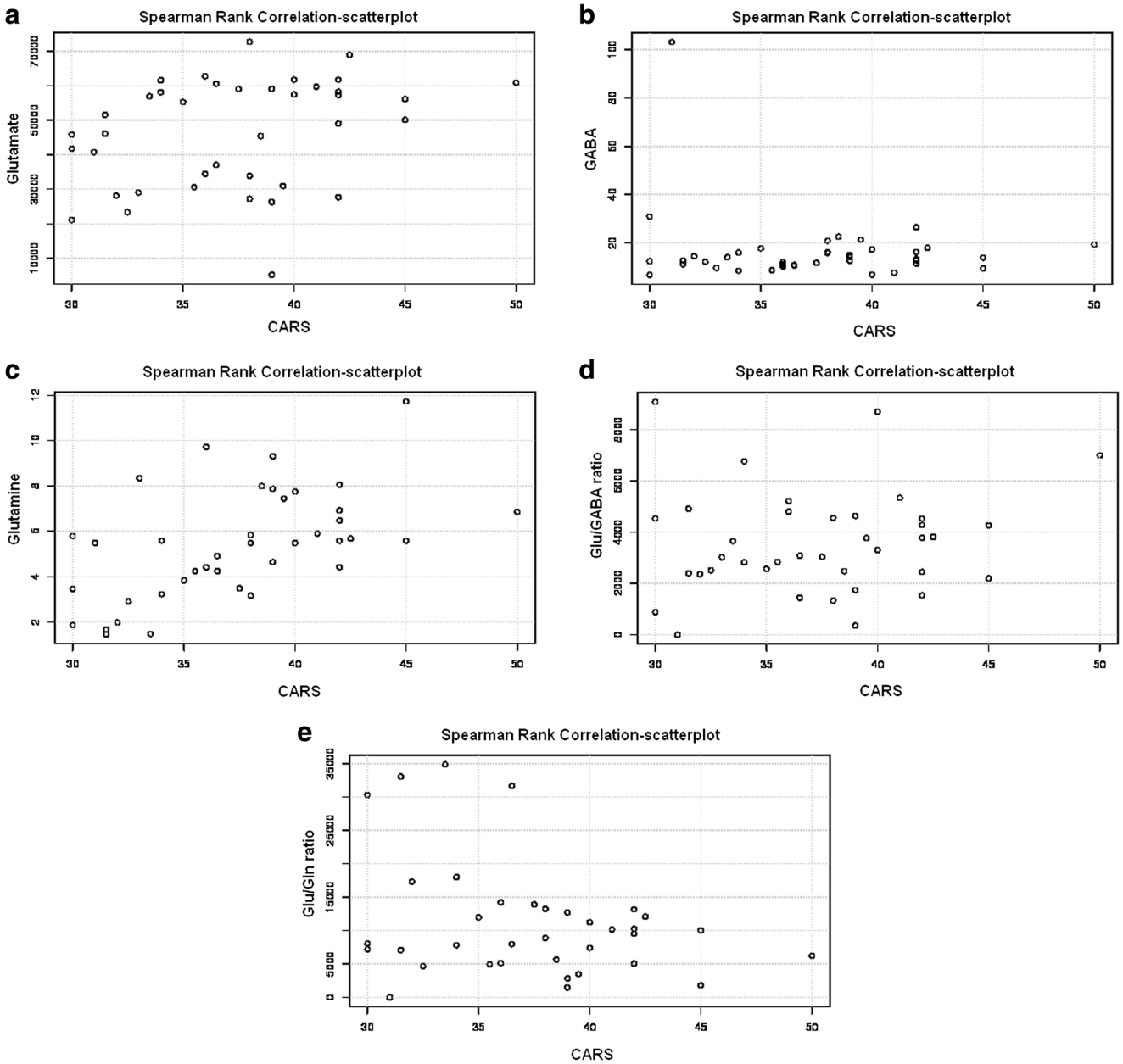

Fig. 1 Spearman rank correlations between CARS scale values and **a** Glutamate, **b** GABA, **c** Glutamine, **d** Glutamate/GABA ratio, and **e** Glutamine/Glutamate ratio

excitotoxicity are some of the major signaling pathways related to ASD (El-Ansary and Al-Ayadhi 2014).

In the present study, impairment of glutamate/GABA – glutamine cycle was found in the patients with ASD, thus confirming previous reports (Coghlan et al. 2012). Unexpectedly, glutamate, which would also be a biomarker of excitotoxicity, did not show a significant change in our statistical analysis, opposite to GABA and glutamine, as their relative concentrations showed significant changes that reflected abnormal glutamate/GABA – glutamine cycle.

Glutamate transporters are abundant in astrocytic processes, and play a predominant role in the glutamate clearance in the CNS. Modulation of glutamate transporters may occur via both slow regulatory mechanism or rapid regulation that occur within minutes (Simard and Nedergaard 2004), allowing glutamate to cause neuronal excitotoxicity before transporters on the astrocyte take up its clearance. Therefore, glutamate excitotoxicity has been proposed as a possible cause of exacerbation of ASD (El-Ansary and Al-Ayadhi 2014).

The present study found no significant association between autism severity as assessed by CARS and SRS and the measured biomarkers glutamate, GABA, glutamine and their relative ratios. In the multiple regression analysis, glutamine appeared as a

Table 2 Results for receiver operating characteristic (ROC) curve analysis between 40 male Saudi children with autism spectrum disorder (ASD) and 38 age- and gender-matched neurotypical controls

Parameter	Group		AUC	Cutoff value	Sensitivity %	Specificity %
Glutamate (Glu) (ng/ml)	ASD children		0.523	55,140.0	45.0%	73.7%
	CARS	Mild to Moderate	0.549	63,195.0	100.0%	18.4%
		Severe	0.602	55,582.5	63.2%	73.7%
	SRS	Mild to Moderate	0.583	37,602.5	60.0%	68.4%
		Severe	0.523	55,140.0	42.9%	73.7%
(GABA) (ng/ml)	ASD children		0.878	10.350	82.5%	86.8%
	CARS	Mild to Moderate	0.880	10.350	85.7%	86.8%
		Severe	0.875	11.841	78.9%	89.5%
	SRS	Mild to Moderate	0.911	8.577	100.0%	71.1%
		Severe	0.922	10.685	92.9%	86.8%
Glutamine (Gln) (ng/ml)	ASD children		0.718	5.997	69.2%	80.0%
	CARS	Mild to Moderate	0.750	5.971	70.0%	80.0%
		Severe	0.671	5.997	66.7%	80.0%
	SRS	Mild to Moderate	0.726	5.512	66.7%	82.5%
		Severe	0.753	5.971	75.0%	80.0%
Glu/GABA	ASD children		0.739	5353.900	89.5%	52.6%
	CARS	Mild to Moderate	0.761	3149.050	65.0%	84.2%
		Severe	0.715	4656.800	83.3%	60.5%
	SRS	Mild to Moderate	0.807	3149.050	77.8%	84.2%
		Severe	0.763	5290.400	92.3%	52.6%
Glu/Gln	ASD children		0.651	9533.20	50.0%	83.3%
	CARS	Mild to Moderate	0.649	13,356.50	36.8%	94.4%
		Severe	0.654	9533.20	52.9%	83.3%
	SRS	Mild to Moderate	0.577	13,023.50	33.3%	94.4%
		Severe	0.667	11,976.50	58.3%	86.1%

The predictive values of the measured markers are presented as area under the curve (AUC), specificity, and sensitivity

CARS Childhood Autism Rating Scale, *SRS* Social Responsiveness Scale

potential marker of autism severity score, but this evidence might come from the awareness that glutamine is a commonly shared metabolite at the crossroad GABA/glutamate. While this result is interesting, further investigation should confirm this finding. It has earlier been shown that elevated levels of glutamate in serum in ASD patients correlate weakly with autism severity (Shinohe et al. 2006). On the other hand, decreased level of glutamine has been found in children with ASD (Ghanizadeh 2013), so that it has also been suggested as a screening test for ASD in children (Ghanizadeh 2013). The suggested importance of depleted glutamine as a diagnostic marker in ASD is also in agreement with the medical hypothesis of Good (2013). He suggested that the remarkable improvements seen in autistic patients during fever are mostly due to the release of glutamine from muscles to blood and directly to the brain through the concentration-dependent transporter (Bode 2001).

Human blood plasma is a complex biological fluid that contains proteins, peptides, lipids, and metabolites, which reflect physiological activity and pathology in various body organs, including the CNS. In this sense, it is particularly cumbersome and difficult to reach a reliable marker for ASD prognosis. In humans, about 500 ml of cerebrospinal fluid (CSF) is absorbed into the blood daily. Blood is, therefore, a suitable source of neurodegenerative disease biomarkers (Hye et al. 2006). As glutamate and glutamine are actively transported out from the CNS (Hawkins et al. 2006), there is some positive expectation of finding a positive association between plasma and CSF levels of glutamine and glutamate (Fatemi 2015).

A further element to be taken into account in the present study is the pediatric cohort of patients. The functional properties of GABA receptor in the immature brain are significantly different from those found in the adult brain (Owens and Kriegstein 2002). Although GABA functions primarily as an inhibitory neurotransmitter, it is quite surprising to know that it can function as an excitatory neurotransmitter during brain development to influence events such as proliferation, migration, synapse maturation, differentiation, as well as cell death (Owens and Kriegstein 2002; Sibilla and Ballerini 2009). GABA mediates these processes by the activation of

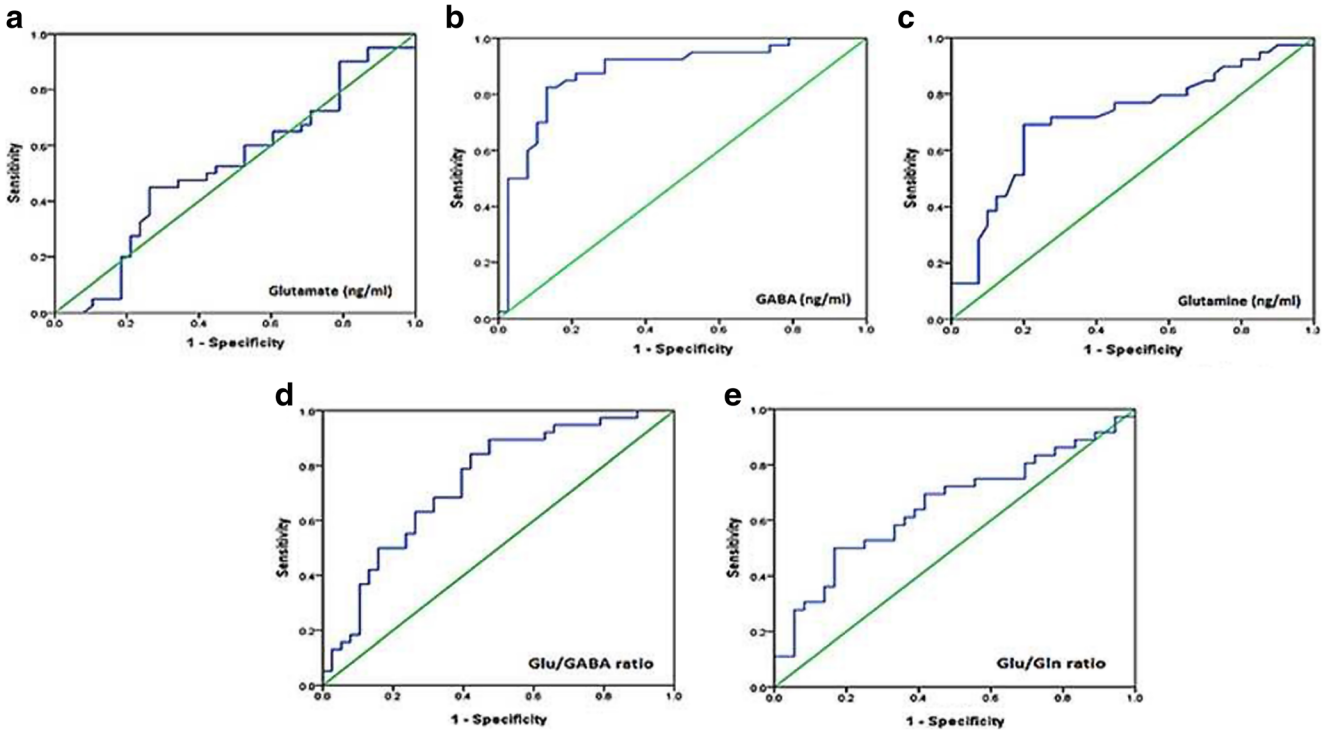

Fig. 2 ROC curves of **a** Glutamate, **b** GABA, **c** Glutamine, **d** Glu/GABA ratio, and **e** Glu/Gln ratio in autistic group

glutamate ionotropic and metabotropic receptors. Relying on this, the significant elevation of GABA found in the present study (Table 1) might be associated with a previous mechanism of excitotoxicity in ASD.

GABA and glutamate derive from each other, and therefore alterations in one of the neurotransmitters can affect the other one. The observed elevation of GABA in the present study can, therefore, be associated with decreased GABA levels in the brain

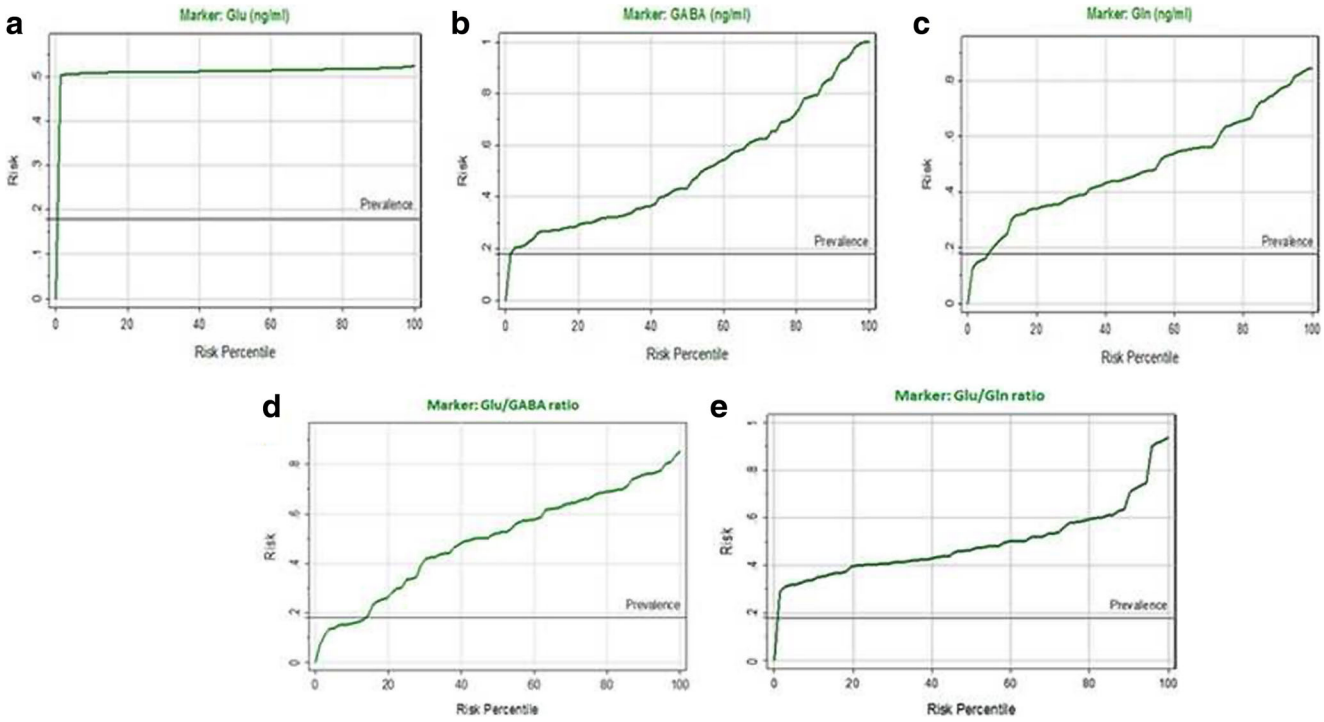

Fig. 3 Predictiveness curve of **a** Glutamate, **b** GABA, **c** Glutamine, **d** Glu/GABA ratio, and **e** Glu/Gln ratio in the autistic group

Table 3 One way MANOVA and multi-regression analysis of 40 male Saudi children with autism spectrum disorder (ASD) investigated in the study and their relationship with autism severity as evaluated by the Childhood Autism Rating Scale (CARS) and the Social Responsiveness Scale (SRS)

Parameter		One way multiple ANOVA		Multiple regression		
1	CARS/SRS/Glutamate	CARS: F value = 13.5	$P < 0.0001$	Multiple R R-squared	0.1695 0.02875	$P = 0.7255$
		SRS F value = 5.046	$P = 0.004$	Adjusted R-sq	−0.05955	
				F-test	0.3256	
2	CARS/SRS/GABA	CARS: F value = 17.274	$P < 0.0001$	Multiple R R-squared	0.2745 0.07533	$P = 0.4225$
		SRS: F value = 30.736	$P < 0.0001$	Adjusted R-sq	−0.008726	
				F-test	0.8962	
3	CARS/SRS/Glutamine	CARS: F value = 9.989	$P = 0.001$	Multiple R R-squared	0.6287 0.3953	$P = 0.03956$
		SRS: F value = 7.182	$P = 0.001$	Adjusted R-sq	0.3403	
				F-test	7.19	
4	CARS/SRS/Glu-GABA ratio	CARS: F value = 3.845	$P = 0.023$	Multiple R R-squared	0.03846 0.001479	$P = 0.9838$
		SRS: F value = 8.241	$P < 0.0001$	Adjusted R-sq	−0.0893	
				F-test	0.0163	
5	CARS/SRS/Glu-Gln ratio	CARS: F value = 6.001	$P = 0.005$	Multiple R R-squared	0.4124 0.1701	$P = 0.1287$
		SRS: F value = 3.037	$P = 0.032$	Adjusted R-sq	0.09462	
				F-test	2.254	
Levene's test for homogeneity of variance				Spearman rank correlation (higher ASD severity)		
1	CARS: $F = 1.089$	SRS: $F = 5.046$	$P = 0.464$; $P = 0.004$	CARS highest scores/Glutamate		
			Variance diff in the SRS/Glu	rho = −0.18736719583786		
				2 sided p value = 0.442422118312541		
2	CARS: $F = 0.447$	SRS: $F = 0.56$	$P = 0.919$; $P = 0.836$	CARS highest scores/GABA		
				rho = −0.0550416800704593		
			(*Homoscedasticity)*	2 sided p value = 0.822911304244861		
3	CARS: $F = 2.397$	SRS: $F = 0.71$	$P = 0.094$; $P = 0.719$	CARS highest scores/Glutamine		
				rho = 0.240216246546711		
			(*Homoscedasticity)*	2 sided p value = 0.336982381293736		
4	CARS: $F = 1.074$	SRS: $F = 1.028$	$P = 0.473$; $P = 0.482$	CARS highest scores/Glu-GABA ratio		
			(*Homoscedasticity)*	rho = −0.129104482867932		
				2 sided p value = 0.609654861517216		
5	CARS: $F = 0.295$	SRS: $F = 0.452$	$P = 0.982$; $P = 0.908$	CARS highest scores/Glu-Gln ratio		
				rho = −0.329224058039744		
			(*Homoscedasticity)*	2 sided p value = 0.196923890319356		

because of a lower number or dysfunctional neuronal GABA receptors (El-Ansary and Al-Ayadhi 2014; El-Ansary 2016). Accordingly, disruption of the cortical GABAergic inhibitory interneurons functioning has been linked to various neurodevelopmental disorders such as schizophrenia, ASD, mental retardation, and epilepsy (Rossignol 2011; Sesarini 2015).

To assess this relationship and its dynamics, ratios between different markers were also considered in this study. The excitation/inhibition ratio is always constant in neurotypical individuals, as excitatory neurons tend to be equal to inhibitory neurons. It is possible that an imbalance of this ratio can be responsible for health conditions like Down syndrome, as well as excessive excitation (Xue et al. 2014).

Recently it was also proposed that frequent association of ASD with seizures as phenotype might arise from an increased excitation/inhibition ratio. This imbalanced ratio can be due to increased glutamate activity, decreased GABA release, or reduced numbers of GABA receptors (Rosenberg et al. 2015). Since there is no efficient receptor/uptake system, GABA accumulates in the extracellular space and reaches a high enough

Table 4 Correlation analysis of 40 male Saudi children with autism spectrum disorder and 38 age- and gender-matched neurotypical controls showing positive and negative correlations between the measured parameters

Parameter	Rho	Two-sided *p* values	
Childhood Autism Rating Scale (CARS) ~ Social Responsiveness Scale (SRS)	0.510**	*0.009*	P[a]
CARS ~ Glutamate (Glu) (ng/ml)	0.344*	*0.032*	P[a]
CARS ~ Glutamine (Gln) (ng/ml)	0.338*	*0.038*	P[a]
CARS- γ-aminobutyric acid (GABA) (ng/ml)	0.145	*0.372*	n.s.
γ-aminobutyric acid (GABA) (ng/ml) ~ Glutamine (Gln) (ng/ml)	−0.384**	*0.001*	N[b]
Glu/GABA ~ Glu/Gln	0.394**	*0.001*	P[a]
CARS-Glu/GABA ratio	0.127	*0.447*	n.s.
CARS-Glu/Gln ratio	−0.178	*0.299*	n.s.

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

[a] Positive Correlation

[b] Negative Correlation

level to exert its excitatory and depolarizing effects on distal neurons (LeBlanc and Fagiolini 2011).

Moreover, the glutamate/glutamine ratio as a measure of buffering glutamate pool through its conversion to glutamine resulted significantly higher in ASD patients compared to neurotypical controls. Since these two amino acids play roles in intermediary metabolism, glutamine and glutamate measurements alone are not a particularly useful index of glutamatergic synaptic measure. On the other hand, the glutamate/glutamine ratio can be more accurate as a functional synaptic measure because it reflects the relative amounts of metabolites. Given glutamate synthesis in neurons and glutamine synthesis in astrocytes, the glutamate/glutamine ratio is a potentially useful index for quantifying neuronal–astrocyte interactions and the balance of glutamatergic metabolites (Hall et al. 2015). In the present study, the glutamate/glutamine ratio was 56.98% elevated in the ASD children compared to the neurotypical controls (Table 1). The increase may reflect increased glutamate neurotransmission in the ASD patients compared to the controls.

Receiver operating characteristic (ROC) curves are usually used in biomarker research for the evaluation of the diagnostic and predictive value of a biomarker. ROC curves are also used in medicine to determine a cutoff value for a clinical test. For example, a cutoff value of 10.350 was identified for GABA level in the ASD group. A test value of GABA below 10.350 is considered normal, while above abnormal. GABA compared to the other measured amino acids (glutamate and glutamine) is considered as a good biomarker to indicate ASD. GABA showed in the case of the SRS scale 100% sensitivity (AUC = 0.911), which is regarded as an excellent biomarker.

Aside from the speculative description of what might occur in ASD children concerning the glutamate/GABA-glutamine signaling, more research is needed to enhance our knowledge this topic. For example, the genetic background of the patients should receive further attention. A limitation of the present study is sample size. We attempted to obtain 100 individuals in order to enhance statistical power, yet, due to difficulties in retrieving accessible and reliable data on both CARS and SRS we were compelled to restrict investigation numbers.

The results of the present study strongly suggest that only glutamine levels, among the different markers investigated in the study, appeared to reliably correlate with the severity of autism, evidenced in the multiple regressions between CARS/SRS. Furthermore, the present study indicates that amino acids related to glutamatergic signaling are a factor in the etiology of ASD in the Saudi population. Disruption in the relative ratios of the evaluated parameters might relate to overstimulation of glutamate signaling, which subsequently leads to glutamate excitotoxicity.

Acknowledgements This research project was supported by a grant from the research center of the Center for Female Scientific and Medical Colleges at King Saud University.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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