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ORIGINAL ARTICLE

Predictive value of selected biomarkers related to metabolism and oxidative stress in children with autism spectrum disorder

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Abstract Autism spectrum disorder (ASD) as a neurodevelopmental disorder is characterized by impairments in social interaction, communication, and restricted, repetitive behavior. Several and reproducible studies have suggested that oxidative stress may represent one of the primary etiological mechanism of ASD that can be targeted for therapeutic intervention. In the present study, multiple regression and combined receiver operating characteristic (ROC) analysis were used to search for a relationship between impaired energy and oxidative metabolic pathways in the etiology of ASD and to find the linear combination that maximizes the partial area under a ROC curve for a pre-identified set of markers related to energy metabolism and oxidative stress. Thirty children with ASD and 30 age and gender matched controls were enrolled in the study. Using either spectrophotometric or ELISA-colorimetric assay, levels of lipid peroxides, vitamin E, vitamin C, glutathione (GSH)/glutathione disulfide (GSSG) together with the enzymatic activity of catalase, plasma glutathione peroxidase (GPx), and blood superoxide dismutase (SOD), were measured in peripheral blood samples, as biomarkers related to oxidative stress. Creatine kinase, ectonucleotidases (ADPase and ATPase) Na^+/K^+ (ATPase), lactate, inorganic phosphate, and levels of adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) together with adenylate energy charge, were also measured as markers of impaired energy metabolism. Statistical analysis using ROC curves, multiple and logistic regression were performed. A remarkable increase in the area under the curve for most of the combined markers, representing both energy impaired metabolism or oxidative stress, was observed by using combined ROC analyses. Moreover, higher specificity and sensitivity of the combined markers were also reported. The present study indicated that the measurement of the predictive value of selected biomarkers related to energy metabolism and oxidative stress in children with ASD using ROC analysis should lead to the better identification of the etiological mechanism of ASD associated with metabolism and diet. Agents with activity against the impaired metabolic pathway associated with ASD including the metabolic defects and involved enzymes hold a promise as a novel therapy for ASD.

Keywords Autism · Autistic children · ROC analysis · ROC curve · Oxidative stress

Introduction

Autism spectrum disorder (ASD) is a developmental brain disorder clinically presented as impairments in social interaction, communication skills with a typical repetitive behavior. To identify individuals with ASD and initiate interventions at the earliest possible age, biomarkers that measure neurological brain damage are clearly desirable. Currently, diagnosis of ASD is still phenotype-based, based on autistic features rather than an insightful laboratory test. As a matter of fact, ASD still lacks an adequate medical treatment in spite of a large number of recorded biomarkers (Loth et al. 2016).

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Several and reproducible studies suggested that oxidative stress might represent one of the most confirmed etiological mechanism of ASD that can be targeted for therapeutic intervention. Many previous studies demonstrated that the depletion of plasmatic reduced glutathione (GSH), increases the ratio of oxidized/reduced glutathione (glutathione disulfide (GSSG)/GSH) (Chauhan and Chauhan 2006; Al-Gadani et al. 2009; Al-Yafee et al. 2011; Ghanizadeh et al. 2012; El-Ansary 2016). Also, abnormal activity of glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD) as markers of the cell endowment in the antioxidant system are often impaired (Al-Gadani et al. 2009; Ghanizadeh et al. 2012). On the other hand, increased oxidative stress-related parameters have been recorded in individuals with ASD (Al-Gadani et al. 2009; Qasem et al. 2016). In relation to the neuroprotective role of GSH against oxidative stress and neuroinflammation, its use to decrease oxidative stress might be a potential treatment for this disorder (Díaz-Hung et al. 2016; Wink et al. 2016).

Growing bodies of evidence demonstrate the impairment of energy metabolism as another etiological mechanism is contributed in autism pathology, and many studies have reported mitochondrial dysfunction and abnormal level of adenosine triphosphate (ATP) in the blood and brain autopsy of individuals with ASD. A remarkably lower serum oxidized nicotinamide adenine dinucleotide (NAD^+) and ATP concentrations together with impaired NAD^+/reduced NAD (NADH) ratio were recorded in patients with ASD compared to neurotypical controls (Giulivi et al. 2010; Rossignol and Frye 2012; Theoharides 2013). A significant negative correlation was reported between plasma GSH, SOD, catalase activity, and serum NAD^+ and ATP levels and Childhood Autism Rating Scale (CARS) scores, as a measure of severity. While no significant correlation was observed between plasma total antioxidant capacity and autism severity, there was a strong relationship between plasma GPx, serum NADH, and severity of the autistic phenotype (Poling et al. 2006; Essa et al. 2013; Frye et al. 2013). In another study phosphocreatine (Pcr) depletion in ASD children was related to its use to maintain brain ATP levels. Depletion of Pcr was found to be positively correlated impaired social interaction as an autistic feature (Fujii et al. 2010).

Moreover, the abnormal concentration of ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inorganic phosphate (Pi) had also been recorded (Al-Mosalem et al. 2009). Authors suggested that the clinical manifestation observed in ASD might be secondary to the impairment of brain bioenergetics (Minshew et al. 1993; Chugani et al. 1999; Adams et al. 2011). Glutathione depletion reported in ASD patients followed by chronic gastrointestinal (GI) problems was related to mitochondrial dysfunction (Nissenkorn et al. 1999; Sherer et al. 2002; Gu et al. 2013) because the GI tract is highly dependent on glutathione to work efficiently (Hoensch et al. 2002). These studies are consistent with impaired mitochondrial function and document that some individuals with ASD have overall lowered cellular energetic balance and deficient reserve mitochondrial energy capability, which might lead to cognitive impairment, language deficits, and abnormal energy metabolism. Based on this fact, it is possible that increased susceptibility to oxidative stress in patients with ASD will occur due to alterations in antioxidant enzymes leading to impaired energy metabolism due to mitochondrial dysfunction.

Biomarkers are becoming essential for the diagnosis and treatment of a wide range of diseases (Smith and Smith 2012). Evaluating these biomarkers as a proper tool for a correct diagnosis of diseases is of great importance also with regard to improvement of the statistical technique. In biomarker research, it is common that several biomarkers may clinically relate to a particular disease and each single marker does not have adequate diagnostic power. Receiver operating characteristics (ROC) curve is an analytical tool where both sensitivity (true positive rate) and the complement to specificity (false-positive rate) are plotted across a series of cutoff values representing the whole range of values of a given biomarker of a disease, regarding its analytical performance. By definition, ROC curves can help researchers and investigators to identify the usefulness of a test to be insightful for the severity of disease and ruling out the disease in normal samples (Hajian-Tilaki 2013). A Bayesian consequence of this is that, as disease prevalence has no effect on sensitivity and specificity (Van Stralen et al. 2009). Also, the accuracy of ROC curve is independent of disease prevalence. A biomarker with greater discrimination or predictive power has a ROC graph very close to the upper left-hand corner of the plotted curve. Therefore, the closer the ROC plot of the biomarker to the upper left-hand corner, the greater is its discriminating capacity. On the contrast, the closer the curve to the reference line (also called diagonal line) of the graph, the lower the discriminating value of the disease marker. The overall discrimination power of a given biomarker is measured by calculating the area under the ROC curve (AUC). The AUC may be used as a perfect estimate of diagnostic accuracy. The AUC usually range from 0.5 (no discriminant capacity) to 1.0 (perfect discriminant capacity). An effective way to improve the diagnostic accuracy is to combine multiple markers. It is known that the AUC is highly recommended as a diagnostic tool to measure the usefulness of many markers.

Therefore, the current study aimed at finding a relationship between impaired energy and oxidative metabolic pathways in the etiology of ASD and to retrieve the linear combination that maximizes the partial area under a ROC curve (pAUC) for a pre-identified set of markers related to energy metabolism and oxidative stress (Al-Gadani et al. 2009; Al-Mosalem et al. 2009).

Materials and methods

In the present study, primary data of selected biomarkers related to energy status and antioxidant status of patients with ASD were reanalyzed in an attempt to use new statistical tools such as multiple regressions and combined ROC to increase the predictive values of previously published markers (Al-Gadani et al. 2009; Al-Mosalem et al. 2009).

Subjects

The protocol of the present study was ethically approved by the College of Medicine, King Saud University ethical committee according to the most recent Declaration of Helsinki (WMA 2013). The subjects enrolled in the study were 30 children with ASD (22 males and eight females) from 29 families ranging in age from 3 to 15 years, and 30 neurotypical children of the same age (20 males and ten females) as a control group. Autism spectrum disorder is much more prevalent in males than in females, and the sex ratio in the present study reflects the distribution of ASD in a local pediatric population. All subjects that were enrolled in the study (30 ASD children and 30 neurotypical control males) had filled informed consent from their tutors/parents, who therefore agreed to the study and signed it. Children were enrolled through the Autism Research and Treatment Center (ART Center) in King Khalid University Hospital in Riyadh. The ART Center population consisted of children diagnosed with ASD. The diagnosis of ASD was confirmed in all subjects using the Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS) (Rutter et al. 2005, 2012) as well as the Developmental Diagnostic Dimensional Interview (3Di) (Skuse et al. 2004). The average age of all children with ASD recruited for the present study was about 3–11 (IC_{95}) years old. The neurotypical controls were enrolled from the pediatric clinic at King Saud Medical City in Riyadh with average age 3–11 (IC_{95}) years old. The exclusion criteria included diagnosis of fragile X, dysmorphic features, other serious neurological, psychiatric, or known physical illness. All participants were screened via a parental interview for current and past physical illness. Children with known endocrine, cardiovascular, pulmonary, liver, kidney or other medical diseases were excluded from the study. Moreover, those treated with antioxidant supplements or psychotropic drugs were also excluded.

Blood samples

After 12 h fasting, blood samples from all participants were drawn into three ml blood collection tubes containing EDTA. Samples were immediately centrifuged at 4 °C at 3000 g for 20 min and stored at −80 °C until analysis.

Biochemical analyses

Plasma levels of lipid peroxides, vitamin E, vitamin C, glutathione together with the enzymatic activity of catalase were measured using spectrophotometric analysis, while plasma GPx and blood SOD were measured using ELISA kits, products of Randox. Creatine kinase, Na+/K+ (ATPase), lactate, Pi, AMP, ADP, and ATP were measured spectrophotometrically. Ectonucleotidases (ADPase and ATPase) were measured using ELISA kits, products of BioVision, USA. The adenylate energy charge (AEC) was calculated using the equation of Atkinson and Walton (1967):

$$\mathrm{AEC} = \left[\mathrm{ATP}\right] + 0.5\left[\mathrm{ADP}\right] \Big/ \left[\mathrm{AMP}\right]+[\mathrm{ADP}]+[\mathrm{ATP}]$$

Statistics

Statistical analysis was performed with IBM SPSS software, version 16 (IBM Inc., Armonk, USA). The obtained data were presented as mean ± SD and an ANOVA assay. Independent Fisher's t-test was used for statistical comparisons with $P \leq 0.05$ considered as significant. The Shapiro-Wilk test was used to test the normal distribution. Multiple regression analysis was used to find the correlation between the selected parameters using the IBM SPSS software. It is usually used to predict the value of a variable (dependent) based on the value of two or more other variables (independent). In multiple regressions, R2 describes the percentage of change in the dependent variable explained by the change in the independent variables together, which sometimes called the predictor variables. An R2 of 1.00 indicates that 100% of the changes in the dependent variable is directly related to the independent variables. Conversely, an R2 of 0.0 indicates the absence of variation in the dependent variable due to the independent variables. In the present study as the studied markers are not in the same unit of measures, a standardized regression coefficient, beta (ß), was used. The ß coefficients values show the positive or negative directions through which an independent variable relative to the other independent variables is contributed in the change of the dependent variable. R2 and (ß) coefficient were enough to interpret the obtained multiple regression data. Stepwise multiple regression analyses were performed for lipid peroxides, GSH, Na+/K+ ATPase and AEC as four dependent variables. Finally, multiple logistic regression analysis for the studied markers were performed as four categorized models. The same statistical packages provide further statistics that may be used to measure the usefulness of the models. Odds ratios (ORs) obtained from logistic regression describe associations of biomarkers with clinical status. ROC curves were constructed for each

Table 1 Primary data of selected biomarkers related to energy status and anti-oxidant status in patients with autism spectrum disorder (ASD) and healthy controls (control) (Al-Gadani et al. 2009; Al-Mosalem et al. 2009)

Parameter	Group	N	Mean ± S.D.	Percent Change	*P* value
Creatine kinase (U/L)	Control	18	132.17 ± 50.42	62.74% ↑	0.011
	ASD	14	215.10 ± 100.64		
Ectonucleotidase (ATPase) (μmol/min/ml)	Control	21	0.045 ± 0.022	44.12% ↑	0.003
	ASD	22	0.065 ± 0.020		
Ectonucleotidase (ADPase) (μmol/min/ml)	Control	24	0.074 ± 0.035	32.51% ↑	0.014
	ASD	23	0.098 ± 0.029		
Na^+/K^+ (ATPase) (μmol/min/ml)	Control	23	0.005 ± 0.002	226.69% ↑	0.001
	ASD	22	0.016 ± 0.005		
Inorganic phosphate (μmol/ml)	Control	19	3.59 ± 1.19	27.74% ↓	0.005
	ASD	22	2.59 ± 0.96		
Lactate (mmol/L)	Control	14	0.82 ± 0.27	61.97% ↑	0.016
	ASD	13	1.33 ± 0.63		
Adenosine triphosphate (ATP) (μmol/ml)	Control	18	2.37 ± 0.79	22.05 ↓	0.031
	ASD	23	1.85 ± 0.70		
Adenosine diphosphate (ADP) (μmol/ml)	Control	16	0.89 ± 0.41	18.22% ↓	0.266
	ASD	22	0.73 ± 0.46		
Adenosine monophosphate (AMP) (μmol/ml)	Control	18	0.267 ± 0.129	15.85% ↓	0.329
	ASD	23	0.225 ± 0.142		
Adenylate energy charge	Control	19	0.81 ± 0.08	1.00% ↑	0.760
	ASD	30	0.81 ± 0.09		
ADP/ATP	Control	18	0.407 ± 0.228	7.40% ↓	0.665
	ASD	23	0.376 ± 0.211		
Catalase (U/dl)	Control	28	39.43 ± 9.10	7.94% ↓	0.204
	ASD	29	36.30 ± 9.26		
Glutathione peroxidase (U/dl)	Control	27	144.56 ± 49.19	81.67%↑	0.001
	ASD	27	262.62 ± 92.48		
Superoxide dismutase (U/dl)	Control	25	1.153 ± 0.221	33.52% ↑	0.001
	ASD	24	1.540 ± 0.441		
Glutathione (μg/ml)	Control	27	33.24 ± 10.24	36.70% ↓	0.001
	ASD	27	21.04 ± 6.86		
Lipid peroxides (μmol/ml)	Control	27	10.21 ± 3.34	58.60% ↑	0.001
	ASD	27	16.19 ± 4.34		
Vitamin C (oxidized) (mg/dl)	Control	28	7.77 ± 1.63	18.00% ↓	0.003
	ASD	24	6.37 ± 1.61		
Vitamin C (reduced) (mg/dl)	Control	28	2.80 ± 0.83	7.36% ↑	0.401
	ASD	24	3.01 ± 0.92		
Vitamin C (total) (mg/dl)	Control	29	10.79 ± 2.09	0.07% ↓	0.990
	ASD	28	10.78 ± 2.58		
Vitamin E (mg/dl)	Control	28	1.93 ± 0.40	65.42% ↓	0.001
	ASD	20	0.67 ± 0.26		

logistic regression model. The area under the curve (ROC-AUC) was compared between each marker and marker combination using a non-parametric method (Campillo-Gimenez et al. 2013).

Results

Table 1 shows preliminary data presented as mean ± S.D. for all the measured parameters, according to previous reports (Al-Gadani et al. 2009; Al-Mosalem et al. 2009). A ROC analysis was used to assess the usefulness of these biomarkers in the early diagnosis of ASD (Table 2). It can be easily noticed that Na+/K+ ATPase, GPx, GSH, lipid peroxides, and vitamin E recorded AUC values between 0.7–0.977. These AUCs were accompanied with satisfactory values of specificity and sensitivity. A direct relationship was observed between the AUC as a diagnostic tool and the accuracy of the marker measured as specificity and sensitivity. Tables 3, 4, 5 and 6 show the relationship between lipid peroxides, GSH, Na+/K+ (ATPase), and AEC as dependent variables against the rest of the measured parameters as predictor variables. It can be

Table 2 Receiver operating characteristic curve of tested parameters in a group of 30 children with autism spectrum disorder

Marker	Area under the curve	Cut-off value	Sensitivity %	Specificity %
Creatine kinase	0.675	182.138	52.9%	76.2%
Ectonucleotidase (ATPase)	0.627	0.035	90.0%	36.7%
Ectonucleotidase (ADPase)	0.657	0.073	76.7%	53.3%
Na+/K+ (ATPase)	0.795	0.0105	75.9%	96.6%
Inorganic phosphate	0.661	2.922	63.3%	78.3%
Lactate	0.724	0.892	73.3%	66.7%
Adenosine triphosphate (ATP) conc.	0.526	2.340	66.7%	52.2%
Adenosine diphosphate (ADP) conc.	0.621	0.600	56.7%	73.9%
Adenosine monophosphate (AMP) conc.	0.586	0.092	33.3%	91.3%
Adenylate energy charge	0.517	0.922	13.3%	100.0%
ADP/ATP	0.600	0.494	76.7%	47.8%
Catalase	0.577	44.405	90.0%	27.6%
Glutathione peroxidase	0.806	172.446	73.3%	73.3%
Superoxide dismutase	0.648	1.578	52.0%	85.7%
Glutathione	0.722	24.720	66.7%	76.7%
Lipid peroxides	0.797	10.850	90.0%	60.0%
Vitamin C (oxidized)	0.618	7.350	66.7%	60.0%
Vitamin C (reduced)	0.541	4.800	20.0%	100.0%
Vitamin C (total)	0.509	7.500	16.7%	96.7%
Vitamin E	0.977	1.154	93.3%	93.3%

easily noticed that lipid peroxides as the dependent variable were associated with Pi as a predictive value with the R2 value of 0.171, which means that 17.1% of the increase of lipid peroxidation (Table 1) is probably due to impaired energy

Table 3 Multiple regression using stepwise method for lipid peroxides (μmol/ml) as a dependent variable in a group of 30 autistic children

Predictor Variable	Beta	*P* value	Adjusted R^2	Model	
				F value	*P* value
Creatine kinase	−0.044	0.387	0.300	1.519	0.372
Ectonucleotidase (ATPase)	−70.099	0.170			
Ectonucleotidase (ADPase)	2.872	0.940			
Na+/K+ (ATPase)	34.489	0.885			
Inorganic phosphate	−0.666	0.740			
Lactate	3.219	0.209			
Adenosine triphosphate (ATP)	0.088	0.979			
Adenosine diphosphate (ADP)	0.131	0.966			
Adenosine monophosphate (AMP)	1.776	0.894			
Adenylate energy charge	−14.246	0.592			
ADP/ATP	7.088	0.270			
Catalase	0.123	0.635			
Glutathione peroxidase	−0.041	0.337			
Superoxide dismutase	0.182	0.970			
Glutathione	0.216	0.390			
Vitamin C (oxidized)	−1.934	0.762			
Vitamin C (reduced)	0.111	0.979			
Vitamin C (total)	2.436	0.740			
Vitamin E	0.288	0.898			

Table 4 Multiple regression using stepwise method for glutathione (μg/ml) as a dependent variable in a group of 30 autistic children

Predictor Variable	Beta	*P* value	Adjusted R^2	Model	
				F value	*P* value
Creatine kinase	0.165	0.051	0.144	1.204	0.478
Ectonucleotidase (ATPase)	124.649	0.240			
Ectonucleotidase (ADPase)	21.147	0.782			
Na^+/K^+ (ATPase)	86.406	0.857			
Inorganic phosphate	−4.560	0.210			
Lactate	−1.029	0.858			
Adenosine triphosphate (ATP)	7.215	0.237			
Adenosine diphosphate (ADP)	-3.012	0.623			
Adenosine monophosphate (AMP)	-32.675	0.163			
Adenylate energy charge	68.848	0.144			
ADP/ATP	-9.908	0.463			
Catalase	−0.763	0.076			
Glutathione peroxidase	0.133	0.076			
Superoxide dismutase	5.792	0.537			
Lipid peroxides	0.872	0.390			
Vitamin C (oxidized)	17.793	0.098			
Vitamin C (reduced)	9.279	0.213			
Vitamin C (total)	−21.071	0.084			
Vitamin E	−0.559	0.901			

metabolism presented as a lower level of Pi. Table 4 shows that 16.8% of GSH depletion is related to the AEC as a measure of the energy status of the cell (R2 value of 0.168). Na+/K+ ATPase demonstrates a remarkable association with lactate as a predictor variable (R2 value of 0.319) and both are related to the abnormal energy metabolism in patients with

Table 5 Multiple regression using stepwise method for Na+/K+ (ATPase) (μmol/min/ml) as a dependent variable in a group of 30 autistic children

Predictor Variable	Beta	*P* value	Adjusted R^2	Model	
				F value	*P* value
Creatine kinase masurement	0.000	0.615	−0.552	0.569	0.821
Ectonucleotidase (ATPase)	0.029	0.822			
Ectonucleotidase (ADPase)	−0.016	0.853			
Inorganic phosphate mesurement	0.002	0.573			
Lactate determination	0.004	0.537			
Adenosine triphosphate (ATP)	-0.003	0.725			
Adenosine diphosphate (ADP)	-0.001	0.855			
Adenosine monophosphate (AMP)	0.020	0.497			
Adenylate energy charge	0.005	0.939			
ADP/ATP	0.002	0.915			
Catalase	0.000	0.546			
Glutathione peroxidase	0.000	0.465			
Superoxide dismutase	−0.003	0.806			
Glutathione	0.000	0.857			
Lipid peroxides	0.000	0.885			
Vitamin C (oxidized)	−0.011	0.435			
Vitamin C (reduced)	−0.007	0.431			
Vitamin C (total)	0.012	0.438			
Vitamin E	−0.005	0.291			

Table 6 Multiple regression using stepwise method for adenylate energy charge as a dependent variable in a group of 30 autistic children

Predictor Variable	Beta	*P* value	Adjusted R^2	Model	
				F value	*P* value
Creatine kinase	−0.001	0.256	0.061	1.079	0.531
Ectonucleotidase (ATPase)	−1.190	0.253			
Ectonucleotidase (ADPase)	−0.559	0.436			
Na+/K+ (ATPase)	0.358	0.939			
Inorganic phosphate	0.034	0.356			
Lactate	0.003	0.960			
Adenosine triphosphate (ATP)	-0.043	0.496			
Adenosine diphosphate (ADP)	0.024	0.686			
Adenosine monophosphate (AMP)	0.129	0.615			
ADP/ATP	0.006	0.968			
Catalase	0.006	0.235			
Glutathione peroxidase	0.000	0.285			
Superoxide dismutase	−0.063	0.486			
Glutathione	0.007	0.144			
Lipid peroxides	−0.005	0.592			
Vitamin C (oxidized)	−0.106	0.376			
Vitamin C (reduced)	−0.049	0.531			
Vitamin C (total)	0.130	0.338			
Vitamin E	0.010	0.815			

ASD (Table 5). An expected association between AEC as the dependent variable and [ADP]/[ATP], and [AMP] was also obtained and recording R2 values of 0.29, and 0.389 respectively. With the use of logistic regression as an analytical tool, four models of combining ROC were produced (Table 7). The combining of ADPase, Na+/K+ ATPase, and oxidized vitamin C were demonstrated (Table 7). By comparing the ORs of the three markers and their corresponding 95% confidence interval (CI), again Na+/K+ ATPase demonstrates the highest discriminating power compared to ADPase while vitamin C shows the least value with OR less than one (OR = 0.591). These models (I-IV) were effective in increasing the AUCs of

Table 7 Stepwise logistic regression in a group of 30 autistic children

	Regression coefficient	Standard error	Odds ratio	95% CI for odds ratio		*P* value
				Lower	Upper	
Adenosine diphosphate (ADP)	-2.675	2.231	0.069	0.001	5.463	0.231
ADP/ATP	6.523	5.928	680.510	0.006	7.56×10^6	0.271
Adenylate energy charge	−12.235	13.126	0.000	0.000	7.24×10^4	0.351
Adenosine monophosphate (AMP)	-7.906	6.818	0.000	0.000	234.302	0.246
Adenosine triphosphate (ATP)	4.639	3.160	103.393	0.211	5.06×10^3	0.142
Catalase	−0.116	0.110	0.890	0.718	1.104	0.289
Creatine kinase	0.022	0.023	1.022	0.977	1.070	0.341
Ectonucleotidase (ADPase)	24.020	30.301	2.70E + 10	0.000	1.68×10^{35}	0.428
Ectonucleotidase (ATPase)	17.550	32.428	4.19E + 07	0.000	1.68×10^{34}	0.588
Glutathione peroxidase	0.024	0.027	1.024	0.970	1.081	0.388
Glutathione	0.006	0.128	1.006	0.783	1.293	0.962
Inorganic phosphate	−2.221	1.761	0.109	0.003	3.425	0.207
Superoxide dismutase	4.369	4.783	78.937	0.007	9.30×10^4	0.361
Vitamin C (oxidized)	−0.516	0.641	0.597	0.170	2.094	0.420

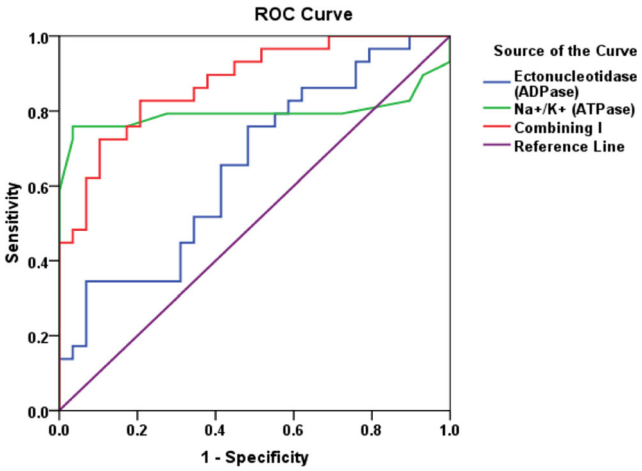

Fig. 1 Receiver operating characteristic (ROC) curve drawn using energy related markers for discriminating autism spectrum disorder (ASD). ROC curve analyses revealed that the plasma levels of (ADPase) and Na^+/K^+ (ATPase) were fair and good biomarkers for differentiating ASD (30 ASD children) and control (30 neurotypical children) groups, with the area under the ROC curve (AUC) values of 0.657 and 0.795, respectively. When the two markers were combined by logistic regression model, the AUC values for differentiating ASD and control groups were 0.876 showing much higher discriminating power

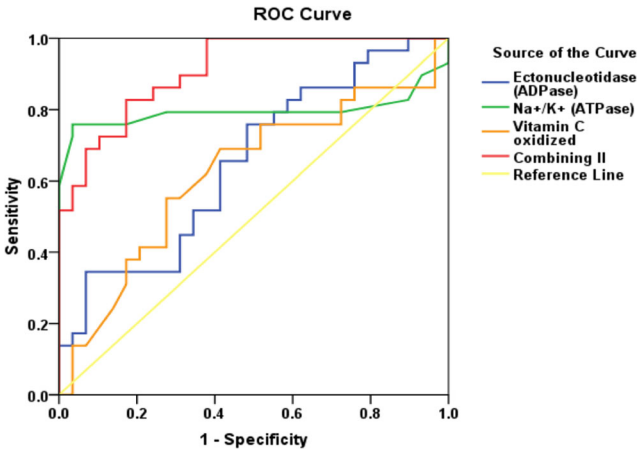

Fig. 3 Receiver operating characteristic (ROC) curve drawn using energy related markers for discriminating autism spectrum disorder (ASD). ROC curve analyses revealed that the plasma levels of (ADPase) and Na+/K+ (ATPase) and vitamin C as antioxidant for differentiating ASD (30 ASD children) and control (30 neurotypical children) groups, with area under the ROC curve (AUC) values of 0.657 and 0.795, and 0.618 respectively. When the three markers were combined by logistic regression model, the AUC values for differentiating ASD and control groups were 0.0.911 showing an excellent discriminating power

the combined variables (Figs. 1, 2, 3, 4, 5, 6 and 7). This was confirmed through the excellent predictiveness curves (Figs. 2, 4, 6, 8, 9, 10) for the four combined models.

Discussion

ROC analysis in the current study (Table 2), categorizes the measured parameters into worthless markers (ATP, AMP, and AEC, catalase, reduced and total vitamin C), fare markers (creatine kinase, NTPDases, Pi, ADP, SOD, and oxidized glutathione, good markers (Na^+/K^+ ATPase, lactate, GPx, GSH, and lipid peroxides), and an excellent marker (vitamin E). Based on this only six out of the 13 measured parameters demonstrate discriminative power between control neurotypical participants and patients with ASD, while vitamin E shows excellent predictive power with AUC (almost equal with one).

Little information related to the mechanism of Pi uptake into brain cells is available. An inorganic phosphate transporter (PiT) which is shown to carry glutamate into synaptic vesicles was identified (Bellocchio et al. 2002), but its transporting capacity is still to be confirmed (Takamori et al. 2000; Kowaltowski et al. 1996). Table 3 shows the relationship between lipid peroxide as a dependent marker of oxidative stress and Pi as a predictive marker. The remarkable decrease of Pi in spite of the high significant increase of RBCs

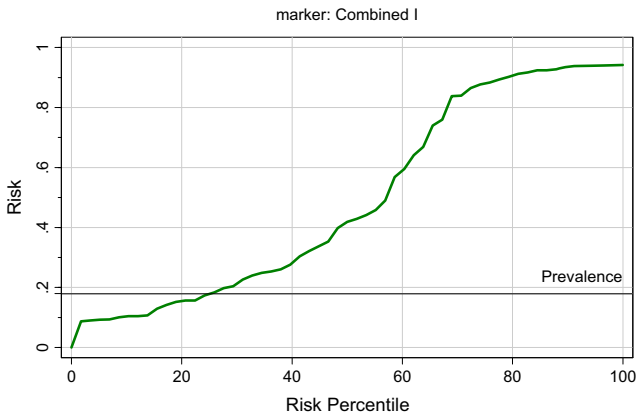

Fig. 2 The predictiveness curve as a measure of the performance of combined ATPase, and Na^+/K^+ (ATPase) in autism risk prediction in the Saudi population. The combined markers showed adequate predictive power

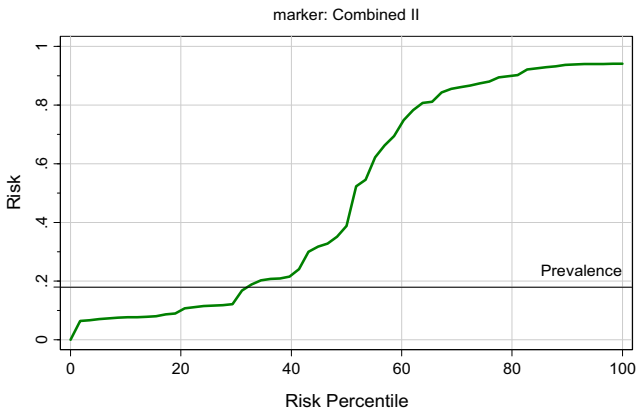

Fig. 4 The predictiveness curve as an assessment of the performance of combined ATPase, Na^+/K^+ (ATPase), and vitamin C in autism risk prediction in the Saudi population. The combined markers showed an excellent predictive power

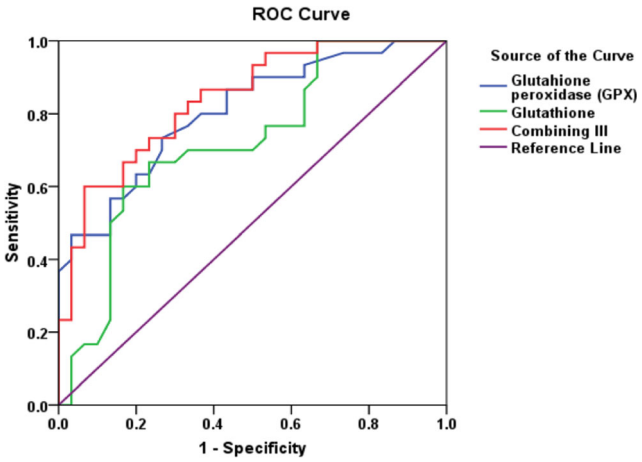

Fig. 5 Receiver operating characteristic (ROC) curve analysis using antioxidant related markers for discriminating autism spectrum disorder (ASD). ROC curve analyses revealed that the plasma levels of glutathione peroxidase and glutathione both are good biomarkers for differentiating ASD (30 ASD children) and control (30 neurotypical children) groups, with area under the ROC curve (AUC) values of 0.806 and 0.722, respectively. When the two markers were combined by logistic regression model, the AUC values for differentiating ASD and control groups were 0.840 showing much higher discriminating power

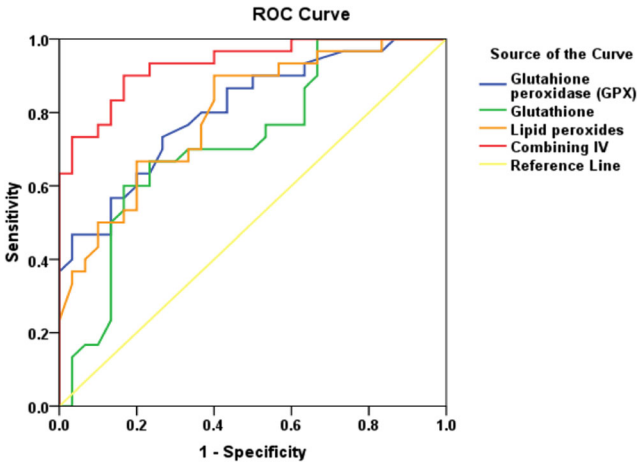

Fig. 7 Receiver operating characteristic (ROC) curve analysis using antioxidant related markers for discriminating autism spectrum disorder (ASD). ROC curve analyses revealed that the plasma levels of glutathione peroxidase, glutathione as antioxidant and lipid peroxide as oxidative stress marker are effective in differentiating ASD (30 ASD children) and control (30 neurotypical children) groups, with area under the ROC curve (AUC) values of 806, 0.722, and 0.797 respectively. When the three markers were combined by logistic regression model, the AUC values for differentiating ASD and control groups were 0.932 showing an excellent discriminating power

activity of Na^+/K^+ ATPase can be related to glutamate excitotoxicity as an etiological mechanism in ASD. Glutamate excitotoxicity was linked to unusual ability to transport glutamate back to the vesicles on pre-synaptic neuron or astrocytes. The reported low Pi in plasma can be accompanied by concomitant influx from blood to brain across the disrupted blood-brain barrier. This can support the association between the contribution of Pi in the production and elevation of lipid peroxides reported in the present study (Table 3). It is well known that lipid peroxides and H_2O_2 production by mitochondria incubated in the presence of Pi increase with increasing Pi concentrations within the CNS cells (De la Fuente et al. 2014).

It is well documented that all cellular bioenergetic processes are coupled with each other through adenosine nucleotides (AMP, ADP, and ATP), which are consumed or synthesized by different enzymatic reactions. The ratio of ATP, ADP, and AMP is practically greater than the absolute concentration of ATP. The AEC measures the energy status of the cell. It is a scientific term proposed by Atkinson and Walton (1967) and presented as [ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP]). Theoretically, the AEC is ranging between zero and one, but under the normal physiological condition, it is usually stabilized within a narrow range (0.85–0.95) (Holmsen and Robkin 1977; Raimundo 2014).

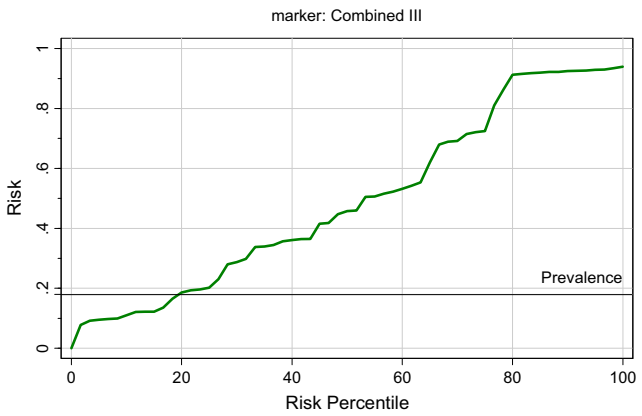

Fig. 6 The predictiveness curve as an assessment of the performance of combined glutathione peroxidase, and glutathione in autism risk prediction in the Saudi population. The combined markers showed a very good predictive power

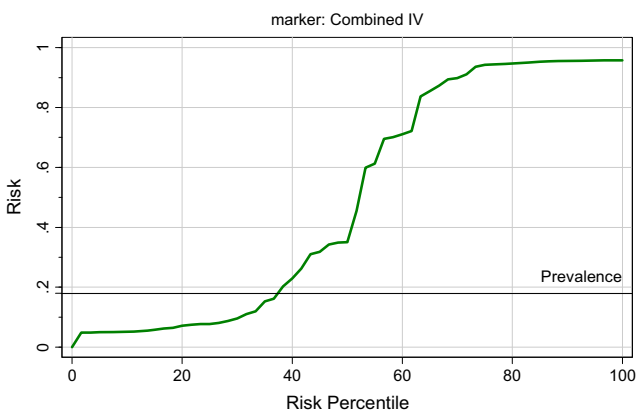

Fig. 8 The predictiveness curve as an assessment of the performance of combined glutathione peroxidase, glutathione, and lipid peroxides in autism risk prediction in the Saudi population. The combined markers showed a perfect predictive power

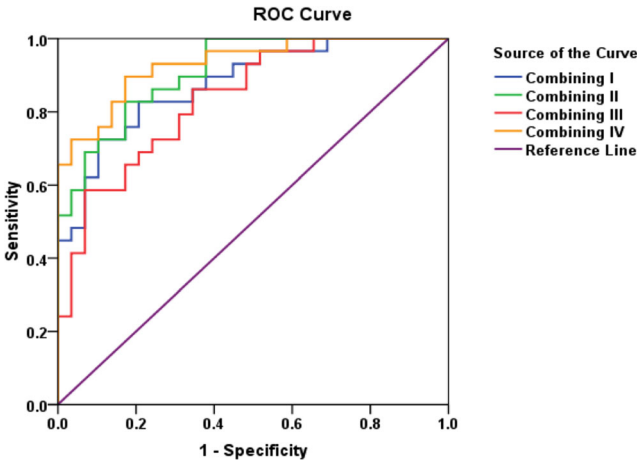

Fig. 9 Receiver operating characteristic (ROC) curve analysis using the four combining models (I-IV) with area under the ROC curve (AUC) values of 0.876, 0.911, 0.84, and 0.932 respective

The significant decrease of ATP reported in the present study and the unchanged AEC, together, can support the previous report of Al-Gadani et al. (2009) that ASD patients from Saudi Arabia are under H_2O_2 stress due to the over-expression of SOD with slightly lower catalase activity. Hydrogen peroxide (H_2O_2) can be used as a tool to specifically reduce the ATP level in platelets without altering ATP turnover and AEC (Sies 2014). The non-significant decrease of AEC reported in the present study does not contradict the significant reduction of ATP level and its effect on brain cells. It is well known that AEC transiently returned to approximately 90% of control, but ATP content recovered only to 40% in argon-mediated hippocampal and cortical neurons in vitro ischemia. This can strongly support the hypothesis of Atkinson and Walton (1967) that energy charge rather than ATP is the relevant regulatory parameter for control of cell functions. The reported association between GSH as dependent variable and AEC as a predictor variable (Table 6), can highlight the role of GSH in the stabilization of AEC especially under the oxidative effect of H_2O_2 as a mitochondrial signal related to AEC stabilization and ATP depletion (Ayer et al. 2010; Yoboue et al. 2012).

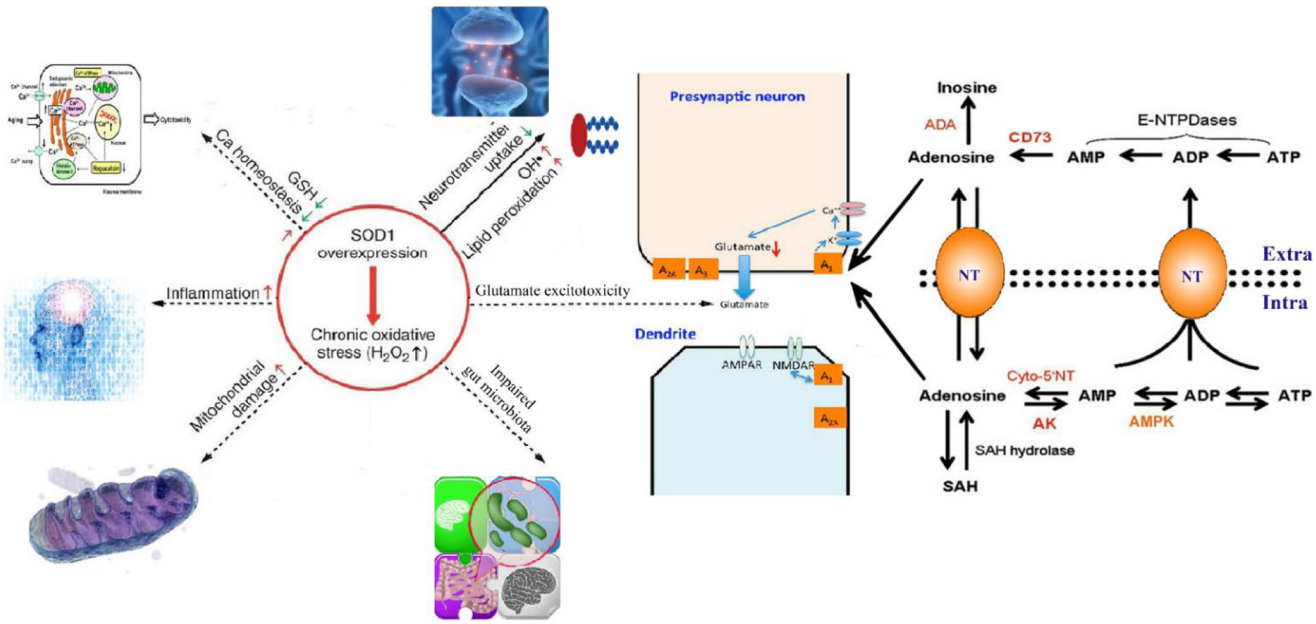

Fig. 10 The cartoon illustrates the main pathoethiological mechanisms underlying the relationship between oxidative stress, inflammation, impaired energy metabolism, and glutamate excitotoxicity in autism. The left part shows how oxidative stress causes an overexpression of the superoxide dismutase (SOD), exacerbating the oxidative stress response with an increase in hydrogen peroxide (H_2O_2). This circumstance is also associated with depletion of glutathione (GSH), impairment in calcium homeostasis and consequently mitochondrial damage. An increased response to inflammation is also a possible cause of this mechanism, which may lead to lipid peroxidation and inhibition in the neurotransmitter uptake mechanism. Chronic causes of an impaired oxidative stress response are represented by glutamate excitotoxicity (also due to disorders in the neurotransmitter (NT) reuptake) and gut microbiota immune imbalance. In the right part, it is represented a model by which glutamate excitotoxicity may be exacerbated by the adenosine signaling pathway, caused by an impaired nucleotide phosphatase (NTP) function as a consequence of a chronic oxidative stress and metabolic disorder. In this part, it is showed how impaired energy balance causes biochemical dephosphorylation of ATP to AMP by the extracellular nucleotidases (E-NTPDases), which is uptaken by the 5'-nucleotidase or CD73 to form adenosine, which is lately inactivated to inosine by the adenosine deaminase (ADA). Adenosine is also produced by the intracellular pathway of the adenosine kinase (AK) and adenosine phosphokinase (AMPK), closely linked to energy balance and cell survival and by the breakdown of S-adenosyl-homocysteine by the S-adenosyl-L-homocysteine hydrolase (SAH). Adenosine plays a major pre-synaptic action while a minor postsynaptic effect in the modulation of glutamate neurotransmission, where it can play a significant role in blocking a large part of the glutamate-induced Ca^{2+} rise. Adenosine, through the A2A receptors, has a fundamental role in modulating glutamate release and glutamate excitotoxicity and its excess due to impaired nucleotides (NTPs) metabolism may lead to impairments in the purinergic signaling, particularly in the hypothalamus and basal ganglia

Table 8 Combined receiver operating characteristic curve of parameters in a group of 30 autistic children

Group		Area under the curve	Cutoff value	Sensitivity %	Specificity %
Ectonucleotidase (ADPase)	1	0.657	0.073	76.7%	53.3%
Na+/K+ (ATPase)	2	0.795	0.0105	75.9%	96.6%
Combining	1 + 2	0.876	-------	82.8%	79.3%
Vitamin C (oxidized)	3	0.618	7.350	66.7%	60.0%
Combining	1 + 2 + 3	0.911	-------	82.8%	82.8%
Glutathione peroxidase	4	0.806	172.446	73.3%	73.3%
Glutathione	5	0.722	24.720	66.7%	76.7%
Combining	1 + 2 + 3 + 4 + 5	0.840	-------	80.0%	70.0%
Lipid peroxides	6	0.797	10.850	90.0%	60.0%
Combining	1 + 2 + 3 + 4 + 5 + 6	0.932	-------	90.0%	83.3%

The relationship presented in Table 5 between Na^+/K^+ ATPase as dependent variable and lactate as a predictor or independent variable can be supported and related to glutamate excitotoxicity as an etiological mechanism in ASD. Pellerin and Magistretti (1997) proposed that on activation of a particular brain area, glutamate is released from glutamatergic presynaptic terminals, to reach their receptors on the postsynaptic neuron. Its excitatory action is terminated via uptake by Na^+-dependent transporters located on astrocytes. Na^+ entry resulting from glutamate transport would activate Na^+/K^+ ATPase, to rapidly provide enough ATP for the astroglial pump. This in turn would increase glucose utilization with a subsequent accumulation of lactate.

Recent researches focus on combining multiple biomarkers to diagnose different diseases more efficiently through improving the sensitivity and specificity. This might help in the future to produce a multi-biomarkers product that can help early diagnosis of ASD as a puzzling disorder (Kim et al. 2013). Logistic regression as the best marker combination for differentiating the disease from neurotypical control was performed (Tables 7). In the present study, the ORs can be used to determine whether impaired biomarkers can be used as risk factors for the development of autistic phenotype. The 95% CI is used to measure the precision of the OR. While the odds ratio for both markers is statistically significant, the confidence interval suggests that Na^+/K^+ ATPase is more contributed as a risk factor to develop autistic features showing more precise OR. A larger study is needed to generate a more accurate estimate of the role of ADPase as a member of ectonucleotidases in the pathogenicity of ASD. The effectiveness of combining ROC in increasing the predictive value of the measured parameters can be easily observed through the remarkable increase of AUCs values of the ROC analysis. While AUCs of 0.627, 0.618 were reported for ADPase and oxidized vitamin C respectively, a value of almost 0.8 (0.795) was recorded for Na^+/K^+ ATPase. Based on the fact that, Na^+/K^+ ATPase is one of the most important enzymes related to energy status of the organism, mitochondrial dysfunction may be one of the most recognized etiological mechanism in ASD. Combining these three markers by logistic regression remarkably increase the AUC to reach 0.911 and collectively described as excellent diagnostic or discriminating markers (Table 8, and Figs. 1 and 3). This can find support through the fact that vitamin C was effective in modulating the apomorphine-induced stereotypic behavior induced in rats through the potentiation of dopaminergic activity. Moreover, the combined and more precise effect of Na^+/K^+ ATPase as a marker of increased ATP turnover (hyperpurinergia) in individuals with ASD, can find support in previous studies which hypothesized hyperpurinergia as a mechanism occurs in ASD patients in an attempt to tolerate the abnormal metabolism and behavior. This is in good agreement with the recent work of Naviaux et al. (2014) who found that disturbances in social behavior and metabolism in maternal immune activation (MIA) mouse model of autism are not permanent but can be reversed with anti-purinergic therapy (APT).

Table 7 show the usefulness of logistic regression as a tool in the research on biomarkers. Combining GSH, GPx, and lipid peroxides noticeably increase the AUC to reach 0.932 and collectively described as perfect diagnostic markers (Table 8 and Figs. 5 and 7). This is consistent with the report of Ghanizadeh et al. (2012) which ascertained the imbalance of oxidative (lipid peroxides) and antioxidative stress systems (GSH and GPx) in ASD and the possibility of using GSH, a neuroprotective against oxidative stress and neuroinflammation as a potential treatment for this disorder (Chauhan et al. 2012; Rose et al. 2012; Hodgson et al. 2014). This was clearly seen in the perfect predictiveness curves of the combined ROC models (Figs. 2, 4, 6 and 8). Recently intranasal administration of GSH elevated brain GSH levels and demonstrated a mild symptomatic improvement in Parkinson's disease symptoms (Mischley et al. 2016). This might give hope of applying the same strategy in treating patients with ASD. The relationship between oxidative stress, inflammation,

impaired energy metabolism, and glutamate excitotoxicity as four pathoetiological mechanisms in autism is illustrated and explained in Fig. 10.

Conclusion

In spite of the excellent diagnostic value of ROC analysis in the evaluation of the discriminating power of biomarkers, the present study revealed that the combination of different biomarkers related to energy metabolism and oxidative stress and/or antioxidant status produced an accurate sensitivity and specificity for the diagnosis of ASD. Therefore, the present work has indicated the possibility to use logistic regression and combining ROC as a simple clinical method that might help in the early diagnosis of ASD.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest with respect to the authorship, and/or publication of this article.

Ethical approval All procedures performed were in accordance with the ethical standards of the institutional and/or national research committee, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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