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Preliminary evaluation of a novel nine-biomarker profile for the prediction of autism spectrum disorder

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Abstract

Background

Autism spectrum disorder (ASD) is a complex group of heterogeneous neurodevelopmental disorders the prevalence of which has been in the rise in the past decade. In an attempt to better target the basic causes of ASD for diagnosis and treatment, efforts to identify reliable biomarkers related to the body's metabolism are increasing. Despite an increase in identifying biomarkers in ASD, there are none so far with enough evidence to be used in routine clinical examination, unless medical illness is suspected. Promising biomarkers include those of mitochondrial dysfunction, oxidative stress, energy metabolism, and apoptosis.

Methods and participants

Sodium (Na+), Potassium (K+), glutathione (GSH), glutathione-s-transferase (GST), Creatine kinase (CK), lactate dehydrogenase (LDH), Coenzyme Q10, and melatonin (MLTN) were evaluated in 13 participants with ASD and 24 age-matched healthy controls. Additionally, five ratios, which include Na^+/K^+, GSH:GST, CK:Cas7, CoQ10: Cas 7, and Cas7: MLTN, were tested to measure their predictive values in discriminating between autistic individuals and controls. These markers, either in absolute values, as five ratios, or combined (9 markers + 5 ratios) were subjected to a principal component analysis and multidimensional scaling (MDS), and hierarchical clustering, which are helpful statistical tools in the field of biomarkers.

Results

Our data demonstrated that both PCA and MDS analysis were effective in separating autistic from control subjects completely. This was also confirmed through the use of hierarchical

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clustering, which showed complete separation of the autistic and control groups based on nine biomarkers, five biomarker ratios, or a combined profile. Excellent predictive value of the measured profile was obtained using the receiver operating characteristics analysis, which showed an area under the curve of 1.

Conclusion

The availability of an improved predictive profile, represented by nine biomarkers plus the five ratios, inter-related different etiological mechanisms in ASD and would be valuable in providing greater recognition of the altered biological pathways in ASD. Our predictive profile could be used for the diagnosis and intervention of ASD.

Introduction

Autism spectrum disorder is characterized by symptoms, such as impairment of social interaction, and repetitive behaviors or restricted interests [1]. Recently, the prevalence of ASD has dramatically increased, reaching 1:37 children in the United States [2]. The severity of autistic features as well as the incidence of comorbid illnesses, which include intellectual disability, anxiety, epilepsy, and gastrointestinal disorders, greatly differ among individuals with autism [3–6]. ASD is currently diagnosed by observing common autistic behaviors in children [7]. Although expert clinicians can diagnose autism in children as young as 24 months the average age at which autism is diagnosed is still considerably high and may reach that of four years [8]. Centers for Disease Control and Prevention., 2009). Families often wait a long time before receiving a definitive diagnosis owing to the small number of well-trained clinicians capable of performing an accurate and realistic assessment [9]. Early diagnosis is important because not only intensive behavioral therapies are effective in decreasing disability in many children with ASD [10,11], but also because the benefit of early intervention is greater the earlier the intervention is started.

Based on our understanding of the etiological mechanisms of ASD, we previously demonstrated that use of selected sets of biomarkers related to impaired lipid metabolism and neuroinflammation were effective for separating autistic from healthy control participants and for correctly predicting the severity of ASD. We proved that effectiveness of identified libraries relied on the fact that they were helpful in correctly discriminating the study population as control or autistic patients and in categorizing autistic patients with different degree of sensory profile impairments [12,13].

It is well accepted that metabolism-related biomarkers are more directly related to the unique metabolic signature of an individual with ASD, than are the genomic, gut microbiome-related, and environmental biomarkers such as neurotoxins and diet [6, 14, 15]. ASD-specific reductions in multiple metabolites with concomitant falling in intelligence quotient have been reported in several brain regions [15]. Metabolic analysis can offer important biomarkers that might help in the identification of the impaired biological processes in ASD. Still, it is important to highlight that there are presently no evidence-based approvals for metabolic or dietary treatments for people with ASD [16,17].

Mitochondrial dysfunction is a well-studied etiological mechanism of ASD. Multiple studies have been performed to understand the role of mitochondrial dysfunction. Shoffner et al [18] reported high levels of lactate, pyruvate, and alanine in the blood, urine, and cerebrospinal

fluid, together with an increase in the mitochondrial complex I in almost half of their participants with ASD.

In 2011, Chauhan et al [19] reported a significant reduction in the activities of the mitochondria electron respiratory chain complexes (ETC) II, III, and IV in different brain regions of children with ASD. Unexpectedly, the levels of these complexes were unchanged when adults with ASD and healthy subjects were compared. Interestingly, these results suggested that low levels of ETC complexes could re-adjust to reach the normal range as these children approached adulthood [19].

These early observations were confirmed by our research group [20]. We previously recorded abnormal levels of the mitochondrial plasma markers pyruvate, lactate dehydrogenase, creatine kinase, glutathione-S-transferase (GST), caspase 7 and respiratory complex I (RCI) in children with ASD compared to those of age- and gender-matched control subjects. Moreover, our study demonstrated that most severely affected children had both RC I and GST abnormalities and that caspase 7, a marker of mitochondrial dysfunction, was the most discriminating biomarker between patients with ASDand controls [20].

Interestingly, Nguyen et al [21] proved that dopaminergic neurons derived from children with ASD displayed decreased neuritis development, concomitant with reduced mitochondrial membrane potential, intracellular calcium level, ATP generation, and total number of mitochondria within the neuritis.

The current study was motivated by observations that mitochondrial dysfunction, as a repeatedly recorded etiological mechanism of ASD, can be easily related to glutamate excitotoxicity, oxidative stress, apoptosis, and impaired gut microbiota among other patho-etiological causes. [22–24]. To record a panel of mitochondria-related markers or a metabotype that might help in identifying children at high risk of presenting clinical features of ASD at very early age, we tested the suitability of using the principal component analysis (PCA), Monte Carlo simulation, and hierarchical clustering.

Based on the availability of potential treatment options for mitochondrial dysfunction-related diseases, investigation into the molecular abnormalities underlying the link between mitochondrial dysfunction and other etiological mechanisms of ASD could result into better therapeutic interventions for patients with ASD.

Materials and methods

Participants

The study protocol was approved by the ethics committee of medical College, King Saud University according to the most recent Declaration of Helsinki (Edinburgh, 2000). Two groups of participants were recruited for the study consisting of 13 autistic patients and 24 age and

Gender matched healthy control. All participants gave written informed consent provided by their parents and agreed to participate in the study. The study participants were enrolled in the study through the ART Center (Autism Research & Treatment Center) clinic. The ART Center clinic sample population consisted of children diagnosed with ASD. The diagnosis of ASD was confirmed in all study subjects using the Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS) and 3DI (Developmental, dimensional diagnostic interview) protocols. The ages of autistic children included in the study were between 2–12 years old. All were simplex cases (i.e. family has one affected individual). All are negative for fragile x syndrome gene. The control group was recruited from pediatric clinic at King Saud medical city whose mean age ranged from 2–14 years. Subjects were excluded from the investigation if they had dysmorphic features, or diagnosis of fragile X or

other serious neurological (e.g., seizures), psychiatric (e.g., bipolar disorder) or known medical conditions.

All participants were screened via parental interview for current and past physical illness. Children with known endocrine, cardiovascular, pulmonary, liver, kidney or other medical disease were excluded from the study. All patients and controls included in the study were on similar but not identical diet and none of them were on any special high fat or fat restricted diet.

Measures of disease severity among autistic patients

Disease severity was measured using the Childhood Autism Rating Scale (CARS). To obtain a CARS score, each child was rated on a scale of 1 (normal) to 4 (severely abnormal) with respect to each of 15 criteria (relating to others; imitation; emotional response; body use; object use; adaptation to change; visual response; listening response; taste, smell, and touch responses; fear and nervousness; verbal communication; non-verbal communication; activity level; level and reliability of intellectual responses and general impressions). A final score was obtained by computing the sum of the 15 individual scores, resulting in a combined score that could range from 15 to 60. Scores below 30 were considered non-autistic; 30–36.5 were considered mild to moderate ASD and scores greater than 36.5 were considered severe ASD [25].

Sample collection

After overnight fasting, blood samples were collected from autistic children and healthy controls by a qualified lab technician into 3-ml blood collection tubes containing EDTA. Immediately after collection, blood was centrifuged at 4˚C at 3000 g for 20 minutes. The plasma was decanted, dispensed into four 0.75 ml aliquots (to avoid multiple freeze-thaws cycles) and stored at −80˚C until analysis.

Ethics approval and consent

This work was approved by the ethics committee of King Khalid Hospital, King Saud University (Approval number: 11/2890/IRB). A written consent was obtained from the parents of all participants recruited in the study as per the guidelines of the ethics committee.

Biomarkers selection and measurements

The selected biomarkers were measured in the plasma samples of both autistic patients and control. After initial assessment of the overall discriminatory power of 9 biomarkers through its maximal area under the curve (AUC), as the best discriminatory power that the biomarker can achieve, the presented 9 biomarkers and 5 relative ratios were selected based on their recorded satisfactory (AUC), specificity and sensitivity when analyzed using receiver operating characteristics.

- **Measurement of K^+ and Na^+ levels**
  Potassium and sodium colorimetric kits, products of United Diagnostics Industry (UDI, Dammam, KSA) were used to investigate plasma K^+ and Na^+ plasma levels according to the manufactures' instructions.
- **Measurement of GST activity and GSH concentration**
  GST activity and total GSH concentration were calorimetrically determined in all blood samples according to Mannervik [26] and Beutler et al. [27] respectively.

- **Measurement of CK and LDH activities**
  Plasma CK activity was evaluated in serum samples by using CK kit, a product of BioSystems (Barcelona, Spain) according to the method of Schumann et al. [28]. Enzyme activity is expressed in U/L with a detection limit of 9.2 U/L = 153 nkat/L. However, LDH activity was assayed spectrophotometrically in all blood samples by using LDH kinetic Kit, a product of United Diagnostics Industry (UDI, Dammam, KSA). According to Amador et al. [29] and Wacker et al. [30], the "forward" reaction (lactate + NAD^+ to pyruvate + NADPH + H^+) was followed and NADH formation rate, indicated by an increase in absorbance at 340nm, was recorded.

- **Caspase 7 level measurement**
  Human Caspase-7 ELISA kit, a product of CUSABIO (China) was used to investigate Cas7 level in all blood samples according to the manufacturer's instructions. This kit employs the competitive inhibition enzyme immunoassay technique. The wavelength was detectable at 540–570 nm while the detection limit was from 62.5 to 400 pg/ml.

- **Measurement of CoQ10 and MLTN levels**
  Human Coenzyme Q10 and Human Melatonin ELISA Kits, products of MyBiosource (San Diego, California, USA) were used to evaluate the quantity of CoQ10 or melatonin in blood samples, respectively. The competitive inhibition enzyme immunoassay technique was employed and the optical density was detectable at 540 nm. The detection range was 6.25 pg/ml-400 pg/ml for MLTN while the minimum measurable level of CoQ10 was 3.12 ng/ml.

Statistical analysis

PCA and multidimensional scaling (MDS) were performed using Bionumerics version 6.6 (Applied Maths, ustin, TX) or IBM SPSS version 22 as previously described [13]. Briefly, the inputs into PCA and MDS were a covariance matrix and a similarity matrix, respectively. Similarity matrices were constructed from all possible pairwise similarities calculated using Canberra distances (Eq 1). PCA reduces the number of variables by condensing correlated variable. Therefore, correlation between some of the variables must exist for the analysis to be meaningful. The presence of correlated variables was tested by Bartlett's test of sphericity with a p-value threshold of <0.001. Kaiser-Meyer-Olkin (KMO) measure was used to test adequacy of the sample sizes. The number of statistically significant components in PCA was determined using Parallel Analysis (Monte Carlo simulation) using Brian O'Connor's syntax for SPSS[27].

$$D = \frac{1}{n}\sum_{i=1}^{n}\frac{|Xi - Yi|}{|Xi + Yi|} \quad \text{Eq 1}$$

Where: "D" is the Canberra distance metric, "n" is the number of variables, "i" is the i^{th} variable, and "X" and "Y" are two participants.

Hierarchical clustering was performed using Bionumerics version 6.6 as previously described [13]. Briefly, pairwise similarities were calculated using Canberra distances and dendrograms were constructed using Unweighted Pair Group Method with Arithmetic Mean algorithm. A two-tailed t-test was used to determine the significance of differences observed in biomarker values between autistic and control participants. A p-value of <0.05 was considered significant. T-test was performed using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA). Correlation was estimated by Spearman Correlation Coefficient, and a p-value is assigned based on permutation analysis. Correlation analyses were performed using GraphPad Prism version 6. For analyses involving computation of a Z-score, Z-scores were calculated

according to the formula of Eq 2 using Excel.

$$Z = \frac{(X - \mu)}{\sigma} \quad \text{Eq 2}$$

Where Z is the Z-score, X is the observed value, μ is the mean, and σ is the standard deviation.

Results

Initial evaluation of the data

Nine biomarkers were evaluated in 13 autistic participants and 24 age-matched healthy controls, and they were all significantly different between the two groups. We selected five ratios between pairs of physiologically related biomarkers that were different between the autistic and control groups to test their potential in predicting ASD (Fig 1).

Based on the nine biomarkers alone, the five ratios alone, or all biomarkers and ratios combined, both PCA and multidimensional scaling (MDS) showed complete separation of autistic and control participants (Fig 2). Bartlett's test of sphericity showed that correlations between

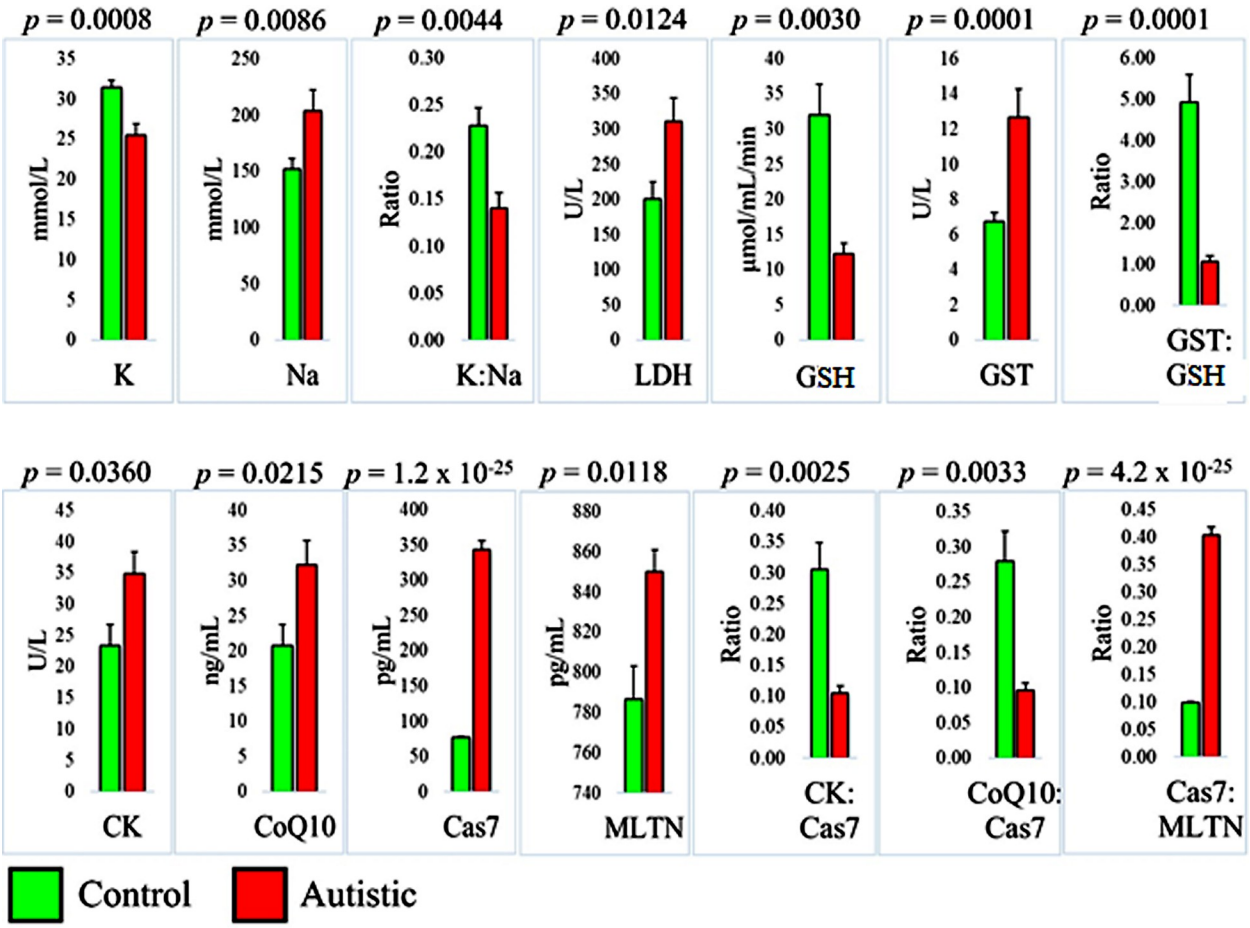

Fig 1. Differences between autistic individuals (n = 13) and aged-matched healthy controls (n = 24) with regard to 9 biomarkers and 5 biomarker ratios. K: potassium, Na: sodium, LDH: lactate dehydrogenase, GSH: glutathione, GST: glutathione S-transferase, CK: creatine kinase, CoQ10: co-enzyme Q10, Cas7: caspase 7, and MLTN: melatonin. Statistical significance was determined using a two-tailed student's t-test.

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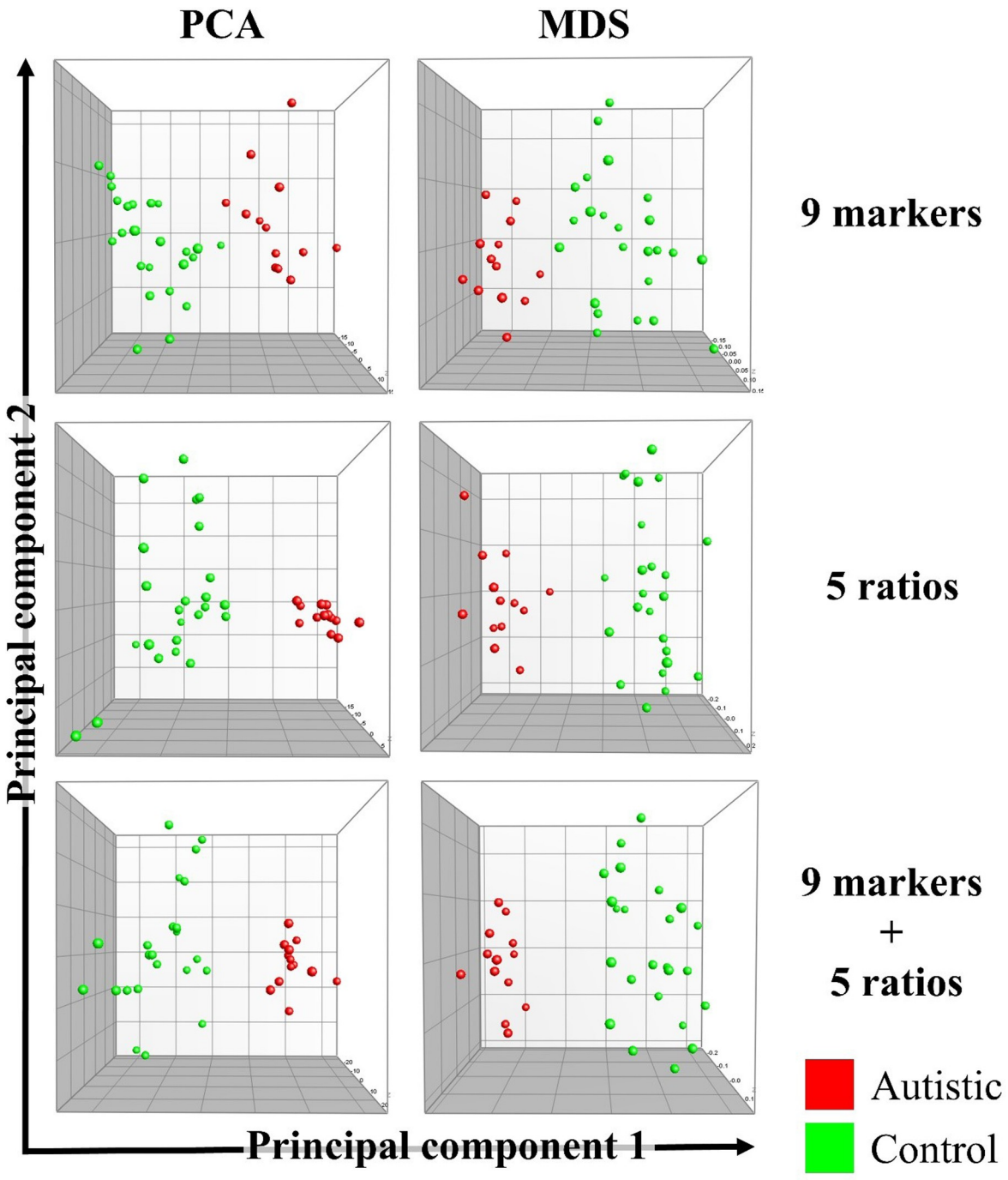

Fig 2. Complete separation of autistic individuals (n = 13) and age-matched healthy controls (n = 24) using principal component analysis (PCA) and multidimensional scaling (MDS). The 9 biomarkers used (top row) were potassium (K), sodium (Na), lactate dehydrogenase, glutathione (GSH), glutathione S-transferase (GST), creatine kinase (CK), co-enzyme Q10 (CoQ10), caspase 7 (Cas7), and melatonin (MLTN). The 5 ratios (middle row) were K:Na, GST:GLTN, CK:Cas7, CoQ10:Cas7, and Cas7:MLTN. A combined profile including the 9 biomarkers and the 5 ratios was also tested (bottom row).

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variables do exist with extremely small p values (2×10^{-61} to 8×10^{-6}), which confirmed the appropriateness of using PCA.

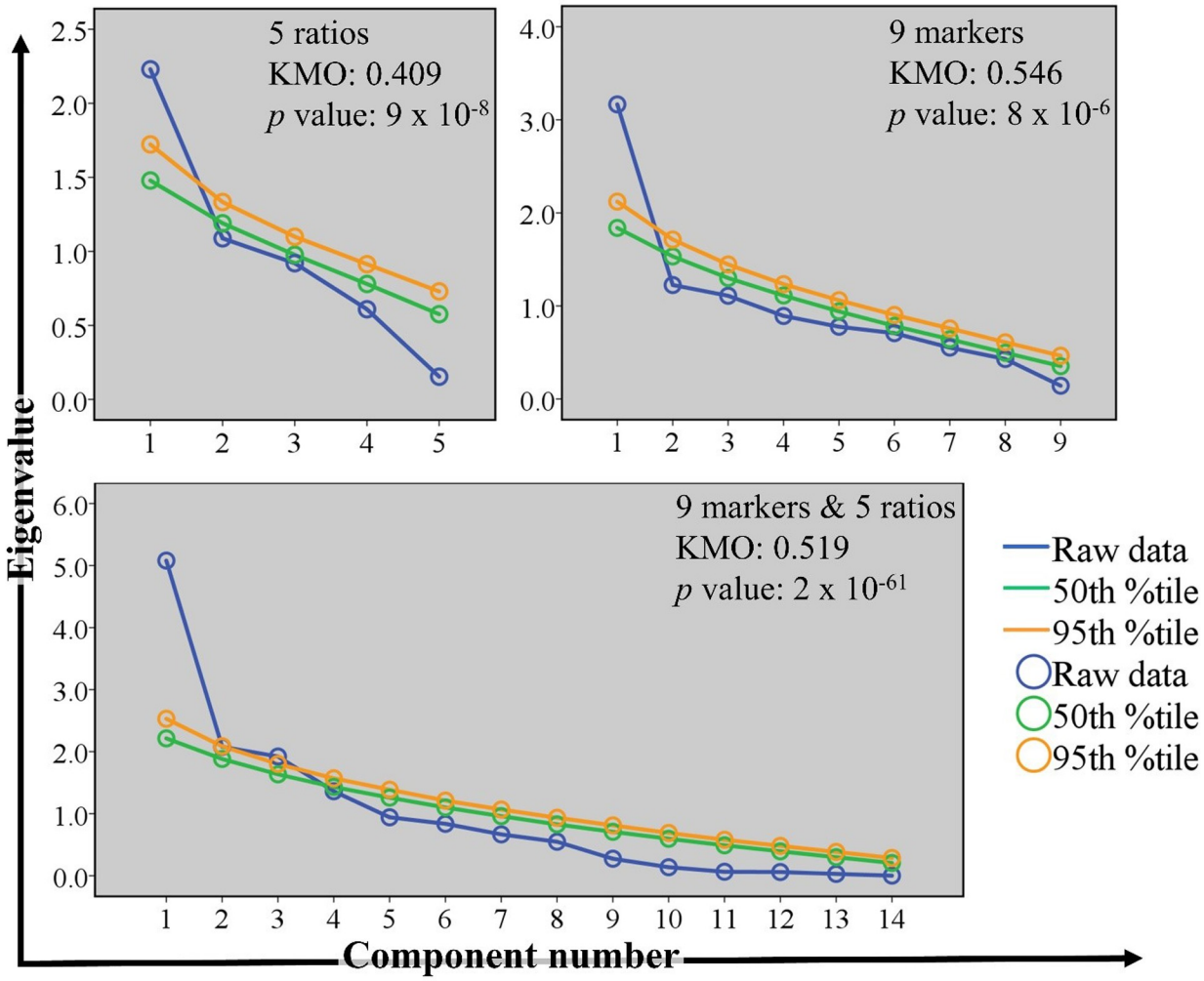

Fig 3. Verification of the suitability of using principal component analysis (PCA). Scree plots were generated using Monte Carlo simulation. The eigenvalues of individual principal components computed from the observed (raw) data were compared to the corresponding simulated eigenvalues. Statistically significant principal components have greater eigenvalues than the corresponding 50th and 95th percentile simulated eigenvalues. Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy was used to evaluate sample size. Bartlett's test of sphericity was used to reject the null hypothesis that the correlation matrices used in PCA were equal to an identity matrix. The *p* values shown represent the likelihood that the null hypothesis is true. The scree plots correspond to PCA results shown in Fig 2.

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The results of the KMO measure of sampling adequacy indicated that a larger sample size was needed for PCA. Groups were mainly separated on the first component (shown in Fig 2 on the × axes), which was shown to be significant using Monte Carlo simulation (Fig 3).

In addition to MDS results, which completely agreed with PCA results, we wanted to confirm further the unbiased partitioning of autistic and healthy participants using hierarchical clustering. In Fig 4, we show complete separation between the autistic and control groups using hierarchical clustering based on nine biomarkers, five biomarker ratios, or a combined profile (Fig 4).

Evaluating the predictive power of biomarkers based on the area under a receiver operating characteristic (ROC) curve (AUC). From the data shown in Table 2 and Fig 5, it is clear that caspase 7 (Cas7) was a very strong predictor of ASD, with an AUC of 1.00, which is equivalent to 100% specificity and 100% sensitivity. Glutathione S-transferase (GST) and potassium (K)

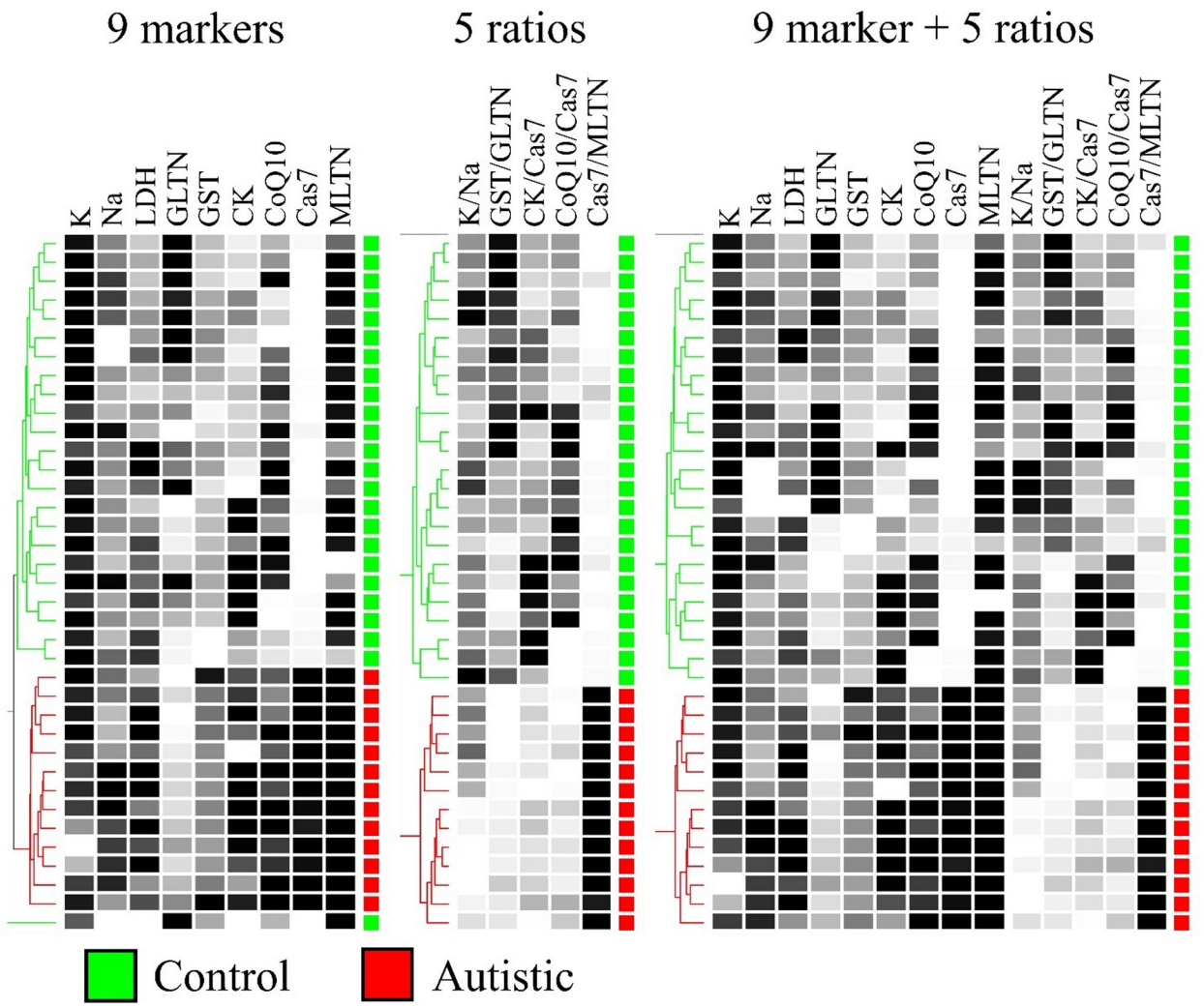

Fig 4. Hierarchical clustering of participants based on 9 biomarkers, 5 biomarker ratios, or both. Data were collected from 13 autistic patients and 24 age-matched controls. Pairwise similarities were based on Canberra distances (Eq 1) and dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. K: potassium, Na: sodium, LDH: lactate dehydrogenase, GSH: glutathione, GST: glutathione S-transferase, CK: creatine kinase, CoQ10: co-enzyme Q10, Cas7: caspase 7, and MLTN: melatonin.

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were among other strong predictors of ASD with AUCs of 0.97 and 0.81, respectively. All other biomarkers were at least reasonable predictors with AUCs ranging from 0.71 to 0.76. Using ratios did not seem beneficial since it either lowered or did not affect AUC values. For example, Cas7 had an AUC of 1.00, which is equal to or greater than the AUC obtained with any ratio of any other analyte to Cas7 (Table 2, Fig 5). On the other hand, combining biomarkers into profiles using PCA appeared to boost AUC values. PCA was performed on groups of biomarkers and biomarker ratios; and the loadings of the first component (PC1)—the component on whose coordinates autistic and control participants were separated were used—as the predictor in ROC analysis. We obtained an AUC of 1.00 when using all 9 biomarkers, 5 ratios, or the biomarkers and ratios combined. Since Cas7 and its ratio to melatonin both showed an AUC of 1.00 when tested individually, it was not clear if lumping them with additional variables in a profile had any advantage. For this reason, we created a 7-biomarker profile lacking both Cas7 and GST, which also had a notably high AUC. Doing so resulted in an AUC of 0.94, which is greater than the AUCs obtained using any of the 7 individual biomarkers alone.

Table 1. Summary of participants' data. Recruited volunteers included 24 healthy controls (identification numbers begin with the letter C) and 13 autistic patients (identification numbers begin with the letter A). CARS: Childhood Autism Rating Scale, K: potassium (mmol/L), Na: sodium (mmol/L), LDH: lactate dehydrogenase (U/L), GSH: glutathione (μmol/mL/min), GST: glutathione S-transferase (U/L), CK: creatine kinase (U/L), CoQ10: co-enzyme Q10 (ng/mL), Cas7: caspase 7 (pg/mL), MLTN: melatonin (pg/mL).

ID	Age in years	CARS	K	Na	LDH	GSH	GST	CK	CoQ10	Cas7	MLTN	K:Na	GST:GSH	CK:Cas7	CoQ10:Cas7	Cas7:MLTN
C1	5		40	149	132	22	8	13	21	81	793	0.27	2.74	0.16	0.26	0.1
C4	4		30	157	554	25	9	10	48	69	869	0.19	2.83	0.14	0.69	0.08
C6	7		35	163	138	60	6	13	16	73	833	0.22	9.36	0.18	0.22	0.09
C9	7		39	154	152	17	6	53	2	83	826	0.25	2.64	0.65	0.03	0.1
C10	5		25	159	488	29	9	17	21	71	676	0.16	3.01	0.24	0.29	0.1
C15	9		39	131	79	16	6	17	28	81	836	0.3	2.8	0.21	0.35	0.1
C16	7		29	162	99	60	7	10	12	84	719	0.18	8.76	0.12	0.14	0.12
C19	9		32	209	105	64	5	10	45	79	882	0.15	11.86	0.13	0.57	0.09
C25	6		28	126	285	8	2	20	9	86	777	0.23	4.8	0.23	0.11	0.11
C29	9		26	127	165	23	3	13	14	70	796	0.2	7.45	0.19	0.21	0.09
C34	8		31	240	125	5	5	13	32	86	842	0.13	0.97	0.15	0.37	0.1
C35	5		30	181	290	8	1	17	7	88	636	0.17	5.14	0.19	0.08	0.14
C36	6		27	119	224	14	8	50	31	66	584	0.23	1.71	0.75	0.47	0.11
C37	7		26	140	277	24	8	53	4	82	829	0.19	3.19	0.65	0.05	0.1
C38	7		35	153	99	4	7	40	21	83	808	0.23	0.65	0.48	0.25	0.1
C39	5		31	131	270	8	7	23	48	72	793	0.24	1.18	0.32	0.67	0.09
C43	5		29	152	171	10	7	60	16	78	869	0.19	1.38	0.77	0.2	0.09
C46	11		34	76	158	55	10	13	2	79	833	0.45	5.26	0.17	0.03	0.1
C47	8		38	75	231	56	9	10	21	69	826	0.52	6.15	0.15	0.3	0.08
C49	7		31	245	224	55	8	43	28	74	676	0.13	6.64	0.58	0.38	0.11
C50	5		24	72	13	54	8	7	12	75	836	0.34	6.58	0.09	0.16	0.09
C51	2		28	139	224	51	4	7	45	65	719	0.2	11.62	0.1	0.69	0.09
C52	9		32	208	132	38	8	23	6	70	882	0.15	4.67	0.33	0.08	0.08
C54	5		33	188	171	62	9	23	9	71	740	0.18	7.02	0.33	0.13	0.1
A1	5	44	29	148	257	23	30	37	34	359	848	0.19	0.76	0.1	0.09	0.42
A4	4	42	26	149	218	9	10	7	22	366	848	0.17	0.91	0.02	0.06	0.43
A19	9	49	31	176	138	6	18	30	22	372	839	0.18	0.33	0.08	0.06	0.44
A22	9	35	28	167	257	12	12	33	17	386	817	0.17	0.99	0.09	0.04	0.47
A29	9	40	29	125	415	6	12	47	19	349	940	0.23	0.53	0.13	0.05	0.37
A34		30	31	126	521	7	12	27	45	340	823	0.25	0.58	0.08	0.13	0.41
A36	6	35	14	208	257	20	9	40	26	305	845	0.07	2.18	0.13	0.08	0.36
A37	7	36	21	200	382	15	10	50	50	279	888	0.1	1.39	0.18	0.18	0.31
A39	5	33	25	351	396	12	10	43	42	438	912	0.07	1.24	0.1	0.1	0.48
A46	11	32	26	225	171	16	11	20	53	377	814	0.12	1.44	0.05	0.14	0.46
A47	8	41	26	283	198	11	11	43	39	295	817	0.09	1	0.15	0.13	0.36
A50	5	30	18	218	356	11	9	30	27	290	842	0.08	1.2	0.1	0.09	0.35
A51	2	39	28	276	468	12	10	47	24	310	817	0.1	1.23	0.15	0.08	0.38

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Evaluating the predictive power of biomarkers using library-based assignment

Cas7 was the only biomarker that achieved 100% rate of correct assignment (RCA) in both autistic and control groups. GST was the next best with 91% overall RCA. Consistent with our ROC analysis results, there was no consistent benefit gained by combining biomarker pairs in

Table 2. Estimating the predictive power of variables using the area under a receiver operating characteristic curve (AUC). The *p* value indicates asymptotic significance with the null hypothesis being that the true AUC is equal to 0.5. The far-right column shows whether the variable is elevated or decreased in autistic patients (ASD) compared to healthy controls. PC1: first principal component in a principal component analysis used as a multivariate biomarker profile. The number of individual biomarkers used in each profile is indicated. The 9 biomarkers (K, Na, LDH, GLTN, GST, CK, CoQ10, Cas7, and MLTN); 7 biomarkers (K, Na, LDH, GSH, CK, CoQ10, and MLTN); 5 ratios (K:Na, GST:GLTN, CK:Cas7, CoQ10: Cas7, and Cas7:MLTN); 9 biomarkers and 5 ratios; or K, GST, and Cas7.

Variable	AUC	*p* value	In ASD
Potassium (K)	0.813	0.001923	Decreased
Sodium (Na)	0.71	0.037175	Elevated
K:Na ratio	0.803	0.002643	Decreased
Lactate dehydrogenase (LDH)	0.758	0.010436	Elevated
Glutathione (GSH)	0.756	0.010923	Decreased
Glutathione S-transferase (GST)	0.973	0.000003	Elevated
GST:GSH ratio	0.91	0.000047	Decreased
Creatine kinase (CK)	0.716	0.031757	Elevated
Co-enzyme Q10 (CoQ10)	0.744	0.015611	Elevated
Caspase 7 (Cas7)	1	<0.000001	Elevated
Melatonin (MLTN)	0.739	0.01778	Elevated
CK:Cas7 ratio	0.893	0.000097	Decreased
CoQ10: Cas7 ratio	0.798	0.003089	Decreased
Cas7:MLTN ratio	1	<0.000001	Elevated
PC1 9 biomarkers	1	<0.000001	Elevated
PC1 7 biomarkers	0.936	0.000015	Elevated
PC1 5 ratios	1	<0.000001	Elevated
PC1 9 biomarkers + 5 ratios	1	<0.000001	Elevated

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ratios. For example, like Cas7 alone, Cas7:melatonin (MLTN) ratio yielded a 100% overall RCA, but creatine kinase (CK):Cas7 and co-enzyme Q10 (CoQ10):Cas7 ratios had overall RCAs of 80% and 69%, respectively. Furthermore, potassium (K) and sodium (Na) had slightly lower RCAs than that of the K:Na ratio in the control group, but K had an equal RCA to that of K:Na ratio in the autistic group. Using biomarker profiles, however, increased the RCA to 100% independently of whether the profiles contained 9 biomarkers, 5 ratios, or both (Fig 6).

Discussion

Neurological disorders are known to induce alterations in concentrations, regulation ratios, and total profiles of different metabolites or biomarkers that could be used to diagnose or distinguish different diseases. Metabolic ratios between concentration levels of related metabolites have been used to describe different biological states in human populations. Taking into account all the heterogeneous etiological mechanisms of ASD, it is reasonable that biomarker ratios together with biomarker profile hold the potential to be more discriminatory than assessing any of the individual biomarkers alone [31–33].

In neurodevelopmental disorders such as ASD, early disease detection is a crucial step in patient care. Therefore, avoiding delayed diagnosis is essential but the absence of sensitive and specific biomarkers makes ASD very challenging [8]. Various classes of protein biomarkers in blood plasma, especially early in life, are promising tools for early detection of ASD. Among the repeatedly etiological mechanisms leading to ASD is mitochondrial dysfunction. Combining prospective biomarkers and targeted intervention strategies in clinical trials for ASD offers a promising method for controlling the heterogeneity of enrolled participants, which may increase the power of studies to identify favorable effects of intervention while also improving our understanding of this disorder [34].

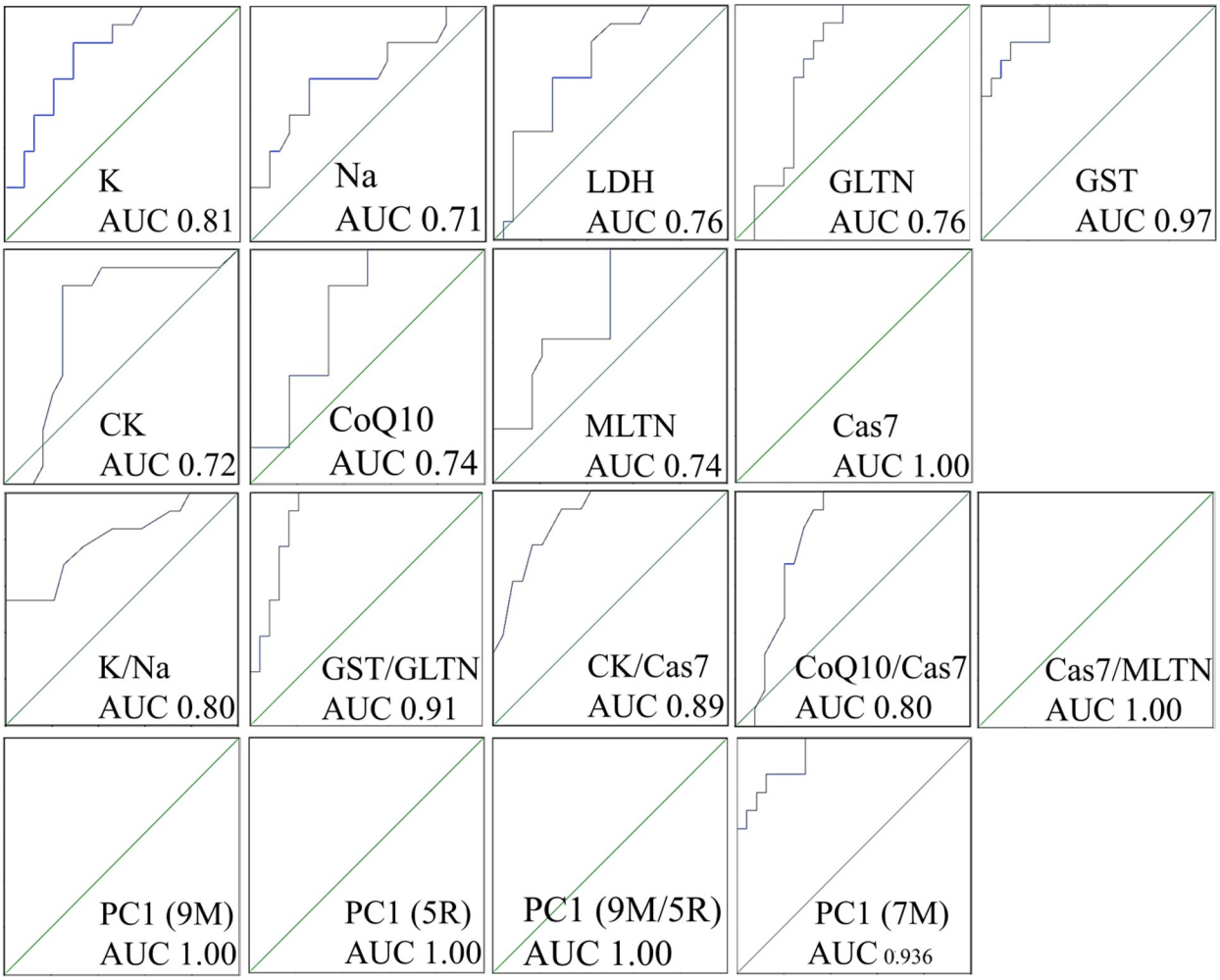

Fig 5. Receiver operating characteristic analysis to evaluate the predictive power of individual and multivariate combined biomarkers using the area under a receiver operating characteristic curve (AUC) method. 9M: 9-biomarkers (K, Na, LDH, GLTN, GST, CK, CoQ10, Cas7, and MLTN); 5R: 5 ratios (K:Na, GST:GLTN, CK:Cas7, CoQ10:Cas7, and Cas7:MLTN); 9M/5R: 9 biomarkers and 5 ratios; and 7M: 7 biomarkers (K, Na, LDH, GSH, CK, CoQ10, and MLTN). PC1: first principal component in a principal component analysis used as a multivariate biomarker profile.

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In the present study, in spite of the heterogeneity of the data of the selected variables, Fig 1 presents high significant differences between patients with autism and control participants for the 9 absolute and the 5 relative variables, which are all directly or indirectly related to mitochondria function.

This study uses PCA and clustering methodology to measure the role of mitochondrial dysfunction—related variables in discriminating between individuals with ASD and matched control participants. The data give a valued addition to the biomarker field by providing a unique shift from an absolute to a relative perspective in understanding and relating mitochondrial dysfunction to ASD. Fig 2 shows the appropriateness of both PCA and MDA in separating autistic patients from controls, using nine biomarkers, five biomarker ratios, or a combination profile.

A ratio was created with K^+ to Na^+, as these ions are part of the Na^+/K^+ ion pump (ATPase), a component of the mitochondria respiratory chain known to be negatively correlated with lipid peroxides as marker of oxidative stress, another etiological mechanism in ASD [35–36].

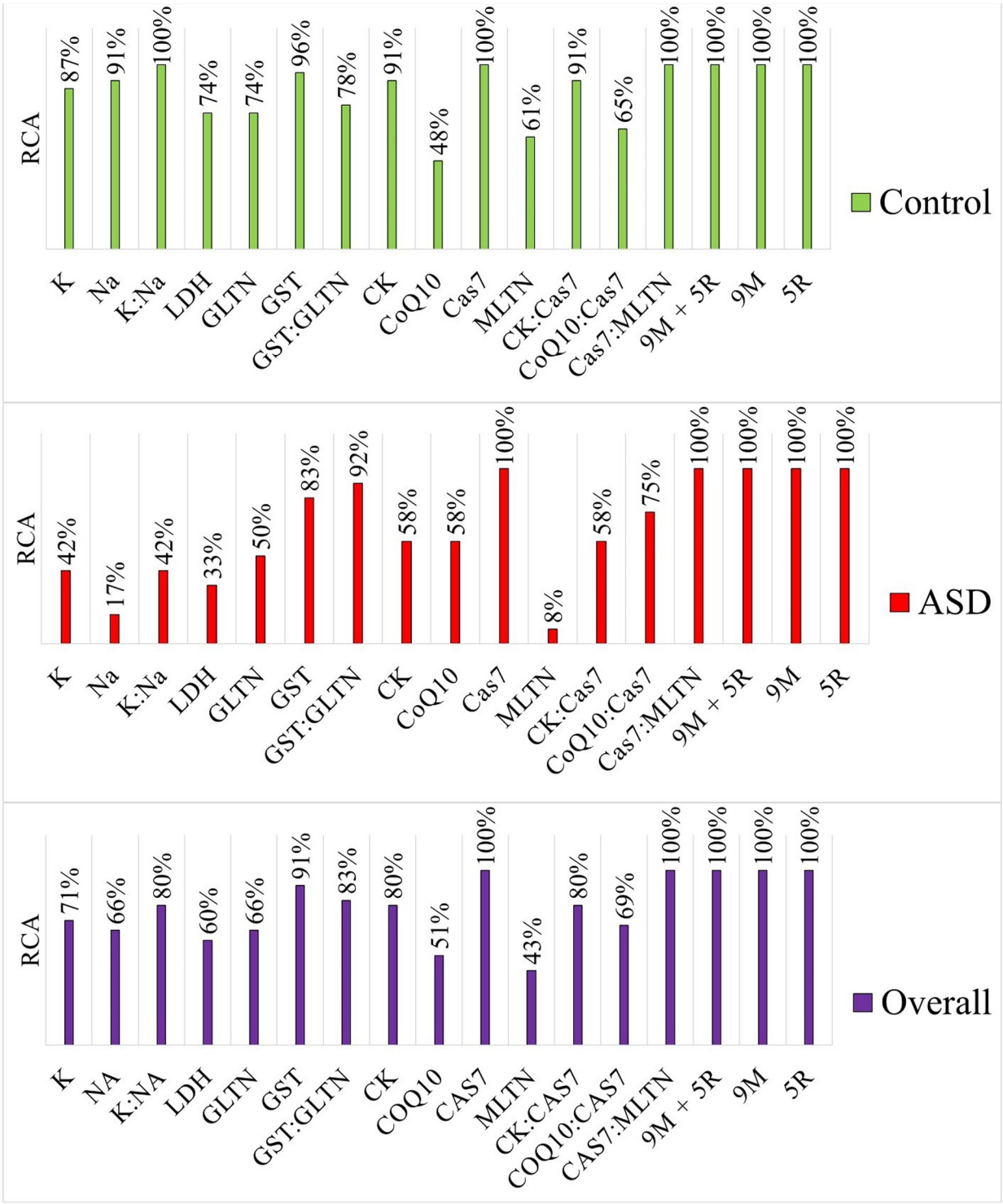

Fig 6. Estimating the predictive power of variables using library-based assignment. A library containing 12–13* autistic and 23–24* healthy participants was used for identification. K: potassium, Na: sodium, LDH: lactate dehydrogenase, GLTN: glutathione, GST: glutathione S-transferase, CK: creatine kinase, CoQ10: co-enzyme Q10, Cas7: caspase 7, MLTN: melatonin, RCA: rate of correct assignment, and ASD: autism spectrum disorder. *To identify any given participant, the participant was removed from the library and then submitted as unknown. Accordingly, autistic participants were identified against a library of 12 autistic and 24 control participants, while control participants were identified using a library of 13 autistic and 23 healthy participants.

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Mitochondria as organelles lack the ability to synthesize reduced glutathione (GSH), use numerous antioxidants to scavenge free radicals and be protected against oxidative stress. This highlights the critical role of GSH mitochondrial import carriers for normal function [37–38]. In case of GSH depletion, the vulnerability of mitochondria to oxidative stress is increased and mitochondrial dysfunction occurs [38]. In the present study, the significantly lower GSH:GST ratio in autistic patients compared to controls suggests the role of GSH and GST, as non-enzymatic and enzymatic antioxidants respectively, in mitochondrial dysfunction, which may underlie the etiological mechanism of ASD. This can find support in the present study of Faber et al [39] in which they reported much higher total glutathione and much lower glutathione status (GSH/GSSG) in patients with ASD due to chronic exposure to environmental toxins.

Creatine is partially synthesized in mitochondria by creatine kinase (CK), which provides the energy buffer to sustain cellular energy homeostasis [40, 41]. The brain, as a high-energy demand organ, is rich in creatine and has a large number of mitochondria. Under mitochondrial dysfunctional stress, creatine synthesis and utilization are usually disturbed, with creatine possibly cleared in the blood. The remarkably higher plasma CK and lower CK:Cas7 ratio presented in Fig 1 and used for the PCA (Fig 2) can help to suggest the role of mitochondrial dysfunction in apoptosis as another etiological mechanism of ASD presented in the present work by caspase 7. This explanation can find support in the recent work of Castora [24] which prove that, in ASD, there are often deficits in respiratory chain complexes that can reduce ATP generation and produce increased levels of reactive oxygen species (ROS) which activates the mitochondrial permeability transition pore (mPTP) and the release of cytochrome c, prompting apoptosis.

In PCA, observed variables are replaced by artificial variables (principal components). The goal is to condense observed variables into fewer PCs that account for as much variance as possible. This results in a model drawn in a new set of coordinates, the PCs, where observed variables contribute to each PC. There are a couple of problems with this manipulation: 1) in the total absence of correlation between all observed variables, there is no way to condense them into fewer ones in any meaningful way, and 2) some level of uncertainty and lack of confidence is created unless we have a way to evaluate the model. The first problem is addressed by Bartlett's test of sphericity, which computes a p-value representing the probability of a total lack of correlation in the dataset to be analyzed. We show that this was not an issue in our study, given the very low p-values obtained. PCA is not meaningful with large p-values. The second problem is addressed by Monte Carlo simulation, which iteratively demonstrates the reliability of each PC by generating an eigenvalue at the 50^{th} and 95^{th} percentile levels of confidence. Any meaningful PC's actual eigenvalue should exceed the 50^{th} percentile eigenvalue generated by the iteration process, but a "good" PC should also exceed the 95^{th} percentile iterative eigenvalue. In our data, PC1 exceeded the 95^{th} percentile eigenvalue in all experiments. In addition, PC1 was the most differential PC between autistic and control subjects. We must mention, however, that according to KMO test of sampling adequacy, a larger sample size was most probably needed for our analysis. On the other hand, consistency between the results of PCA, MDS, and hierarchical clustering in showing the unmistakable efficiency of our biomarker profile in differentiating between autistic and control subjects, led us to conclude that our biomarker profile is at least highly promising.

It is well known that CoQ10 is essential for supporting mitochondrial functions such as shuttling electrons, serving as a potent antioxidant, and working as an electron transport chain to generate ATP [42]. In spite of the elevated level of CoQ10 in the plasma of autistic children, the remarkably lower value of CoQ10:Cas 7 (Table 1) can provide biochemical proof for a mitochondrial role in the pathogenesis of ASD [24,43].

The significantly higher Cas7:MLTN in individuals with ASD compared to control, in spite of the significant increase of plasma melatonin, can be explained on the basis that through the disrupted blood brain barrier (BBB) in ASD, melatonin can passively pass from the brain to blood. As high levels of ventricular fluid melatonin are critically needed to protect ventricular-contacting, neural tissue against oxidative stress, efflux of melatonin from brain to blood through the disrupted BBB can be easily related to apoptosis, which occurs in these active neuronal populations.

This might explain the high predictive value of MLTN, Cas 7, and Cas:MLTN, with AUCs of 0.739, 1.0, and 1.0 respectively [44,45]. This can be supported through considering the work of Braam et al [45] which shows a possible relationship between low melatonin metabolism and ASD clinical presentation.

In conclusion, the present study helps to better understand the etiology of ASD, on the basis of the profile of the studied combined biomarkers, which present oxidative stress, energy metabolism, mitochondrial dysfunction, and apoptosis as possible etio-pathological mechanisms. This would enable integration of highly predictive disease biomarkers with existing knowledge and hypothetically provide further awareness on the impaired biological pathways. The availability of improved predictive power by combining biomarkers into profiles that can be measured using simple, non-invasive procedures would be beneficial for better recognition of the biological pathways altered in ASD and could be used for an early diagnosis of and early intervention for this neurodevelopmental disorder [13, 46].

Supporting information

S1 Data. Raw data- PLOS one.
(XLSX)

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