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ORIGINAL ARTICLE

Novel biomarkers of metabolic dysfunction is autism spectrum disorder: potential for biological diagnostic markers

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Abstract Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is behaviorally defined by social and communication impairments and restricted interests and repetitive behaviors. There is currently no biomarkers that can help in the diagnosis. Several studies suggest that mitochondrial dysfunction is commonly involved in ASD pathophysiology, but standard mitochondrial biomarkers are thought to be very variable. In the present study we examine a wide variety of plasma biomarkers of mitochondrial metabolism and the related abnormalities of oxidative stress and apoptosis in 41 ASD patients assessed for ASD severity using the Childhood Autism Rating Scales and 41 non-related age and sex matched healthy controls. Our findings confirm previous studies indicating abnormal mitochondrial and related biomarkers in children with ASD including pyruvate, creatine kinase, Complex 1, Glutathione S-Transferase, glutathione and Caspase 7. As a novel finding, we report that lactate dehydrogenase is abnormal in children with ASD. We also identified that only the most severe children demonstrated abnormalities in Complex 1 activity and Glutathione S-Transferase. Additionally, we find that several biomarkers could be candidates for differentiating children with ASD and typically developing children, including Caspase 7, gluthatione and Glutathione S-Transferase by themselves and lactate dehydrogenase and Complex I when added to other biomarkers in combination. Caspase 7 was the most discriminating biomarker between ASD patients and healthy controls suggesting its potential use as diagnostic marker for the early recognition of ASD pathophysiology. This study confirms that several mitochondrial biomarkers are abnormal in children with ASD and suggest that certain mitochondrial biomarkers can differentiate between ASD and typically developing children, making them possibly useful as a tool to diagnosis ASD and identify ASD subgroups.

Keywords Autism · Mitochondria · Energy metabolism · Lactate dehydrogenase · Complex I · Caspase 7

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Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disability characterized by impaired communication and social skills along with restricted interests and repetitive behaviors (American Psychological Association 2013). Additional features include poor eye contact (Esposito and Venuti 2008), atypical sensory modulatory (Baranek et al. 2005) and disruption in motor and cognitive development (Ozonoff et al. 2008; Esposito et al. 2009). Clinical symptoms are usually manifested by 3 years old; however deficits in communication, social responsiveness, and play may be present as early as 6–12 months of age (Pizzarelli and Cherubini 2011; Jones and Klin 2013).

At this time, the gold-standard therapy is limited to behavioral intervention, although many other emerging therapies are on the horizon (Frye and Rossignol 2016). Early intervention is beneficial for young children with ASD and often decreases ASD symptoms and associated maladaptive behaviors, resulting in improved outcomes (Rogers and Vismara 2008). However, these therapies are most effective if applied early. This is a serious issue since the diagnosis of ASD may be delayed if symptoms are mild or even moderate in severity. Thus, there is a continuing search for diagnostic biomarkers for ASD in order to assist with the differential diagnosis when behavioral assessments are unclear and to identify children early during the presymptomatic phase of the disease (Goldani et al. 2014; Howsmon et al. 2017). In order to identify candidate biomarkers that can be used for early diagnosis, we start by examining biomarkers which can differentiate already diagnosed children with ASD.

Mitochondrial dysfunction is one of the most common metabolic abnormalities associated with ASD (Frye and Rossignol 2011; Rossignol and Frye 2012a; Rossignol and Frye 2012b). There are several biomarkers to identify mitochondrial dysfunction with perhaps the best known being lactic and pyruvic acid (Rossignol and Frye 2012a). Lactate dehydrogenase (LDH) catalyzes the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. When oxidative phosphorylation is reduced, as can occur when oxygen tension of the blood is low, pyruvate, the final product of glycolysis, increases in the cell, resulting in a reduction to lactate by LDH. This is due to the fact pyruvate is the cellular metabolite which is produced in the cytoplasm as an end product of glycolysis and enters the mitochondria to fuel the tricyclic acid cycle (TCAC). The TCAC produces electron carriers that are necessary for the electron transport chain (ETC) to function. Thus, if the ETC is decreased in function, upstream pathways, such as the TCAC are decreased in function, resulting in a decreased consumption of pyruvate and an increase in pyruvate.

Over the last two decades, several reports demonstrated that lactate is an essential cerebral oxidative-energy substrate (Schurr and Payne 2007). The brain can take up lactate from the blood, particularly during intense exercise and recovery (Ide and Secher 2000). In the brain, the astrocyte-neuron lactate shuttle supplies the neurons with lactate for fuel. Astrocytes convert glucose to lactate via glycolysis and export the lactate into the extracellular space through the isoform 1 of monocarboxylate transporter. Neurons then pick up extracellular lactate via the isoform 2 of monocarboxylate transporter and use it as a fuel for mitochondrial respiration. Activity of LDH as a glycolytic enzyme is usually regulated through the availability of lactate and pyruvate as two substrates, together with the NAD+/NADH ratio. The NAD+/NADH ratio is controlled by the activity of the TCAC and ETC, among other biochemical pathways.

The ETC is the major source and target of reactive oxygen species (ROS) (Fernandez-Checa et al. 1998; Trushina and McMurray 2007). The ETC is protected from ROS damage by mitochondrial-specific enzymes such as mitochondrial glutathione peroxidase and non-enzymatic antioxidants like glutathione (GSH) (Mari et al. 2013). Mitochondria lack the ability to synthesize GSH, highlighting the critical role of GSH mitochondrial import carriers (James et al. 2009; Mari et al. 2013). When GSH is depleted in the mitochondria, the vulnerability of the cell to oxidative stress and inflammation is increased (Mari et al. 2013). Additionally, factors increasing the production of ROS (such as environmental toxicants, autoimmune disease, inflammation and infections) can indirectly and directly provoke impairments in ETC activity (Rossignol and Frye 2012a; Rossignol and Frye 2012b; Rossignol et al. 2014), deplete GSH (Calabrese et al. 2005) and stimulate non-mitochondrial and mitochondrial-dependent biochemical cascades causing apoptosis (programmed cell death) (Di Meo et al. 2016). Although apoptosis signaling is known to occur in laboratory models of mitochondrial dysfunction, less is known whether biomarkers of apoptosis can be helpful is detecting mitochondrial dysfunction in the clinical setting.

The mitochondria is commonly regarded as the powerhouse of the cell but it has other important roles that are critical to cellular health, including redox regulation and a major pathway for the activation of apoptosis, also known as program cell death. Recent work by Siniscalco et al. (Siniscalco et al. 2012) has demonstrated elevation in several caspase proteins which signal apoptosis through mitochondrial mechanism in patients with ASD, suggesting that mitochondrial dysfunction may be signaling apoptosis pathways in individuals with ASD. As such we have included Caspase-7 as a novel biomarker of mitochondrial dysfunction in this study.

Understanding how mitochondrial metabolism and ETC function is linked to ASD could help to better understand the key role of these pathways in the etiology of ASD. Two previous studies reported a remarkable association between biomarkers of mitochondrial dysfunction and the severity of ASD measured as social responsiveness scores (SRS) and Childhood Autism Rating Scales (CARS) (Minshew et al.

1993; Mostafa et al. 2005), suggesting that mitochondrial dysfunction is linked to underlying ASD pathophysiology.

In this study we expand on the relationship between mitochondrial biomarkers and ASD by examining a wide variety of biomarkers of mitochondrial function in children with ASD. First, this is the first study to look at LDH, which is an important enzymes for the conversation between lactate and pyruvate. Second, we examine biomarkers of liver and muscle damage which have been reported to be abnormal in children with ASD. Third, we consider the relationship between mitochondrial function and redox pathways by examining both GSH and Glutathione S-Transferase (GST) as well as examining antioxidants (Melatonin) and cofactors (CoQ10) which are important for supporting mitochondrial function. Fourth, we also examined apoptosis pathway which is closely linked to mitochondrial function. Lastly, we examined whether these biomarkers are related to mitochondrial function and whether they could be used as diagnostic markers by examining if they can differentiate between children with ASD and typically developing children.

Materials and methods

Subjects

The protocol followed the ethical guidelines of the current Declaration of Helsinki (World Medical Association 2013). Forty-one male children aged 2–14 years diagnosed with ASD were recruited from the Autism Research and Treatment Centre, Faculty of Medicine, King Saud University, Riyadh, Saudi Arabia. The control group, comprised 41 age and sex-matched apparently healthy children unrelated to the ASD group.

Participants were recruited from the well-baby clinic at King Khaled University Hospital during routine check-up of their growth parameters. All participants had normal results for urine analysis and sedimentation rate and they did not have any neuropsychiatric disorders or clinical indication of infectious disease.

Exclusion criteria included a diagnosis of epilepsy, fragile X syndrome, affective disorders, obsessive-compulsive disorder or any additional neurological or psychiatric diagnosis. Moreover, those on vitamin or natural product supplementation were also excluded.

Autism severity measurement

The CARS was used to measure ASD severity. The Standard Version of the CARS is designed for children of all ages including preschool age (from 2 years) (Schopler et al. 2010). It was developed to objectively distinguish children with ASD from those without ASD and can be used to measure changes in ASD symptoms throughout life. The 15 CARS features include social relations, emotional responses, imitation, using the body, using objects, visual responses, adapting to change, auditory responses, taste-smell-touch, fear-anxiety, verbal communication, non-verbal communication, activity level, intellectual level and homogeneity of operation, and general impression.

The CARS was completed by observers who saw the child in different situations, at different times of the day (during psychological tests, behavior in the institution, parent interview). The features measured by the CARS divided into 4 categories, each with a severity rating from 1 (absence) to 4 (severe) in increments of 0.5. A score between 15 and 30 is consistent with typical development. Mild to moderate ASD is considered with a score between 30 and 37 and severe ASD is considered with a score from 38 to 60. Rating are done through a purely behavioral approach, irrespective of the reason of the behavior. The assessment included quantitative and qualitative judgments of all observed behaviors. Two ASD participants did not have CARS scores.

Sample collection

Following an overnight fasting, 10 ml blood was collected from both groups in test tubes containing heparin as anticoagulant. Centrifugation produced plasma which was frozen at −80 °C until analysis. Because of the limited amount of blood, not all assays could be performed on all participants. Table 1 provides a breakdown of number of subjects that underwent testing for each biomarker as well as their age ranges.

Biomarker assays

Reagents were analytical grade products of Sigma USA or Merck (Germany). Human ETC Complex I ELISA Kit, Human Coenzyme Q10 (CoQ10) ELISA Kit and Human Melatonin ELISA Kit were products of MyBiosource USA; Human Caspase-7 ELISA Kit was a product of CUSABIO China. Lactate and Pyruvate colorimetric kits were products of BioVisionSan Francisco, USA; Creatine Kinase (CK) kit was a product of BioSystems, Barcelona, Spain; Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Lactate dehydrogenase (LDH) kinetic Kits were products of United Diagnostics Industry (UDI), Dammam, KSA. Positive and negative controls were measured to check the validity of the measurement, and to determine the detection limits.

Lactate

Plasma lactate was measured as reported in the manufactures instruction of the diagnostic kit. This kit was a product from BioVision in which lactate reacts specifically with an enzyme

Table 1 Number of participants for each biomarker

Biomarker	Controls (N, Age Range)	ASD Overall (N, Age Range)	ASD Mild/Moderate (N, Age Range)	ASD Severe (N, Age Range)
Lactate	41, 2–14 years	41, 2–14 years	12, 4–11 years	27, 2–14 years
Pyruvate	41, 2–14 years	41, 2–14 years	12, 4–11 years	27, 2–14 years
LDH	41, 2–14 years	41, 2–14 years	12, 4–11 years	27, 2–14 years
CK	25, 2–11 years	24, 2–11 years	8, 4–11 years	16, 2–9 years
AST	10, 4–9 years	13, 4–9 years	6, 5–9 years	7, 4–7 years
ALT	14, 5–9 years	20, 2–9 years	6, 5–9 years	14, 2–9 years
Complex I	40, 2–14 years	40, 2–14 years	12, 4–11 years	26, 2–14 years
GSH	26, 2–11 years	26, 2–14 years	7, 4–11 years	19, 2–14 years
GST	40, 2–14 years	40, 2–14 years	12, 4–11 years	26, 2–14 years
CoQ10	30, 2–14 years	27, 2–11 years	12, 4–11 years	13, 2–14 years
C7	30, 2–14 years	27, 2–11 years	10, 4–11 years	16, 2–9 years
Melatonin	32, 2–14 years	29, 2–14 years	12, 4–11 years	15, 2–14 years

mixture to generate a product, which interacts with a lactate probe to produce color at 570 nm. Detection limit was 0.001–10 mM.

Pyruvate

Plasma pyruvate was measured using the diagnostic kit. The kit was a product of BioVision in which pyruvate is oxidized by pyruvate oxidase via enzyme reactions to generate color at 570 nm. The pyruvate concentration is relative to the color intensity, which is detected with a limit between 1 μM - 10 mM.

Lactate dehydrogenase (LDH)

LDH activity was assayed spectrophotometrically by the "forward" reaction (lactate + NAD^+ to pyruvate + NADPH + H^+). The activity of LDH in serum is directly proportional to NADH formation rate, which is indicated by an increase in absorbance at 340 nm. This activity was measured by the UDI kit (Amador et al. 1963).

Aspartate aminotransferase (AST)

A UV kinetic method was used for the evaluation of AST in serum. The kit is manufactured by UDI and uses a coupled reaction with malate dehydrogenase (MDH) and NADH (Henry et al. 1960). The AST catalyzes the transfer of amino groups between L-aspartate and 2-Oxoglutarate. In the presence of MDH, NADH reacted with the oxaloacetate formed in the first reaction to form NAD^+. AST activity is measured by following the rate of oxidation of NADH at 340 nm. During the lag phase prior of measurement, Lactate dehydrogenase is incorporated in the reagent to convert domestic pyruvate to lactate. AST activity is expressed in U/L.

Alanine aminotransferase (ALT)

Pyruvate is reduced to L-lactate by LDH with the simultaneous oxidation of NADH. ALT activity was measured using the ALT kit (UDI product). Expressed in U/L, ALT activity is proportional to the rate of NADH oxidation as measured by a decrease in absorbance at 340 nm (Karmen et al. 1953).

Creatine kinase (CK)

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

Measurement of complex I

Human ETC Complex I was measured using the MyBioSource product according to the manufacturer's instructions. This kit is suitable to measure the level of ETC Complex I in undiluted original human body fluids and employs a quantitative sandwich enzyme immunoassay technique; using an enzyme-linked immunosorbent assay (ELISA) reader. Color was read at 450 nm with a detection limit of 3.12 ng/ml-100 ng/ml.

Caspase 7

An ELISA kit CUSABIO product was used to measure Caspase 7 according to the manufacturer's instructions. The assay employs the competitive inhibition enzyme immunoassay technique. The detection range was 62.5 pg/ml-400 pg/ml, and the optical density was detectable at (540-570 nm). The concentration of caspase 7 was then determined by comparing O.D.

CoQ10

An ELISA kit product of MyBioSource was used to evaluate the quantity of CoQ10 in blood. The competitive inhibition enzyme immunoassay technique was employed and the wavelength was detectable at 540 nm. The minimum measurable CoQ10 level was 3.12 ng/ml.

Melatonin

Melatonin was measured using the ELISA product of MyBioSource. The intensity of the color developed was measured at 540 nm. The detection range was 6.25 pg/ml-400 pg/ml.

Total glutathione (GSH)

For the determination of total blood GSH, a rapid colorimetric procedure as described by Beutler et al. (Beutler et al. 1963) was used. A yellow color develops when 5.5′ dithiobis-2-nitrobenzoic acid (DTNB) is added to sulfhydryl compounds. This allows the measurement of GSH concentration at 405 nm.

Glutathione S-Transferase (GST)

GST activity was measured using the colorimetric method of Mannervik (Mannervik 1985), based upon that GST-catalyze reaction between GSH and the GST substrate, CDNB (1-chloro-2, 4- dinitrobenzene). The formation of GS-DNB produces a dinitrophenylthioether, which can be detected by spectrophotometer at 340 nm. GST activity was indicate in μmol/mL/min

Statistical analysis

Statistical Package for the Social Sciences (SPSS, IBM, Chicago, USA) and SAS version 9.3 (Cary, NC, USA) were used to analyze data. In general, significant difference was considered at P value <0.05. We present the distribution of the biomarkers fitted to the normal distribution to demonstrate that biomarkers were normally distributed allowing parametric analysis tools to be used. We also calculated the Pearson's correlations to investigate the relationships between the various biomarkers.

We then investigated whether biomarkers were significantly different between the control and ASD individuals and whether these differences were related to ASD severity. We used an analysis of variance (ANOVA) to investigate the overall group differences between ASD and control groups. The ASD group was then divided into two groups, those with mild and moderate CARS scores and those with severe CARS scores. ANOVA examining the differences across these three levels (control, mild-to-moderate ASD, severe ASD) was conducted and for those with a significant effect the Tukey Studentized Range test was used to determine if either or both the ASD groups differed from the controls. The Tukey Studentized Range test was used to control for multiple comparisons and used a corrected alpha of 0.05.

Using the SPSS program the receiver operating characteristic (ROC) curves for all biomarkers were calculated. The true positive rate (Sensitivity) is plotted as a function of the false positive rate (100-Specificity) for different cut-off points of a parameter in a ROC curve. Each point on the ROC curve represents a sensitivity/specificity pair analogous to a particular decision moment. We use the ROC curve to distinguish between ASD and control subjects. In order to maximize both, sensitivity and specificity we calculated the maximum Youden's index (sensitivity + specificity-1) to determine the optimal cutoff value. The area under the curve (AUC) was also calculated to provide a comparison of the performance of several biomarkers. An AUC value close to 1 indicates an excellent diagnostic and predictive marker, while a curve which is close to the diagonal (AUC = 0.5) has no diagnostic utility. AUC close to 1 is always accompanied by satisfactory values of specificity and sensitivity of the biomarker. An AUC 0.9 to 1 is considered excellent, 0.8 to 0.9 is considered good, 0.7 to 0.8 is considered fair, 0.6 to 0.7 is considered poor and 0.5 to 0.6 is considered very poor.

In addition to the ROC curves we also generated predicitiveness curves. Different aspects of the predictive power of the score are represented by the predicitiveness curve allowing a detailed evaluation of the performance of virtual screening methods. Predicitiveness curves are functions of the repartition of the scores and furnish a common scale for the evaluation of virtual screening methods similarly to ROC curve. The dispersion of the scores is well described by predictiveness curves as compared to ROC curves (Empereur-Mot et al. 2015).

SAS was used to create a multivariable discriminant function from the biomarkers using discriminant analysis with cross validation. The cross validation process calculates the discriminant function on all the data except for 1 participant and then predicts if the left out participant is in the ASD or control group, and then repeats the process for each participant. First, biomarkers which were measured for the majority (> 97%) of the patients were entered into the analysis and a final function was created with only the biomarkers which appears to make a significant contribution to the function. We then relaxed the criteria for biomarker inclusion so we could examine a broader range of biomarkers. The Wilks lambda statistic provides a measurement of the overall quality of the Discriminant Function.

Results

Parameter distribution

Figure 1 demonstrates the distribution of the measured parameters in both groups with a superimposed best fit normal distribution curve.

Relationship between biomarkers

Table 2 presents correlations between biomarkers. Notice that GST activity has a particular significant positive correlation with Complex I and Caspase 7 while GSH has a particularly significant negative (inverse) correlation with LDH, CK and Caspase 7.

Group differences

Figure 2 shows the means and standard errors for the biomarkers for the control group and the two groups of ASD participants, those mild-to-moderately effected and those severely affected, although the significance bars demonstrate differences of the two severity group combined. ANOVA demonstrated significant differences between the Controls and ASD participants for several biomarkers including pyruvate [$F(1,80) = 4.02$, $p < 0.05$], LDH [$F(1,80) = 15.59$, $p < 0.0005$], CK [$F(1,47) = 4.63$, $p < 0.05$], Complex I [$F(1,79) = 11.53$, $p = 0.001$], GSH [$F(1,50) = 18.33$, $p < 0.0001$], GST [$F(1,78) = 11.06$, $p = 0.001$], CoQ10 [$F(1,55) = 9.70$, $p < 0.005$], Caspase 7 [$F(1,55) = 875.37$, $p < 0.0001$] and Melatonin [$F(1,59) = 17.27$, $p = 0.0001$] (See Fig. 2).

Relationship to severity

Severity level was used in the one-way ANOVA to determine whether biomarkers for both ASD severity levels differed from control and whether the two ASD severity levels differed from each other. The one-way ANOVA demonstrated that the ASD participants in one or both levels of severity differed from control levels for several biomarkers but the two levels of ASD severity did not differ from each other for any biomarker. The level of severity was significantly related to LDH [$F(2,77) = 8.54$, $p < 0.0005$], Complex I [$F(2,75) = 4.93$, $p < 0.01$], GST [$F(2,75) = 5.72$, $p < 0.005$], GSH [$F(2.49) = 9.00$, $p = 0.0005$], Caspase 7 [$F(2,53) = 424.53$, $p < 0.0001$], CoQ10 [$F(2,52) = 6.88$, $p < 0.005$] and Melatonin [$F(2,56) = 8.16$, $p < 0.001$]. Using the Tukey Studentized Range test, it was found that both mild-to-moderate and severe ASD participants differed from controls for LDH, GSH, Caspase 7 and Melatonin while only the most severe ASD group differed from controls for Complex I and GST and only the mild-to-moderate ASD group differed from controls for CoQ10.

ROC and Predictiveness curves

First we analyzed each biomarker separately to determine the ability to separate the control and ASD groups by generating ROC curves. Table 3 showed the AUC, best cut off to differentiate the two groups and the number of control and ASD above and below the best cutoff values of all biomarkers, as well as the specificity and sensitivity. Figure 3 shows the ROC curves for each biomarker and Fig. 4 shows the predictiveness curves for each biomarker. CK, Caspase 7, CoQ10, GSH and GST have good (>75%) Sensitivity while Caspase 7, Melatonin and GST have good (>75%) Specificity. Caspase 7 and GST were the only biomarkers to have both good sensitivity and specificity. Caspase 7 is the only biomarker with an AUC that is considered excellent and LDH, CoQ10, melatonin, GSH and GST are the biomarkers with the next best AUC values which are all considered fair. AST and ALT showed good specificity, but their AUC values were considered poor or very poor.

Discriminant analysis

Next we examined the ability of a combination of biomarkers to discriminate between the control and ASD groups. Many of the biomarkers were only performed on a subset of patients. Thus, we first selected the biomarkers that were performed on the great majority (>97%) of patients, which included Lactate, Pyruvate, LDH, Complex I and GST. The analysis demonstrated that Lactate and Pyruvate did not significantly contribute to the discriminant function. Recalculating the analysis without Lactate and Pyruvate resulted in a significant Wilks' Lamdba of 0.67 [$F(3,76) = 12.47$, $p < 0.0001$] with significant contributions of LDH [$F(1,78) = 15.00$, $p < 0.0005$], Complex I [$F(1,78) = 11.52$, $p = 0.001$] and GST [$F(1,78) = 11.06$, $p = 0.001$]. The discriminant function resulted in an accuracy of 77.5% with a Sensitivity of 75% and Specificity of 80%. A cross validation analysis demonstrated an accuracy of 74% with a Sensitivity of 70% and Specificity of 77.5%. Figure 5 shows the discriminant function values for all cases.

In order to investigate the contribution of novel biomarkers we relaxed our criteria for missing data and included three addition biomarkers which had up to 31% missing values. These included Caspase 7, CoQ10 and Melatonin. Adding these to the multivariable discriminant analysis resulted in the selection of Caspase 7 [$F(1,55) = 875.37$, $p < 0.001$] as the only necessary biomarker to separate the groups with a Wilks' Lamdba of 0.06 [$F(1,55) = 875.37$, $p < 0.0001$]. This discriminant function resulted in a 100% accuracy with 100% Sensitivity and Specificity for the classification and the cross-validation analysis.

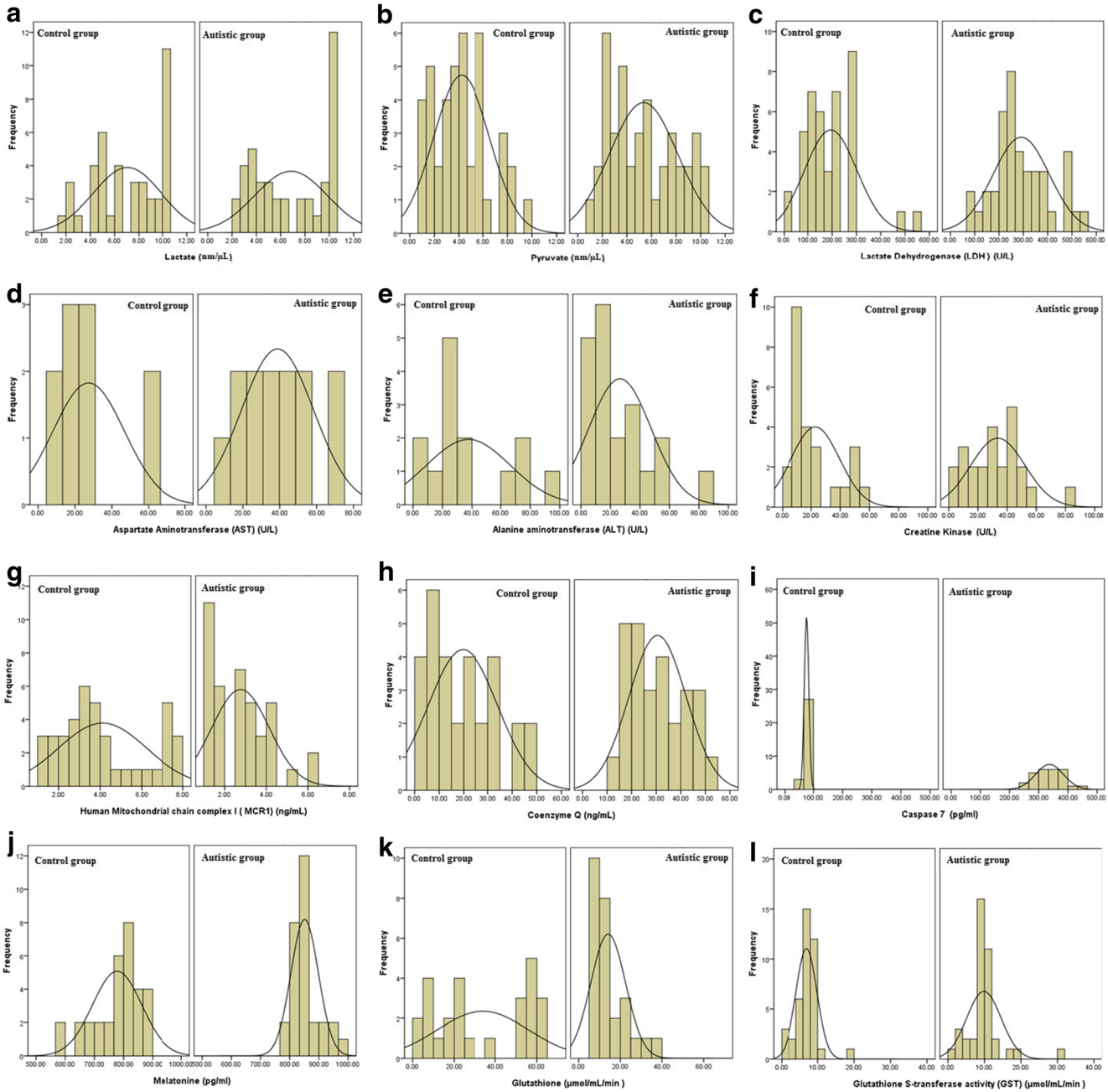

Fig. 1 Normal distribution for control and autistic groups in **a** Lactate, **b** Pyruvate, **c** Lactate Dehydrogenase, **d** Aspartate Aminotransferase, **e** Alanine Aminotransferase, **f** Creatine Kinase, **g** Electron Transport Chain complex I, **h** Coenzyme Q, **i** Caspase 7, **j** Melatonin, **k** Glutathione, **l** Glutathione S-transferase

Discussion

In this study we examined a wide variety of biomarkers of mitochondrial function in children with ASD and compared these values to typically developing controls. We confirmed previous studies indicating abnormal biomarkers in children with ASD including pyruvate, CK, ETC Complex 1, GSH, GST and Caspase 7. We also identified that only the most severe children demonstrated abnormalities in ETC Complex 1 activity and GST. Additionally, we found that several biomarkers could be candidates for differentiating children with ASD and typically developing children, including Caspase 7, GSH and GST by themselves because of their favorable sensitivity and specificity and consistency with previous studies. LDH and Complex I were also good candidates when added to other biomarkers in combination.

It is believed that AST and ALT may be released into the serum because mitochondrial dysfunction causes loss of some cellular integrity of certain organs such as muscle and liver (Rossignol and Bradstreet 2008). In the USA, serum ALT, AST,

Table 2 Correlations between biomarker (only significant correlations presented) with an indication of direction of correlation (last column)

Biomarkers	r value	*P* value.	Direction
Pyruvate ~ Caspase 7	0.269	0.043	P
Lactate Dehydrogenase ~ Glutathione	−0.389	0.004	N
Lactate Dehydrogenase ~ Creatine Kinase	0.388	0.006	P
Lactate Dehydrogenase ~ Caspase 7	0.325	0.014	P
Lactate Dehydrogenase ~ Coenzyme Q	0.284	0.032	P
Complex I ~ Glutathione S-transferase activity	0.324	0.004	P
Glutathione S-transferase activity ~ Caspase 7	0.434	0.001	P
Glutathione ~ Creatine Kinase	−0.413	0.004	N
Glutathione ~ Alanine aminotransferase	0.368	0.030	P
Glutathione ~ Caspase 7	−0.494	0.001	N
Caspase 7 ~ Melatonin	0.428	0.002	P

and lactate were found to be higher than normal in 52%, 36% and 76% of ASD children and mitochondrial disease, respectively (Weissman et al. 2008). Similarly, Poling et al. (Poling et al. 2006) reported elevated serum AST level in 38% of a general ASD clinic. It should be noted that these aforementioned studies were limited because of the use of standard normal ranges rather than contemporaneous control participants. In this study, no significant difference in the

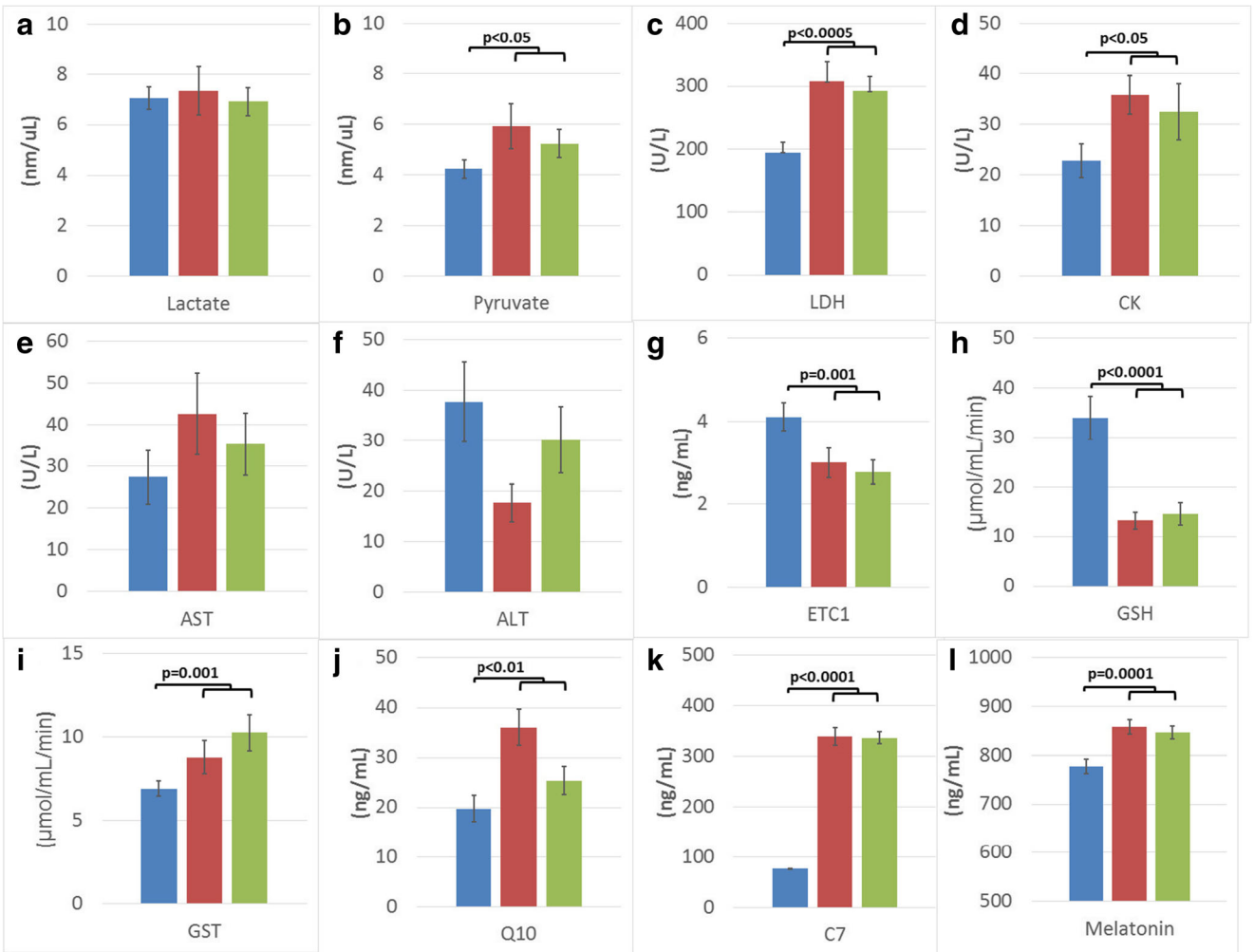

Fig. 2 Mean and standard error of biomarkers of metabolic function in children with mild-to-moderate ASD (*red*) and severe ASD (*green*) as well as typically developing controls (*blue*). *P* values represent the result of analysis of variance examining the difference between the Control and ASD groups including all severities

Table 3 Number of controls and autistic patients outside and inside the best cutoff values as well as area under the curve (AUC) and sensitivity and specificity. Values bolded are those above the threshold to be considered acceptable. After the best cutoff value is a A or B to indicate whether the control group is considered to be above (A) or below (B) the cutoff value as determine by the discriminant analysis

Parameters		Below cutoff	Above cutoff	Cutoff / AUC	Spec / Sens
Lactate (nm/μL)	Control (n = 41)	17% (7)	83% (34)	4.34 B	17%
	Autism (n = 41)	34% (14)	66% (27)	0.51	66%
Pyruvate (nm/μL)	Control (n = 41)	63% (26)	37% (15)	4.53 B	63%
	Autism (n = 41)	44% (18)	56% (23)	0.61	56%
Lactate Dehydrogenase (U/L)	Control (n = 41)	73% (30)	27% (11)	237.3 B	73%
	Autism (n = 41)	32% (13)	68% (28)	**0.74**	68%
Aspartate Aminotransferase (U/L)	Control (n = 10)	80% (8)	20% (2)	30.9 B	**80%**
	Autism (n = 13)	38% (5)	62% (8)	0.58	62%
Alanine Aminotransferase (U/L)	Control (n = 14)	21% (3)	79% (11)	20.3 A	**79%**
	Autism (n = 20)	55% (11)	45% (9)	0.60	55%
Creatine Kinase (U/L)	Control (n = 25)	56% (14)	44% (11)	19.9 B	56%
	Autism (n = 24)	21% (5)	79% (19)	0.66	**79%**
ETC complex I (ng/mL)	Control (n = 40)	43% (17)	57% (23)	3.31 A	57%
	Autism (n = 40)	70% (28)	30% (12)	0.52	70%
Caspase 7 (pg/mL)	Control (n = 30)	100% (30)	0% (0)	171.3 B	**100%**
	Autism (n = 27)	0% (0)	100% (27)	**1.00**	**100%**
Coenzyme Q (ng/mL)	Control (n = 30)	53% (16)	47% (14)	16.34 B	53%
	Autism (n = 27)	4% (1)	96% (26)	**0.74**	**96%**
Melatonin (pg/mL)	Control (n = 32)	81% (26)	19% (6)	837.1 B	**81%**
	Autism (n = 29)	38% (11)	62% (18)	**0.77**	62%
Glutathione (μmol/mL/min)	Control (n = 26)	35% (9)	65% (17)	22 A	65%
	Autism (n = 26)	81% (21)	19% (5)	**0.76**	**81%**
Glutathione-S-Transferase (μmol/mL/min)	Control (n = 40)	85% (34)	15% (6)	8.39 B	**85%**
	Autism (n = 40)	23% (9)	77% (31)	**0.79**	**77%**

concentration of lactate, AST and ALT between the ASD and control groups (Fig. 2).

Data presented in Fig. 2 demonstrate a significant increase in LDH and pyruvate in the ASD group as compared to the control group. The ROC analysis (Table 3) found that LDH was above the optimal cutoff values in 68% of children with ASD but only 27% of controls. This increase of LDH is consistent with altered energy metabolism previously reported in Saudi autistic patients (El-Ansary et al. 2010). On the other hand, the non-significant differences in lactate, AST, and ALT is not consistent with the recent study of Karim et al. (Karim et al. 2016) which show significant elevation of these parameters in ASD children when compared to control children. Future studies can investigate the role of serum lactate, ALT, AST as predictive markers of mitochondrial dysfunction in ASD individuals.

The significant elevation of CK (Fig. 2) found in the present investigation is consistent with the work of Al-Mosalim et al. (Al-Mosalem et al. 2009) and Poling et al. (Poling et al. 2006). However Frye et al. (Frye et al. 2013a, b, c) noted that CK was elevated in only a minority of the ASD patients they studied with mitochondrial disorders and a recent meta-analysis suggested that CK was not one of the biomarkers for mitochondrial disorders that is consistently elevated (Rossignol and Frye 2012a). CK was above the cutoff in 79% of children with ASD but also in 44% of control children. The higher positive rate in the control children resulted in a low AUC despite a good sensitivity.

It is well known that energy is critical for all cellular processes during brain development and function, including ATP formation, synaptogenesis, neurotransmitters synthesis and uptake, regulation of ionic gradients, antioxidant status, and myelination (McKenna et al. 2015). Biomarkers did not differ between severity of ASD measured by CARS but Complex I and GST were found to differ from the control group and only the most severe children with ASD. These findings are consistent with Adams et al. (Adams et al. 2011), which demonstrate the absence of correlation between energy metabolism related parameters and ASD severity as measured by the Autism Treatment Evaluation Checklist, Pervasive Development Disorder Behavior Inventory and Severity of Autism Scale.

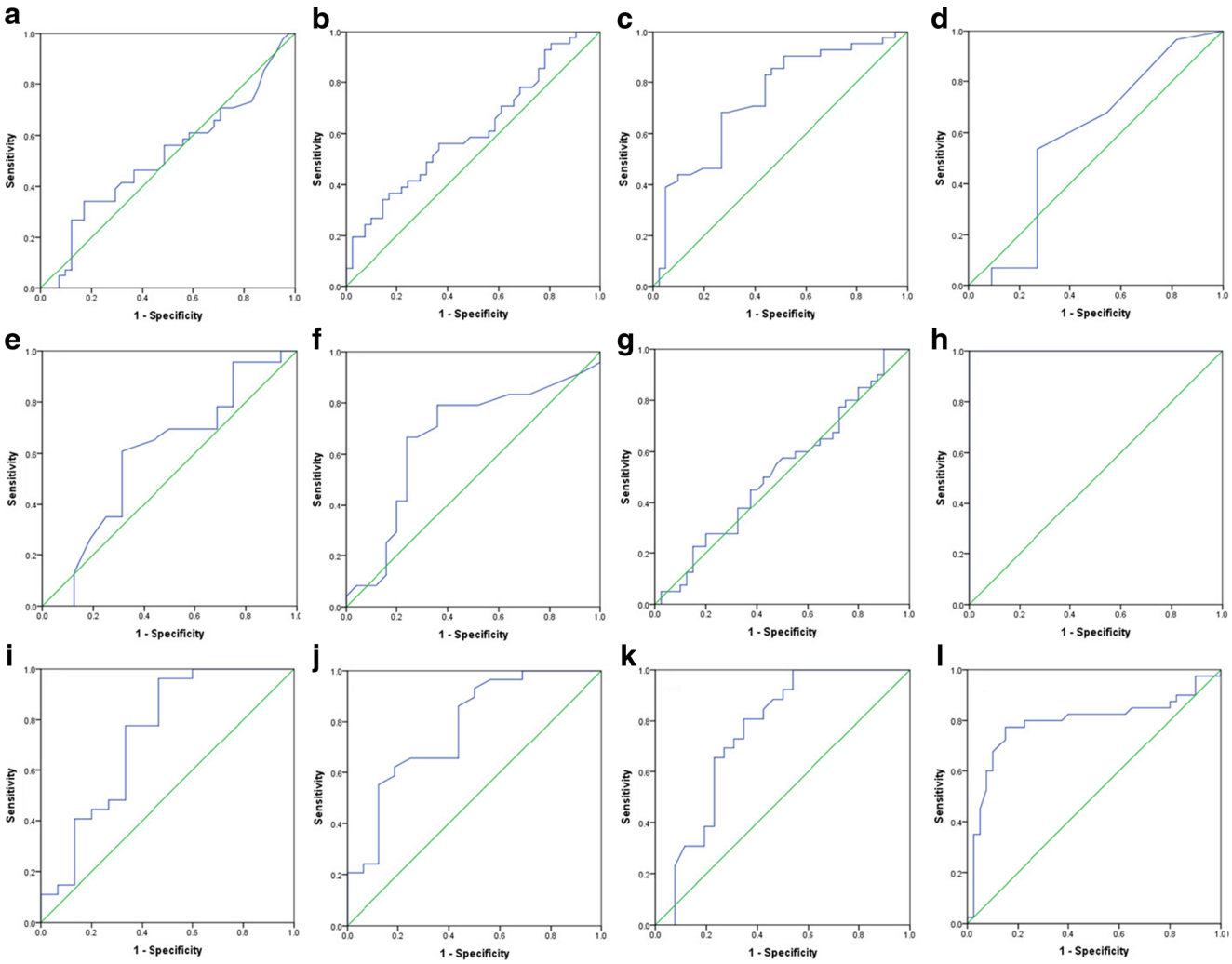

Fig. 3 Receiver Operational Curves for **a** Lactate, **b** Pyruvate, **c** Lactate Dehydrogenase, **d** Aspartate Aminotransferase, **e** Alanine Aminotransferase, **f** Creatine Kinase, **g** Electron Transport Chain Complex I, **h** Caspase 7, **i** Coenzyme Q, **j** Melatonin, **k** Glutathione and **l** Glutathione S-transferase, in relation to differentiation between Control and ASD groups. The curves for Caspase 7 (h) overlaps the x and y axes so it is difficult to see

Mitochondria produce ATP by generating a proton gradient with the help of ETC complexes. The number of mitochondria per cell is roughly linked to the energy demands of the cell. Neurons comprise a relatively high number of mitochondria since the brain has a high energy demand. The alterations in the mitochondrial ETC have been suggested to be involved in the pathogenesis of various neurological diseases, including neurodegenerative (Boekema and Braun 2007; Rezin et al. 2009) and neuropsychiatric disorders (Burchell et al. 2010; Moreira et al. 2010). Figure 2 shows that Complex I was significantly reduced in children with ASD. In addition, the multivariate discriminant function finds that Complex I activity adds to the ability to discriminant between ASD and control groups. Our results are consistent with the work of Guevara-Campos et al. (Guevara-Campos et al. 2013) in which a significant decrease of ETC activity (Complex I + III & IV) was reported in ASD.

Even though CoQ10 is cycled between the ubiquinol and ubiquinone forms, ubiquinol is the protective or antioxidant form of CoQ10. This is explained by the fact that its reduced form allows it to neutralize free radicals and thus prevent them from damaging cellular lipids, proteins, and genetic material. Therefore CoQ10 supplements that contain ubiquinone must first be reduced into ubiquinol in the body in order to exert their antioxidant effects. The findings of an increase in CoQ10 in ASD in this study is consistent with previous study of Wada et al. (Wada et al. 2006) which showed an increase of CoQ10 under oxidative stress in Chronic Obstructive Pulmonary Disease although it is inconsistent with the results of Adams et al. in ASD patients (Adams et al. 2011). Gvozdjáková et al. (Gvozdjakova et al. 2014), demonstrate the beneficial effect of ubiquinol in ASD patients, suggesting that high doses of the reduced form of CoQ10 may be critical for the function of a

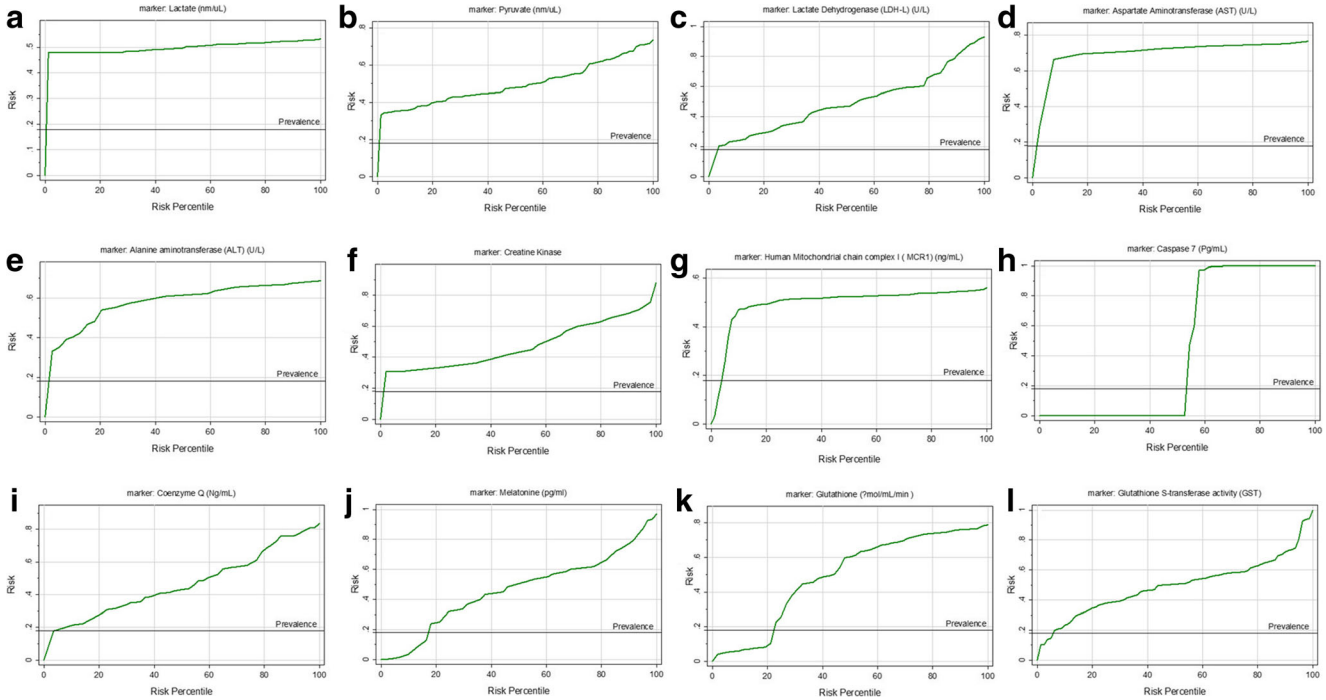

Fig. 4 Predictiveness Curves for **a** Lactate, **b** Pyruvate, **c** Lactate Dehydrogenase, **d** Aspartate Aminotransferase, **e** Alanine Aminotransferase, **f** Creatine Kinase, **g** Electron Transport Chain Complex I, **h** Caspase 7, **i** Coenzyme Q, **j** Melatonin, **k** Glutathione and **l** Glutathione S-transferase, in relation to differentiation between Control and ASD groups

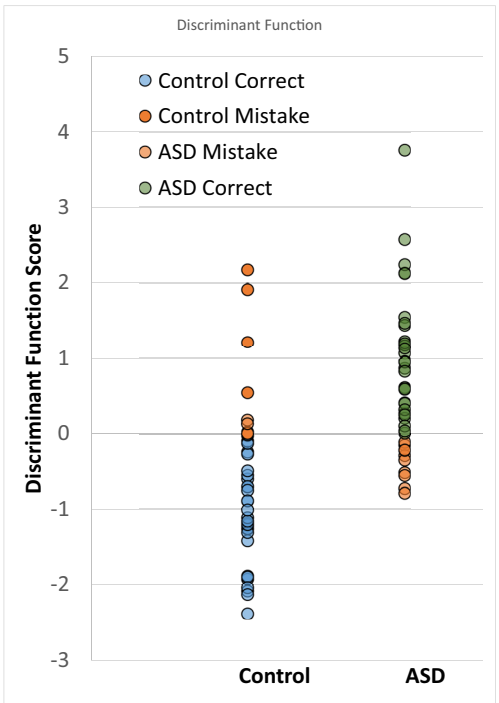

Fig. 5 Classification of control and ASD cases based on discriminant function. Scores below zero predict that the participant should be in the control group while scores above zero predict that participant should be in the ASD group. Thus, individual participant data is color coded to indicate correct and incorrect group assignments

suboptimally functioning mitochondria possibly due to high levels of oxidative stress in children with ASD.

Immune dysfunction and neuro-inflammation are two etiological mechanisms linked to ASD which can alter blood brain barrier (BBB) integrity (Quan 2008; Banks and Erickson 2010). The ASD brain, especially the hypothalamus, contains many mast cells critically located around the BBB and their activation usually leads to BBB disruption (Esposito et al. 2002). Additionally, recent studies have demonstrated how inflammation and autoantibodies can disrupt transport of nutrients into the brain (Frye et al. 2013a, b, c, Desai et al. 2016a, b, Frye et al. 2016a, b, Sequeira et al. 2016), potentially resulting in higher blood levels (Frye et al. 2017). Based on this phenomenon, the remarkable increase of plasma CoQ10 as a component of mitochondrial ETC might be accompanied by its depletion in the brain. This in turn induces oxidative stress in the brains of patients with ASD. This explanation can find support in the recent study of Zhang et al. (Zhang et al. 2011) which showed that brain mast cell activation leads to mitochondrial translocation to the cell surface. This is usually followed by secretion of extracellular mitochondrial DNA (mtDNA) (Angelidou et al. 2011), consistent with studies showing elevated serum mtDNA concentrations (Zhang et al. 2010). This notion should be tempered with the caveat that we do not have direct evidence for this notion with regard to CoQ10. Thus, the increase in CoQ10 in the blood could also be found in the brain. It is possible that decreased function

of the ETC in ASD individuals with mitochondrial dysfunction could result in less consumption and higher serum levels. Indeed, further research will be needed to address these possibilities.

Melatonin efficacy in treating several neuropsychiatric diseases could be due to its various biological effects, some of which are mediated via activation of mitochondrial receptors and others of which that are due to strong antioxidant activities which protect nuclear DNA and mtDNA from reactive oxygen species (ROS) (Reiter et al. 2007). Under normal physiological conditions, melatonin has been reported to easily cross the BBB and modify neurons' electrical activities by inhibiting glutamatergic and improving γ-aminobutyric (GABA) neurotransmission (Banach et al. 2011). Based on the fact that glutamate excitotoxicity and imbalanced excitatory/inhibitory ratio is one of the most confirmed pathological mechanisms in ASD, the significant elevation of plasma melatonin (Figs. 1 and 2) which could be concomitantly accompanied by low brain level due to the BBB disruption and may be linked to glutamate excitotoxicity (El-Ansary and Al-Ayadhi 2014). It should be noted, however, that melatonin has been demonstrated to be decreased in children with ASD in both blood and urine (Pagan et al. 2011; Tordjman et al. 2012; Pagan et al. 2014). The possibility of the discrepancy could be related to the specific population studied and/or the fact that in addition to disruption in absolute melatonin levels, children with ASD have disruptions in the circadian melatonin rhythm (Rossignol and Frye 2011; Rossignol and Frye 2014), making the measurement of absolute melatonin levels sensitive to time of day.

Under normal physiological conditions, the mitochondria maintain a membrane potential ($\Delta\Psi_m$) and shuttles electrons across the ETC with minimal ROS production, which can occur at Complex III (Saraste 1999). During apoptosis, cytochrome c is lost from the mitochondria. Cytochrome c is required for electron transfer from Complex III to IV. Thus, apoptosis can result in a loss of electron transfer in the ETC and result in an increase in ROS production (Cai and Jones 1998). The significant elevation of caspase 7 found in this study (Figs. 1 and 2) demonstrated the pro-apoptotic mechanism in relation to mitochondrial dysfunction. Table 3 demonstrates that 100% of ASD patients have elevated caspase 7 levels while none of the control participants have elevated concentrations of caspase 7. This can find support in the recent study of Brentnall et al. (Brentnall et al. 2013) which suggest that caspases (e.g caspase 3, 7 and 9) have different roles in intrinsic apoptosis and that caspase 7 could play a key role in ROS production. The elevation of caspase 7 showed in this investigation is consistent with the recent work of Siniscalco et al. (Siniscalco et al. 2012) in which the authors show the increase of protein levels of caspase-3, −7 and −12 in ASD patients and suggested the possible role of the caspase pathway in ASD and the use of caspases as potential diagnostic and/or therapeutic tool.

Normal functional mitochondria possess many antioxidant defenses that detoxify superoxide (O_2^-) and H_2O_2. Firstly, (O_2^-) is converted to H_2O_2 by superoxide dismutase (SOD) (Fridovich 1995). H_2O_2 as SOD product can diffuse to the cytoplasm to be reduced to H_2O by catalase, GSH and peroxidase. Moreover, mitochondria use various antioxidant molecules, most important of which is GSH, to minimize or inhibit oxidative processes (Mattson et al. 2008). Despite their damaging effect, ROS act on signaling functions in physiological processes, including learning, synaptic plasticity and memory (Kishida and Klann 2007). This might be correlated to the impairment of cognitive ability measured as the CARS in ASD patients. Based on this, the significant increase of GST together with the remarkable depletion of GSH, both support the fact that Saudi ASD patients are under oxidative stress. The remarkable impairment of these two markers can be clearly seen in Fig. 2. Table 3 demonstrates that these markers are abnormal in a majority of individuals with ASD with both markers having reasonable sensitivity. In fact, GST has both a reasonable specificity and sensitivity and was selected as a biomarker for the multivariate discriminant model. This is consistent with the previous work of Al-Gadani et al. (Al-Gadani et al. 2009) in which authors found that Saudi ASD patients are under H_2O_2 stress due to over-expression of SOD with slightly inhibited catalase as well as by the work of Frye et al. (Frye et al. 2013a, b, c) and several studies by the James group (James et al. 2004, James et al. 2006, James et al. 2008, James et al. 2009, Rose et al. 2012a, b, Rose et al. 2014, Howsmon et al. 2017, Rose et al. 2017, Vargason et al. 2017).

This study has also demonstrated that markers related to energy metabolism (lactate, pyruvate, LDH, AST, ALT, CK, Complex 1, CoQ10), antioxidant status (Melatonin, GST), and apoptosis (caspase 7) are positively correlated. On the other hand, GSH showed negative correlation with caspase 7 and CK which suggests that GSH depletion contributes to apoptosis and energy impairment as two etiological mechanisms in ASD. The negative correlation between LDH, CK, and caspase 7 can relate oxidative stress and energy impairment.

Interneurons are essential for the Glutamatergic/GABAergic balance, which has been shown to be abnormal in ASD. These neurons are highly dependent on energy, so they are highly affected by mitochondrial dysfunction. This suggests that the parameters measured in the present study might be useful for the early diagnosis of ASD as this disorder appears to be highly affected by mitochondrial dysfunction (Inan et al. 2016; Kann 2016). Most notably, Caspase 7 recorded an AUC of 1, showing perfect predictive value in this study.

Conclusion

This study examined biomarkers related to mitochondrial dysfunction, oxidative stress and apoptosis in Saudi children with ASD. It shows the potential usefulness of some of these biomarkers. Caspase 7 was the most discriminating marker between ASD and healthy controls. The mitochondrial abnormality observed in the present study may or may not indicate an etiological role for the mitochondria in ASD. However, mitochondrial dysfunction could greatly amplify and propagate brain dysfunction and could be related to other etiological mechanisms such as oxidative stress, glutamate excitotoxicity, and impaired energy metabolism.

ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; AUC, Area under the curve; ASDs, Autism spectrum disorders; CARS, Childhood Autism Rating Scales; CoQ10, Coenzyme Q; CK, Creatine Kinase; ELISA, enzyme-linked immunosorbent assay; ETC, Electron transport chain; GSH, Glutathione; GST: Glutathione S-Transferase; LDH, Lactate dehydrogenase; ROS, Reactive oxygen species; ROC, Receiver Operating Characteristics.

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Author's contribution MAK, performed all the biochemical assays as part of her PhD thesis, ABB, Supervised the practical work and co-drafted the manuscript, LA provided samples and participated in the diagnosis of the autistic samples, AE designed the study and drafted the manuscript, REF, interpretation of data and critically revised the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Declarations The data will not be shared because autistic patients from Autism Research and Treatment Centre, College of Medicine, King Saud University, did not give consent to the public release of their data but only to participate in the present study.

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Competing interests The authors declare that they have no competing interests.

Ethics approval and consent to participate This study was certified by the local Ethical Committee of Faculty of medicine, King Saud University, Riyadh, Saudi Arabia.

Consent for publication Not applicable.

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