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# Metabolism-Associated Markers and Childhood Autism Rating Scales (CARS) as a Measure of Autism Severity

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## Abstract

Autism spectrum disorder (ASD) is a neuro-behavioral syndrome with a broad spectrum of different mechanisms and etiologies that are caused by abnormal brain development. To date, no highly reliable and effective diagnostic biomarker to assess ASD is available so far. The present study investigated the predictivity potential of some suggested markers in ASD diagnosis focusing onto the relative ratios of several plasma biomarkers of electron transport chain function, and mitochondrial metabolism in 41 patients with ASD evaluated for behavior deficits measured using Childhood Autism Rating Scales (CARS). The control matched for further 41 healthy subjects. The relation of these relative ratios to ASD severity was also examined, as well as their ability to distinguish ASD children from neurotypical children. All predictive ratios were found to be markedly altered and correlated in ASD patients. However, no ratio was connected with autism severity. Interestingly, MRCC-I/caspase-7, GSH/GST, and MRCC-I/COQ10 were the most distinctive relative ratios between neurotypical controls and ASD patients and may thereby be useful biomarkers for early diagnosis of ASD. Overall, this investigation proves that relative ratios of numerous mitochondrial biomarkers might be predictive and efficient to differentiate between neurotypical children and ASD.

**Keywords** Autism · Mitochondria · Energy metabolism · MRCC-I/caspase-7 · GSH/GST · MRCC-I/COQ10

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## Introduction

Autism spectrum disorder (ASD) is a range of complex neurodevelopmental disabilities characterized by impaired social communication and/or interaction, associated with restricted and repetitious behaviors (APA 2013; Baum et al. 2015; Ivanov et al. 2015; Thye et al. 2017). The most frequent comorbidities present in children with ASD are mental retardation, impairment in sensory, gastrointestinal dysfunction, sleep disorder, and autoimmune disease (APA 2013; Brookman-Frazee et al. 2017). Nowadays, the prevalence of ASD reached a dramatic increase worldwide, accounting for 1 of 68 American children aged 8 years who were diagnosed with ASD according to the Autism and Developmental Disabilities Monitoring Network (ADDM) (Christensen 2016). The most common standardized tool used in the ASD assessment process is the Childhood Autism Rating Scale (CARS) that originally was developed by Schopler and Reichler (1971). The CARS is a semi-structured interview

and takes between 20 and 50 min of the susceptive subjects to have the ASD from the age of 24 months. This scale of assessment identifies and distinguishes children with ASD with respect to other children having different developmental disorders. It also assesses the intensity of autistic disorders and measures the behaviors of the child during interactions with the world around him (Schopler et al. 1980; Al-Otaish et al. 2018). Notably, early therapeutic intervention has been found to decrease the burden of ASD both for the children and for their families (Ganz 2007; Dawson et al. 2010).

A blood-based biomarker for ASD would facilitate early intervention with behavioral therapies. Neuronal functions are supported by mitochondrial ATP production. Neurons critically need well functional and intact mitochondria and enough oxygen supply to perform the complex processes such as neurogenesis, excitability, neurotransmission, and synaptic plasticity (Ames 2000; Erecinska et al. 2004; Kann and Kovács 2007). The electron transport chain (ETC), which is on the internal mitochondrial membrane, is involved in mitochondrial respiration. It generates a gradient of protons, which enables the production of energy in the form of ATP via oxidative phosphorylation (Wu et al. 2016; Serasinghe and Chipuk 2017). ATP is essential for all biological processes that guarantee the survival of neurons and the reactions of protein phosphorylation, which mediate synaptic signaling and related long-term changes in neuronal structure and function (Mattson et al. 2008).

The efficiency of oxidative phosphorylation may depend on reducing equivalents delivered inside the mitochondrial respiratory chain (MRC) as well as the activity of enzyme complexes or enzymes that are involved. MRC complex I (MRCC-I) as an enzyme responsible for adaptive changes and physiological setup of oxidative phosphorylation efficiency is known to be impaired in ASD patients (Khemakhem et al. 2017). The enzyme creatine kinase (CK) is crucial for ATP repletion and is associated with the MRCC-I activity. Measurement of MRCC-I/CK might explain how energy impairment can be considered as an etiological mechanism in ASD. Al-Mosalim et al. (2009) reported lower ATP levels in red blood cells together with elevated lactate and CK activities in the plasma of Saudi children with ASD compared with their age-matched neurotypical controls.

Chauhan et al. (2011) also reported marked increased activity of $Na^+/K^+$ ATPase and $Ca^{2+/}Mg^{2+}$ ATPase as well as a significant diminution in the MRC complexes expression in diverse brain regions of patients with ASD compared to age-matched neurotypical controls suggesting these enzymes contribute to the abnormal energy circuit functioning in ASD.

Numerous studies have highlighted the existence of altered levels of biomarkers such as lactate, pyruvate, alanine, MRCC-I, acylcarnitine, CK, caspase-7, coenzyme Q10 (CoQ10), glutathione (GSH), and glutathione-S-transferase (GST) in the blood and urine samples from ASD patients (Rossignol and Frye 2012a). Contradiction in the literature, particularly regarding the presence of elevated biomarkers such as lactate, pyruvate, $K^+$, $Na^+$, and mitochondrial-related enzymes are recently observed in ASD (Hollis et al. 2017; Khemakhem et al. 2017). Based on the scientific observation that MRCC-I, CK, glycolysis, TCA cycle, and fatty acid oxidation as energy generation pathways appeared to be regulated by ATP and ADP levels and most importantly our belief that relative ratios are more predictive compared to their absolute values in the field of biomarkers, it was interesting to measure the predictive values of $K^+/Na^+$, lactate/pyruvate, MRCC-I CK, MRCC-I/caspase-7, MRCC-I/CoQ10, and GSH/GST.

## Materials and Methods

### Subjects

Forty-one male ASD patients aged from 2 to 14 years and 41 age- and sex-matched apparently healthy control children were enrolled from the Autism Research and Treatment Centre (Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia) to carry out the present study in accordance with the ethical principles of the Declaration of Helsinki (WMA 2013, 2014). No clinical indication of infectious disease or neuropsychiatric disorders was detected in any participant. The enrolled children had normal sedimentation rate and urine analysis results. Any participant diagnosed with epileptic seizures, fragile X syndrome, or any psychiatric, neurological, affective, or obsessive-compulsive disorders were excluded from the study. CARS score which measures the autism severity was determined according to Schopler et al. (2010).

### Sample Collection

Ten milliliters of blood samples were collected in test tubes containing heparin as an anticoagulant, from both ASD and control groups following overnight fasting. After centrifugation was the plasma obtained stored at − 80 °C until the analysis.

### Kits and Chemicals

All chemicals that were used in the present study were produced by Merck (Germany) or Sigma Aldrich (Mo, USA) and were of a good analytical grade. Human CoQ10, melatonin, and MRCC-I ELISA kits were purchased from MyBiosource (San Francisco, USA) while human caspase-7 ELISA kit was provided by CUSABIO (Beijing, Republic of China). CK kit was a product of BioSystems (Barcelona, Spain). Lactate and pyruvate colorimetric kits were purchased from BioVision (San Francisco, USA). $K^+$ and $Na^+$ colorimetric kits, lactate

dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) kinetic kits were products of United Diagnostics Industry (Dammam, South Arabia). Negative and positive controls were measured to determine the detection limits of all diagnostic kits that were used and to check the measurement validity.

### Measurement of Pyruvate and Lactate Concentrations

Plasma pyruvate and lactate levels were determined calorimetrically following the manufacturers' instructions of the respective diagnostic kits used. Detection limits are between 1.0 μM–10 mM and 0.001–10 mM for pyruvate and lactate, respectively.

### Measurement of LDH

Plasma LDH activity was assayed spectrophotometrically by following the "forward" reaction (lactate + NAD+ to pyruvate + NADPH + H+) according to Amador et al. (1963) and Wacker et al. (1956). NADH formation rate was indicated by an increase in absorbance at 340 nm, which was directly proportional to LDH activity.

### Measurement of AST and ALT Activities

AST and ALT activities were kinetically evaluated in serum samples following the manufacturer's guidelines with respect to the NADH oxidation rate measured as a decrease in absorbance at 340 nm (Karmen et al. 1953; Henry et al. 1960; Weindling and Henry 1974). AST and ALT activities were expressed in units per liter.

### CK Activity Measurement

The CK activity was investigated in serum samples according to the method of Schumann et al. (2002) by using CK kit, a product of BioSystems. Enzyme activity is expressed in U/L with a detection limit of 9.2 U/L = 153 nkat/L.

### Measurement of MRCC-I Level

Human MRCC-I level was analyzed following the manufacturer's guidelines of the diagnostic ELISA kit used which employs a quantitative sandwich enzyme immunoassay technique. The color developed was read at 450 nm using an ELISA reader, and the detection limit is ranging from 3.12 to 100 ng/ml.

### Caspase-7 Level Measurement

The caspase-7 level was measured according to the manufacturer's instructions of the ELISA kit used. This assay makes use of competitive inhibition enzymatic immunoassay method, and the optical density was detectable at 540 nm. The detection limit is ranging from 62.5 to 400 pg/ml.

### Determination of CoQ10 and Melatonin Levels

The levels of CoQ10 and melatonin were investigated in blood samples by competitive inhibition enzyme immunoassay technique using ELISA kits from MyBioSource. The color developed was read at 540 nm. The minimum detected CoQ10 level was 3.12 ng/ml, whereas the detection range of melatonin level was 6.25–400 pg/ml.

### Measurement of GSH Concentration and GST Activity

Total GSH level and GST activity were calorimetrically measured in the studied blood samples according to Beutler et al. (1963) and Mannervik (1985), respectively.

### Measurement of $K^+$ Level

Plasma $K^+$ level was evaluated according to the method of Terri and Sesin (1958). The turbidity of the colloidal suspension resulting from the reaction between $K^+$ and $Na^+$ tetraphenyl boron in a protein-free alkaline medium was measured at 505 nm.

### Measurement of $Na^+$ Level

Blood $Na^+$ level was determined using a direct enzymatic colorimetric kit from UDI and was expressed in millimoles per liter. $Na^+$-dependent β-galactosidase activity was followed at 405 nm using o-Nitrophenyl-β, D-galactopyranoside as substrate.

### Statistical Analysis

To analyze the data, the Statistical Package for the Social Sciences (SPSS) computer program was used and the data were assessed as means ± S.D. Statistical comparisons using ANOVA and independent *t* tests were performed between control and ASD patients as well as between mild-moderate and severe autism. Significance was considered at a $p$ value < 0.05. A Pearson's correlation test was performed to measure the bivariate correlation between different diagnostic associations, indicating if this covariance was positive or negative. Moreover, a Stepwise Multiple Regression Method (SMR) was applied to the whole parameter association ratios, according to previously published methods (El-Ansary 2016). Receiver operating characteristics (ROC) curve analysis for biomarker evaluation was also carried out. The degree of specificity and sensitivity along with the area under the curve (AUC) and cutoff values were determined. Additionally, predictiveness curves were also performed to describe the dispersion of the scores.

## Results

Table 1 and Fig. 2 show means ± S.D. and percentage of change (%), respectively, for the investigated ratios of the two groups of ASD participants (mild-to-moderately and severely affected) and the control group. ANOVA demonstrated marked differences between the controls and ASD subjects for almost all studied ratios, in particular, MRCC-I/caspase-7 [% = 18.88%, $p < 0.001$], MRCC-I/CK [% = 51.55%, $p = 0.009$], MRCC-I/COQ10 [% = 29.96%, $p < 0.001$], and GSH/GST [% = 32.42%, $p < 0.001$] ratios. Table 1 and Fig. 1 also demonstrate the highly significant lower MRCC-I/CoQ10 ratio (70% less) in ASD patients compared with neurotypical controls. This can be easily related to mitochondrial dysfunction as a repeatedly recorded etiological mechanism of this disorder (Frye and Rossignol 2011; Rossignol and Frye 2012a; Rossignol and Frye 2012b).

Table 2 shows a Pearson's correlation between all the studied ratios. Noteworthy, MRCC-I/caspase-7, MRCC-I/COQ10, lactate/pyruvate, lactate/LDH, and pyruvate/LDH were significantly and positively correlated with GSH/GST, while Tables 3 and 4 represent the multiple regressions using stepwise method for MRCC-I/caspase-7 or GSH/GST, respectively, as a dependent variable (using all data).

Fig. 2 and Table 5 show the ROC curves analysis of each ratio in relation to the differentiation between the control and ASD groups. The greatest value of the AUC was found for the concentration of lactate in CSF (0.994) followed by the level of pyruvate in CSF (0.983). Most interesting is the inverse relationship they reported between lactate CSF/blood ratio and blood lactate (AUC of 1.0). This can support either the non-significant difference in the plasmatic ratio of lactate/pyruvate in ASD patients compared to healthy controls, which can be attributed to the overlapping observed between the individual values of both groups, or the AUC of 0.615 for lactate/pyruvate ratio in plasma of individuals with ASD. Table 5 shows the AUC and the best cutoff values to differentiate the two groups of all ratios, as well as the specificity and sensitivity. Fig. 2 shows the ROC curves for each ratio. $K^+/Na^+$, lactate/pyruvate, MRCC-I/caspase-7, and GSH/GST ratios have very good (> 75%) specificity while pyruvate/LDH, MRCC-I /CK, MRCC-I/CoQ10, MRCC-I/caspase-7, and GSH/GST ratios display very good (> 80%) sensitivity. MRCC-I/caspase-7 and GSH/GST are found to be the only ratios to have both good specificity and sensitivity. MRCC-I/caspase-7 is the only ratio showing an AUC that is considered excellent while $K^+/Na^+$ and GSH/GST are the ratios with the next best AUC values, which are all considered good. Although MRCC-I/CK and MRCC-I/

**Table 1** $K^+/Na^+$, lactate/pyruvate, MRCC-I/CK, MRCC-I/caspase-7, MRCC-I/CoQ10, and GSH/GST ratios of neurotypical controls and CARS autistic groups

| Parameter | | Groups | *N* | Min | Max | Mean ± S.D. | Percent change | *P* value |
|---|---|---|---|---|---|---|---|---|
| $K^+/Na^+$ | Groups | Control group | 41 | 0.13 | 0.52 | 0.222 ± 0.079 | 100.00 | 0.001 |
| | | ASD group | 41 | 0.05 | 0.25 | 0.143 ± 0.054 | 64.46 | |
| | CARS | Mild to moderate | 12 | 0.07 | 0.25 | 0.140 ± 0.064 | 63.06 | 0.875 |
| | | Severe | 26 | 0.05 | 0.25 | 0.140 ± 0.047 | 62.93 | |
| Lactate/pyruvate | Groups | Control group | 41 | 0.31 | 11.07 | 2.481 ± 2.373 | 100.00 | 0.049 |
| | | ASD group | 41 | 0.34 | 8.05 | 1.744 ± 1.497 | 70.31 | |
| | CARS | Mild to moderate | 12 | 0.38 | 2.44 | 1.423 ± 0.627 | 57.37 | 1.000 |
| | | Severe | 26 | 0.34 | 8.05 | 1.857 ± 1.727 | 74.85 | |
| MRCC-I /CK | Groups | Control group | 24 | 0.02 | 0.71 | 0.257 ± 0.197 | 100.00 | 0.009 |
| | | ASD group | 23 | 0.02 | 0.58 | 0.132 ± 0.136 | 51.36 | |
| | CARS | Mild to moderate | 8 | 0.05 | 0.17 | 0.107 ± 0.039 | 41.55 | 0.682 |
| | | Severe | 14 | 0.02 | 0.58 | 0.154 ± 0.169 | 59.89 | |
| MRCC-I /caspase-7 | Groups | Control group | 30 | 0.01 | 0.12 | 0.052 ± 0.028 | 100.00 | 0.001 |
| | | ASD group | 23 | 0.00 | 0.02 | 0.010 ± 0.004 | 18.88 | |
| | CARS | Mild to moderate | 10 | 0.00 | 0.02 | 0.009 ± 0.004 | 17.73 | 0.420 |
| | | Severe | 13 | 0.00 | 0.02 | 0.010 ± 0.004 | 19.85 | |
| MRCC-I/CoQ10 | Groups | Control group | 29 | 0.04 | 1.11 | 0.334 ± 0.304 | 100.00 | 0.001 |
| | | ASD group | 26 | 0.02 | 0.25 | 0.100 ± 0.061 | 29.96 | |
| | CARS | Mild to moderate | 12 | 0.03 | 0.25 | 0.098 ± 0.065 | 29.39 | 0.295 |
| | | Severe | 11 | 0.02 | 0.23 | 0.118 ± 0.058 | 35.23 | |
| GSH/GST | Groups | Control group | 25 | 0.65 | 11.86 | 5.022 ± 3.207 | 100.00 | 0.001 |
| | | ASD group | 26 | 0.33 | 5.80 | 1.628 ± 1.481 | 32.42 | |
| | CARS | Mild to moderate | 7 | 0.58 | 2.18 | 1.289 ± 0.488 | 25.67 | 0.586 |
| | | Severe | 18 | 0.33 | 5.80 | 1.783 ± 1.749 | 35.49 | |

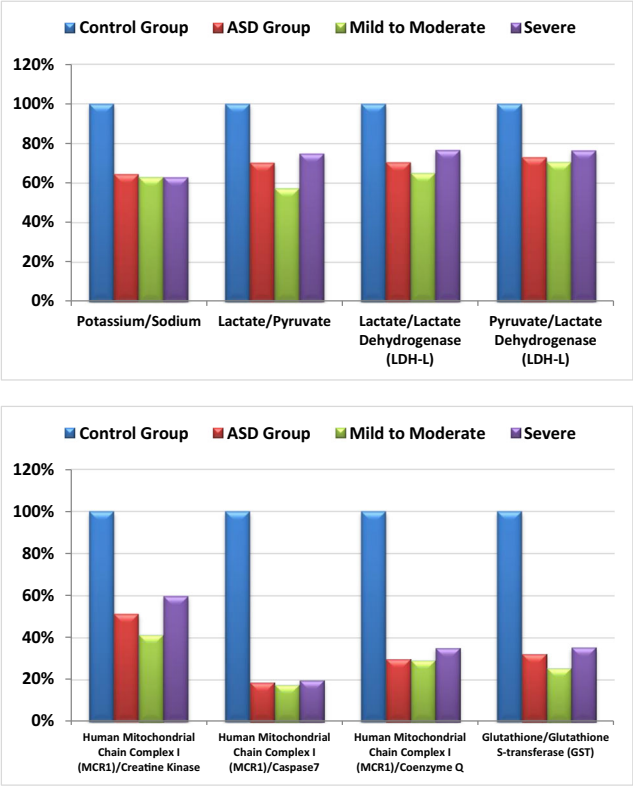



**Fig. 1** Percentage change (%) for neurotypical controls and ASD children in $K^+/Na^+$, lactate/pyruvate, MRCC-I/CK, MRCC-I/caspase-7, MRCC-I/CoQ10, and GSH/GST ratios

CoQ10 have good to excellent sensitivity, their AUC values were considered fair. However, pyruvate/LDH ratio that exhibits excellent specificity (95.1%) is found very poor.

Fig. 3 shows the predictiveness diagrams of the eight studied ratios in relation to the prevalence of ASD in Saudi Arabia, which was most recently recorded as 18 per 10,000 persons (El-Tarras et al. 2012).

**Table 2** Pearson's correlation outputs from the comparison between all the studied parameters ratios in ASD patients, which resulted significantly at $p < 0.05$ and $p < 0.01$

| Parameter | *R*-value | *P* value | Direction |
|---|---|---|---|
| Lactate/pyruvate~Lactate/LDH | 0.501** | 0.001 | P |
| Lactate/pyruvate~Pyruvate/LDH | − 0.432** | 0.007 | N |
| Lactate/LDH~Pyruvate/LDH | 0.530** | 0.001 | P |
| Lactate/LDH~MRCC-I/CK | 0.500* | 0.018 | P |
| Lactate/LDH~GSH/GST | 0.492* | 0.013 | P |
| Pyruvate/LDH~MRCC-I/CK | 0.548** | 0.008 | P |
| MRCC-I/CK~MRCC-I/CoQ10 | 0.585* | 0.017 | P |
| MRCC-I/caspase-7~MRCC-I/CoQ10 | 0.650** | 0.006 | P |
| MRCC-I/caspase-7~GSH/ GST | 0.493* | 0.045 | P |

** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05; *P* positive trend; *N* negative trend

## Discussion

Astrocytes, as neurons supporting cells, are highly sensitive to a $K^+/Na^+$ relative concentration within the brain (Noda and Hiyama 2015). Following neuronal excitation, astrocytic $Na^+/K^+$-ATPase is critically important for the re-accumulation in cells of $K^+$, which extracellularly is moderately increased. Upon increasing the $K^+$ levels, astrocytic $Na^+/K^+$-ATPase is supported by the $Na^+$, $K^+$, and 2Cl-transporter NKCC1. A significant elevation of $K^+$ levels in the brain stimulates the astrocytic $Na^+/K^+$-ATPase. Neuronal $Na^+/K^+$-ATPase is little sensitive to elevated extracellular $K^+$ but is stimulated when $Na^+$ increases intracellularly (Hertz et al. 2015). Based on this evidence, the significant reduction of $K^+/Na^+$ ratio in the ASD patients compared to healthy controls can be related to the previously reported impaired energy metabolism represented by $Na^+/K^+$-ATPase-mediated $K^+$ influx and $Na^+$ efflux and regulation (Al-Mosalim et al. 2009).

Yamada et al. (2012) studied the diagnostic correctness of lactate in cerebrospinal fluid (CSF) and blood, pyruvate, and most importantly lactate/pyruvate ratio to identify children that have mitochondrial diseases that affect the CNS (Yamada et al. 2012). They reported that although lactate in CSF and blood, pyruvate levels, and the ratio of lactate/pyruvate were significantly elevated in the dysfunctional mitochondrial group compared to healthy controls, there was a

**Table 3** Stepwise multiple regression method using the adjusted R square approach for all the data expressed in Table 1 and considering MRCC-I/caspase-7 as the dependent variable

| Predictor variable | Coefficient | *P* value | Adjusted R-square | Model | |
|---|---|---|---|---|---|
| | | | | *F*-value | *P* value |
| MRCC-I/CK | 0.088 | 0.001 | 0.258 | 12.135 | 0.001 |
| MRCC-I/CK<br>MRCC-I/CoQ10 | 0.074<br>0.042 | 0.003<br>0.008 | 0.397 | 11.544 | 0.001 |

significant overlap of different values within the two groups. They added that measurement of these three variables in the CSF is much reliable than blood as a diagnostic tool of mitochondrial dysfunction (Yamada et al. 2012).

However, according to Yamada et al. (2012), the non-significant variation of plasma lactate/pyruvate ratio recorded in the present study does not reject the repeatedly reported mitochondrial dysfunction in ASD patients, due to the more reliable accuracy of CSF and the inverse relationship between CSF and blood.

The lactate/pyruvate ratio should reflect the cytosolic compartment's redox state. A lower lactate/pyruvate ratio in the group with ASD individuals indicates a higher oxidized $NAD^+$/NADH when compared to control participants. This much higher oxidized redox state in ASD might promote glycolytic influx more than oxidative phosphorylation being a supply of ATP. This can find support in our previous work in which lactate oxidase enzyme was found significantly increased in ASD patients compared to control (El-Ansary et al. 2010).

Based on the fact that plasma lactate/pyruvate ratios can be due to pyruvate dehydrogenase complex (PDHC) deficiency, the altered ratio observed in the present study can find support in the previous work of Giulivi et al. (2010) who found that the activity of PDHC in ASD children was nearly half compared to neurotypical controls. PDHC defects lead to the inadequate elimination of lactate and pyruvate, which lead to too low energy production (De Meirleir et al. 1993). Hence, in relation to the impaired lactate/pyruvate ratio reported in the present study, deficiency of PDHC can be a factor related to dysfunction in the ASD brain. Oxidative modifications and oxidative stress related to PDHC are in accordance with increased production of hydrogen peroxide ($H_2O_2$). This is supported by the work of Al-Gadani et al. (2009). The researchers found that Saudi patients with ASD are under $H_2O_2$ stress due to the over-expression of superoxide dismutase together with a remarkable lower activity of catalase (Al-Gadani et al. 2009).

Both clinical and experimental studies demonstrate the relationship between impaired energy metabolism presented by $Na^+/K^+$-ATPase; MRCC-I, MRCC-III, and MRCC-IV; and CK, and many of brain diseases among which is neurodevelopmental disorders and depressive disorders (Rezin et al. 2008; Al-Mosalim et al. 2009). El-Ansary et al. (2010) reported that alteration of selected ions among which are $K^+$ and $Ca^{2+}$, oxidative stress and defective mitochondrial energy production could represent the primary causative factor in the pathogenesis of ASD (El-Ansary et al. 2010). Moreover, Dudley et al. (2016) highlighted the role of significantly decreased phosphocreatine (PCr) levels in gray matter and ATP levels in the white matter of the brains of subjects with bipolar disorder compared with healthy control individuals (Dudley et al. 2016). This can support the significantly lower MRCC-I/CK ratio reported in Table 1 and Fig. 1. A lower ratio can be easily attributed to a much lower MCR1 and higher CK (Khemakhem et al. 2017). The remarkable elevation of CK can be related to the significantly lower ATP previously reported by Al-Mosalim et al. (2009). This can find more support in the early study of Burbaeva et al. (1999) in which they proved the usefulness of CK measurement in blood elements as a diagnostic and/or prognostic marker of neurological diseases.

Mitochondria play a significant role within cells and are involved in many essential processes, such as energy transduction, and apoptosis signaling represented by caspases (3, 7, and 9) (Huang et al. 2011). However, when the respiratory chain and the activity of enzymes involved in oxidative phosphorylation are disrupted (e.g., MRCC-I), it affects the mitochondrial membrane potential and decreases ATP production (Gonzalez-Cabo and Palau 2013). The significant decrease of MRCC-I/caspase-7 ratio reported in the present investigation showing more than 80% reduction of MCR1 concomitant with remarkable activation of caspase-7 (Khemakhem et al. 2017) can be easily related to the clinical presentation of ASD. Notably, brain as an organ of high-energy demands and

**Table 4** Stepwise multiple regression method using the adjusted R square approach for all the data expressed in Table 1 and considering GSH/GST as the dependent variable

| Predictor Variable | Coefficient | *P* value | Adjusted *R*-square | Model | |
|---|---|---|---|---|---|
| | | | | *F*-value | *P* value |
| MRCC-I/CK | 9.263 | 0.001 | 0.264 | 12.495 | 0.001 |

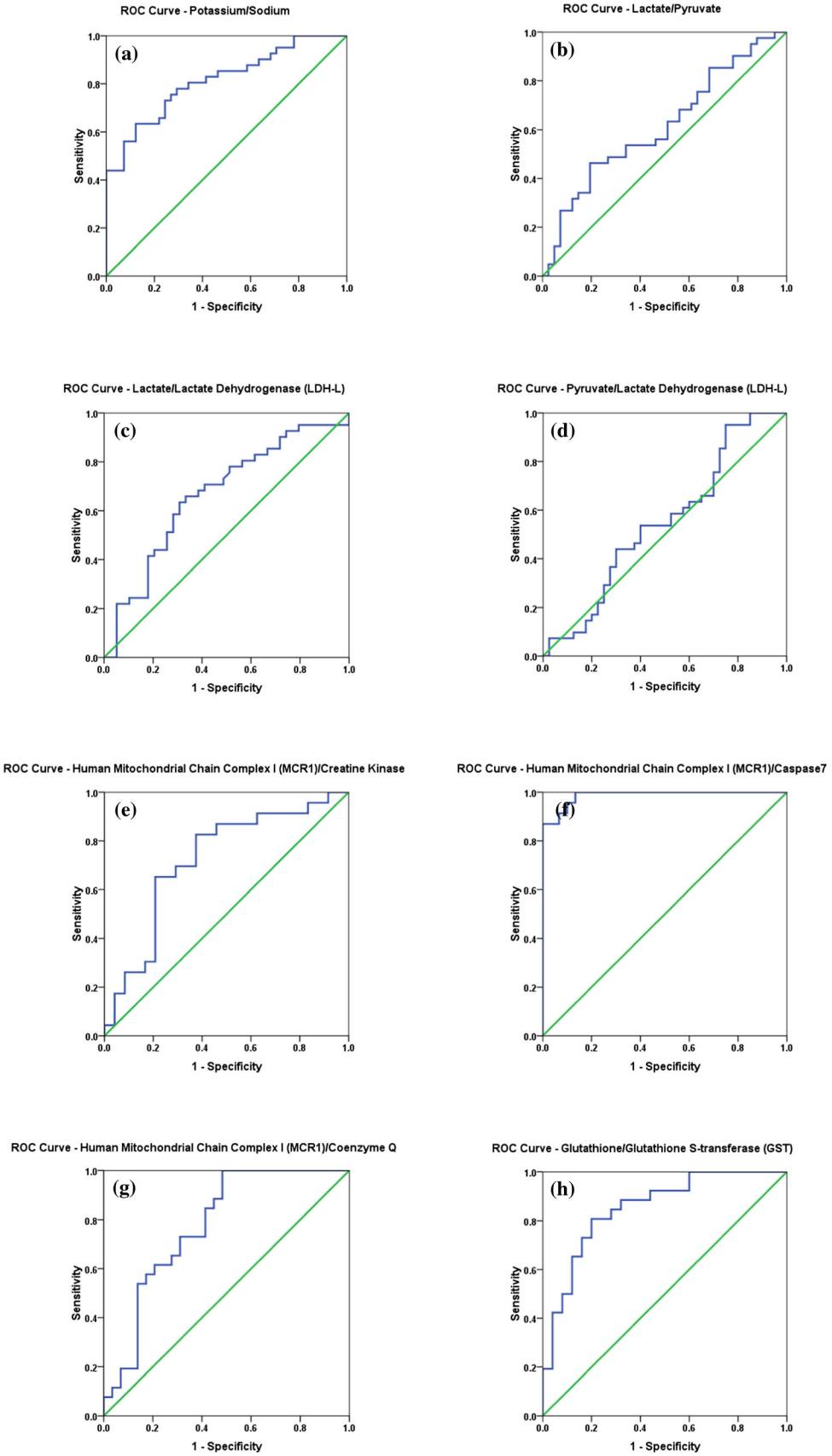



**Fig. 2** **a**–**h** ROC curves for **a** $K^+/Na^+$, **b** lactate/pyruvate, **c** lactate/LDH, **d** pyruvate/LDH, **e** MRCC-I /CK, **f** MRCC-I/caspase-7, **g** MRCC-I/CoQ10, and **h** GSH/GST ratios in relation to differentiation between control and ASD groups

**Table 5** Parameters of ROC curves for biomarkers of mitochondrial dysfunction for differentiating controls from all autism participants and for differentiating controls from two different severity groups separately. The CARS was used to differentiate the autism groups into mild-to-moderate and severe

| Parameter | Groups | | AUC | Cutoff value | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|---|---|
| $K^+/Na^+$ | ASD group | | 0.814 | 0.155 | 63.4 | 87.8 |
| | CARS | Mild to moderate | 0.795 | 0.125 | 58.3 | 100.0 |
| | | Severe | 0.851 | 0.181 | 88.5 | 70.7 |
| Lactate/pyruvate | ASD group | | 0.615 | 1.058 | 46.3 | 80.5 |
| | CARS | Mild to moderate | 0.614 | 2.549 | 100.0 | 31.7 |
| | | Severe | 0.613 | 1.058 | 46.2 | 80.5 |
| Lactate/LDH | ASD group | | 0.666 | 0.031 | 65.9 | 66.7 |
| | CARS | Mild to moderate | 0.669 | 0.029 | 66.7 | 69.2 |
| | | Severe | 0.646 | 0.031 | 61.5 | 66.7 |
| Pyruvate/LDH | ASD group | | 0.548 | 0.043 | 95.1 | 25.0 |
| | CARS | Mild to moderate | 0.540 | 0.041 | 100.0 | 25.0 |
| | | Severe | 0.539 | 0.043 | 92.3 | 25.0 |
| MRCC-I/CK | ASD group | | 0.723 | 0.146 | 82.6 | 62.5 |
| | CARS | Mild to moderate | 0.745 | 0.171 | 100.0 | 54.2 |
| | | Severe | 0.693 | 0.109 | 64.3 | 79.2 |
| MRCC-I/caspase-7 | ASD group | | 0.987 | 0.015 | 87.0 | 100.0 |
| | CARS | Mild to moderate | 0.983 | 0.018 | 100.0 | 90.0 |
| | | Severe | 0.990 | 0.015 | 92.3 | 100.0 |
| MRCC-I/CoQ10 | ASD group | | 0.776 | 0.251 | 100.0 | 51.7 |
| | CARS | Mild to moderate | 0.793 | 0.096 | 75.0 | 79.3 |
| | | Severe | 0.721 | 0.244 | 100.0 | 51.7 |
| GSH/GST | ASD group | | 0.852 | 2.410 | 80.8 | 80.0 |
| | CARS | Mild to moderate | 0.880 | 2.410 | 100.0 | 80.0 |
| | | Severe | 0.840 | 1.314 | 66.7 | 88.0 |

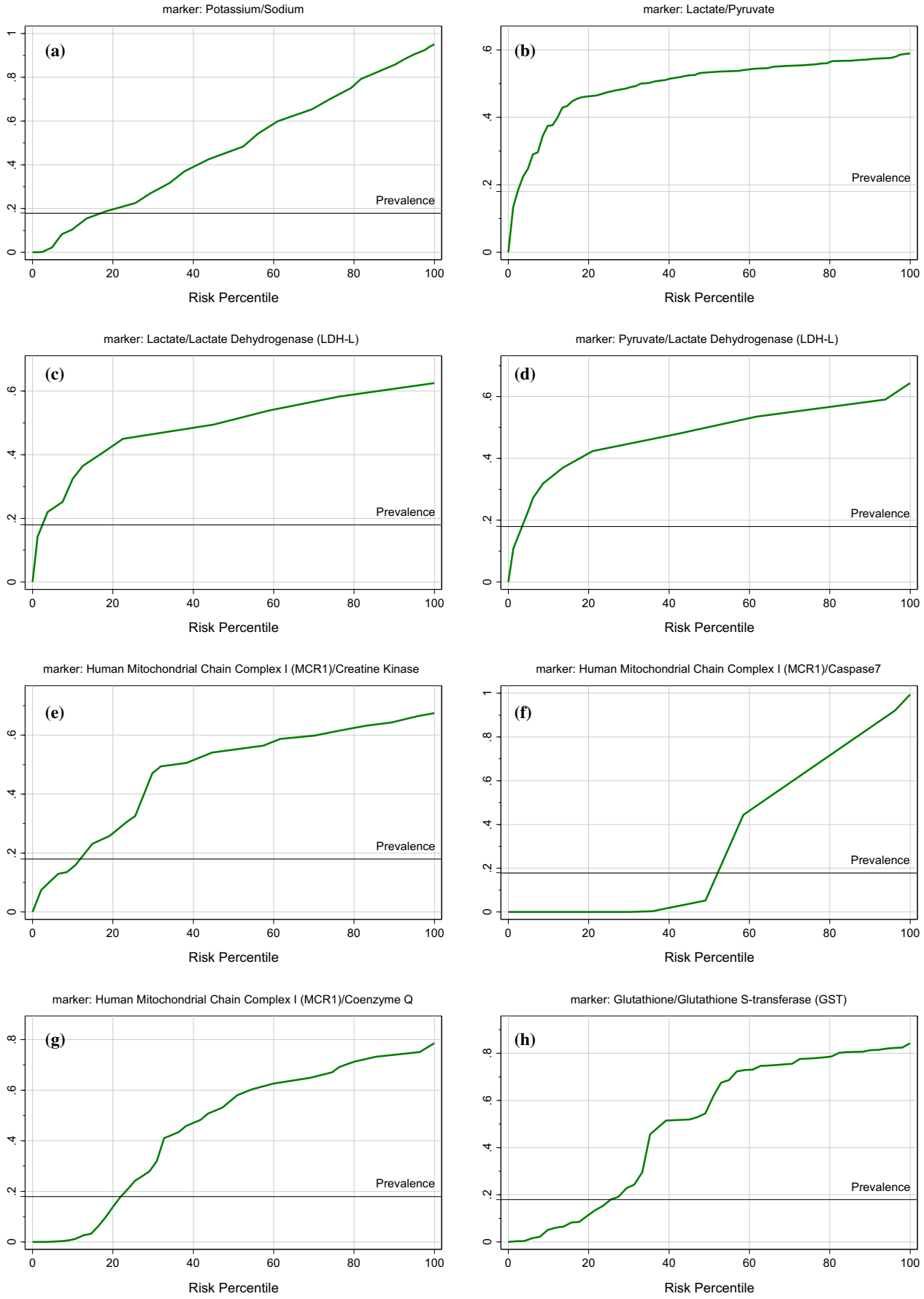
consumption can be greatly affected by the remarkable decrease of MRCC-I/caspase-7 ratio (Islam 2017; Serasinghe and Chipuk 2017). This can find more support in our previous work, which reported that Saudi individuals with ASD are under superoxide and $H_2O_2$ stress, leading to a significant increase of caspases as pro-apoptotic markers (Al-Gadani et al. 2009; El-Ansary et al. 2011; El-Ansary and Al-Ayadhi. 2012).

In the present study, the reported mitochondrial dysfunction represented as significantly lower MRCC-I/caspase-7 ratio can be related to the remarkable increase of superoxide anion previously reported by Al-Gadani et al. (2009). In dysfunctional mitochondria, while electrons are transferred to molecular oxygen in the MRC, a small proportion of electrons "leak," resulting in the production of superoxide anions and stimulation of caspases (Islam 2017).

The significant decrease of CoQ can be easily related to glutamate excitotoxicity as a phenotype of ASD (El-Ansary 2016). Zeron et al. (2004) reported that enhanced apoptosis of glutamate NMDA receptors was related to mitochondrial compromisation and more interestingly can be dramatically reduced with CoQ10 as mitochondrial function promoting agent with antioxidant potency (Zeron et al., 2004). This explanation can find support in the previous work of Tsao and Mendell (2007) in which ASD children are demonstrating a decreased activity of mitochondrial ETC in muscle biopsy, including a partial defect of MRCC-I associated with a CoQ10 deficiency was remarkably improved by CoQ10 supplementation (Tsao and Mendell 2007; Frye and Rossignol, 2014).

Table 1 and Fig. 1 also demonstrate the significant decrease of GSH/GST ratio. Remarkable lower concentrations of both markers were repeatedly reported in ASD (Al-Gadani et al. 2009; Al-Yafee et al. 2011; Khemakhem et al. 2017). The reported depletion of GSH and lower activity of GST as markers for oxidative stress and detoxification capacity can be related to ferroptosis as a non-apoptotic form of cell death that can be initiated by small molecules or conditions that inhibit GSH biosynthesis (Yang and Stockwell 2016). It is well known that both lipophilic antioxidants such as vitamin

**Fig. 3** **a**–**h** Predictiveness diagrams of **a** $K^+/Na^+$, **b** lactate/pyruvate, **c** ▶ lactate/LDH, **d** pyruvate/LDH, **e** MRCC-I/CK, **f** MRCC-I/caspase-7, **g** MRCC-I/CoQ10, and **h** GSH/GST ratios in relation to autism prevalence


marker: Potassium/Sodium
(a)
Prevalence
Risk Percentile
marker: Lactate/Pyruvate
(b)
Prevalence
Risk Percentile
marker: Lactate/Lactate Dehydrogenase (LDH-L)
(c)
Prevalence
Risk Percentile
marker: Pyruvate/Lactate Dehydrogenase (LDH-L)
(d)
Prevalence
Risk Percentile
marker: Human Mitochondrial Chain Complex I (MCR1)/Creatine Kinase
(e)
Prevalence
Risk Percentile
marker: Human Mitochondrial Chain Complex I (MCR1)/Caspase7
(f)
Prevalence
Risk Percentile
marker: Human Mitochondrial Chain Complex I (MCR1)/Coenzyme Q
(g)
Prevalence
Risk Percentile
marker: Glutathione/Glutathione S-transferase (GST)
(h)
Prevalence
Risk Percentile

E and iron chelators could block this process from occurring (Weiwer et al. 2012; Brigelius-Flohe and Maiorino 2013; Dixon and Stockwell 2014; Yang et al. 2014). Certain ASD susceptibility genes including GSTs (GSTM1 and GSTP1), the iron transporter SLC11A3, and the metal-regulatory transcription factor 1 (MTF1) can be directly related to impaired detoxification pathways in ASD (Rossignol et al. 2014). This suggestion can find support in Table 2, which demonstrates the high significant positive and negative correlations between the studied ratios. Mitochondrial dysfunction presented by MRCC-I/caspase-7 and MRCC-I/COQ10 and impaired energy metabolism presented by lactate/pyruvate, lactate/LDH, and pyruvate/LDH were positively correlated with ferroptosis as GSH/GST as a new suggested mechanism in ASD. This can be confirmed by the multiple regression analysis using MRCC-I/caspase-7 and GSH/GST as dependent variables (Tables 3 and 4). The association between MRCC-I/caspase-7 with MRCC-I/CK and MRCC-I/COQ10 shown in Table 3 ascertains mitochondrial dysfunction as an etiological mechanism, while the association between GSH/GST and MRCC-I/CK shows that oxidative stress is involved in the reported mitochondrial dysfunction (Table 4).

Table 5 and Fig. 2 present the ROC analysis of the studied ratios. Among these ratios, MRCC-I/caspase-7, GSH/GST, and MRCC-I/COQ10 recorded remarkably predictive values measured as high AUC and satisfactory sensitivity and specificity. These predictive values can be more easily observed in Fig. 3f–h as excellent predictiveness curves with those of high risk and low risk far from the prevalence line.

## Conclusion

Collectively, based on the obtained data demonstrating channelopathy ($K^+/Na^+$), impaired energy metabolism (Pyruvate/lactate, MRCC-I/CK), mitochondrial dysfunction-induced oxidative stress and apoptosis (MRCC-I/caspase-7 and MRCC-I/COQ10), and ferropoptosis (GSH/GST), we can suggest relative ratios as more accurate biomarkers in ASD.

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### Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**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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