Mitochondrial Dysfunction in Autism

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A UTISM SPECTRUM DISORDERS (ASD) are characterized by impaired social interaction, problems with verbal and nonverbal communication, and repetitive or severely restricted activities and interests.[1] Limited scientific advances have been made regarding the causes of autism, with general agreement that both genetic and environmental factors contribute to this disorder.

Some reports have suggested that mitochondrial dysfunction and altered energy metabolism may influence the social and cognitive deficits in autism[2-4]; however, most reports involved only 1 or a few isolated cases.[2,5] A school-based study of 69 children aged 10 to 14 years with confirmed autism or ASD found mitochondrial respiratory chain dysfunction in 7.2%.[4] Considering the limitations of elevated lactate-to-pyruvate ratios and the bias toward skeletal muscle disturbances in the study sample, some cases were likely overlooked, and the incidence in the affected population could actually be higher than reported. To our knowledge, there are no systematic studies aimed at investigating changes in mitochondrial function, mitochondrial DNA (mtDNA) copy number, mtDNA deletions, and oxidative stress in chil-

Context Impaired mitochondrial function may influence processes highly dependent on energy, such as neurodevelopment, and contribute to autism. No studies have evaluated mitochondrial dysfunction and mitochondrial DNA (mtDNA) abnormalities in a well-defined population of children with autism.

Objective To evaluate mitochondrial defects in children with autism.

Design, Setting, and Patients Observational study using data collected from patients aged 2 to 5 years who were a subset of children participating in the Childhood Autism Risk From Genes and Environment study in California, which is a population-based, case-control investigation with confirmed autism cases and age-matched, genetically unrelated, typically developing controls, that was launched in 2003 and is still ongoing. Mitochondrial dysfunction and mtDNA abnormalities were evaluated in lymphocytes from 10 children with autism and 10 controls.

Main Outcome Measures Oxidative phosphorylation capacity, mtDNA copy number and deletions, mitochondrial rate of hydrogen peroxide production, and plasma lactate and pyruvate.

Results The reduced nicotinamide adenine dinucleotide (NADH) oxidase activity (normalized to citrate synthase activity) in lymphocytic mitochondria from children with autism was significantly lower compared with controls (mean, 4.4 [95% confidence interval {CI}, 2.8-6.0] vs 12 [95% CI, 8-16], respectively; $P = .001$). The majority of children with autism (6 of 10) had complex I activity below control range values. Higher plasma pyruvate levels were found in children with autism compared with controls (0.23 mM [95% CI, 0.15-0.31 mM] vs 0.08 mM [95% CI, 0.04-0.12 mM], respectively; $P = .02$). Eight of 10 cases had higher pyruvate levels but only 2 cases had higher lactate levels compared with controls. These results were consistent with the lower pyruvate dehydrogenase activity observed in children with autism compared with controls (1.0 [95% CI, 0.6-1.4] $nmol \times [min \times mg\ protein]^{-1}$ vs 2.3 [95% CI, 1.7-2.9] $nmol \times [min \times mg\ protein]^{-1}$, respectively; $P = .01$). Children with autism had higher mitochondrial rates of hydrogen peroxide production compared with controls (0.34 [95% CI, 0.26-0.42] $nmol \times [min \times mg\ of\ protein]^{-1}$ vs 0.16 [95% CI, 0.12-0.20] $nmol \times [min \times mg\ protein]^{-1}$ by complex III; $P = .02$). Mitochondrial DNA overreplication was found in 5 cases (mean ratio of mtDNA to nuclear DNA: 239 [95% CI, 217-239] vs 179 [95% CI, 165-193] in controls; $P = 10^{-4}$). Deletions at the segment of cytochrome *b* were observed in 2 cases (ratio of cytochrome *b* to *ND1*: 0.80 [95% CI, 0.68-0.92] vs 0.99 [95% CI, 0.93-1.05] for controls; $P = .01$).

Conclusion In this exploratory study, children with autism were more likely to have mitochondrial dysfunction, mtDNA overreplication, and mtDNA deletions than typically developing children.

JAMA. 2010;304(21):2389-2396 www.jama.com

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dren with autism who have been clinically well defined.

Mitochondrial function in disease often has been investigated in muscle biopsies[6]; however, the detection of mitochondrial defects in readily available cells from body fluids (lymphocytes and platelets[7]) would be valuable under the assumption that many such defects may not be confined to muscle or the brain, tissues in which mitochondrial diseases are most strongly evident. Considering that the energy requirement for lymphocytes is derived almost equally from glycolysis and oxidative phosphorylation,[8] we tested the hypothesis that children with full syndrome autism have dysfunctional mitochondria in peripheral blood lymphocytes.

METHODS

This study was conducted using a subset of children from the Childhood Autism Risks From Genetics and Environment (CHARGE) study. The focus of the CHARGE study is on modifiable factors in autism etiology and markers of biological dysregulation that may provide mechanistic clues. The recruited children were aged 2 to 5 years and resided with a biological parent in a well-defined catchment area of more than 22 counties in northern California and parts of Los Angeles County, California. Between 2003 and 2010, cases were recruited through the California Department of Developmental Services system, Medical Investigations of Neurodevelopmental Disorders Institute clinics, referrals by clinicians, and self-referrals.

General population controls were sampled from birth files with frequency matching to the projected distribution of sex, age, and geographic area among cases of autism. Environmental, lifestyle, reproductive, maternal medical, and detailed demographic information was collected through an extensive telephone interview with the primary caregiver. Participants' mothers classified parents into race and ethnicity categories identical to those used in the US Census and these same categories were used to define child race and ethnicity.

Diagnoses were confirmed through clinical examinations using the Autism Diagnostic Inventory-Revised (ADI-R)[9] and the Autism Diagnostic Observation Schedule (ADOS).[10] The ADI-R provides a standardized, semistructured interview and a diagnostic algorithm for the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition, Text Revision) and the *International Statistical Classification of Diseases, 10th Revision* definitions of autism.[11] The ADOS is a semistructured, standardized assessment in which the researcher observes the social interaction, communication, play, and imaginative use of materials for children suspected of having autism or ASD.

A final CHARGE study diagnosis of autism was defined as meeting criteria on the communication, social, and repetitive behavior domains of the ADI-R and scoring at or above the cutoff for autistic disorder on the ADOS (module 1, 2, or 3). The Social Communication Questionnaire was used to screen for ASD among those recruited as general population controls. Children who scored above the screening cutoff were fully assessed using the ADI-R and the ADOS. The cognitive and adaptive functions of all children were evaluated with, respectively, the Mullen Scales of Early Learning[12] and the Vineland Adaptive Behavior Scales[13]; and children from the general population scoring at 71 or above on both of these scales who did not have an ASD qualified as typically developing controls. The child's medical history was taken and a developmental-behavioral pediatrician conducted an examination for physical or neurological abnormalities.

Further details on the CHARGE study protocols have been published.[14] The study protocol follows the ethical guidelines of the Declaration of Helsinki[15] and was approved by the institutional review board of the University of California, Davis, School of Medicine. All participants enrolled in the study had written informed consent provided by a parent.

For this study, we selected 10 individuals who met criteria for full syndrome autism on both the ADI-R and ADOS and 10 typically developing control children. Children were recruited consecutively and examined during the time windows when our laboratory staff was available to conduct assays of mitochondrial function; this was necessary to ensure fresh biological specimens. Scheduling of examinations in the clinic is essentially random, depending only on a match between the parent's availability and a clinic slot, with all children being seen by clinical staff having identical expertise. Because ASD represents a fairly diverse phenotype, we studied children meeting criteria for full syndrome autism and neurotypically developing children (controls) without a clinical diagnosis of full syndrome autism, ASD, or developmental delays.

We also attempted to achieve comparable age, sex, and race/ethnicity across groups to minimize confounding sociodemographic factors that may be surrogates for genetic, epigenetic, or cultural background. Consent already had been obtained for these children to participate in a further follow-up at the Medical Investigations of Neurodevelopmental Disorders Institute with a blood draw. To minimize storage time of samples on ice and prevent travel-induced artifact, it was predetermined that the children live within an hour drive of the laboratory where all of the mitochondrial functional studies would be performed. All of the children were genetically unrelated, both within and between diagnostic groups. Prior to obtaining the additional blood draw and associated data, families were administered another informed consent for this substudy.

Given that mitochondrial myopathies are generally accompanied by increases in the plasma lactate and in the lactate-to-pyruvate ratio,[16] the glycemia, pyruvate, and lactate levels in plasma were evaluated in all participants (eMethods at http://www.jama.com).

Mitochondrial-dependent oxygen consumption was measured in freshly isolated lymphocytes permeabilized with digitonin. These biological samples

behaved in an analogous fashion to isolated mitochondrial preparations, but had the advantage that experiments could be performed without large numbers of cells. Oxygen uptake by lymphocytic mitochondria was performed in the presence of substrates linked to either reduced nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide and inhibitors to test the fidelity of various segments of the electron transport chain (eTable 1 at http://www.jama.com).

Individual complex and pyruvate dehydrogenase complex (PDHC) activities were evaluated by spectrophotometric assays and normalized to citrate synthase. Mitochondrial dysfunction can often be separated from the simple decrease in numbers by normalization of the respiratory flux to the activity of citrate synthase,[16] which is thought to be relatively stable and proportional to the mitochondrial numbers. Further experimental details are included in eMethods at http://www.jama.com.

Considering that some types of mitochondrial defects are accompanied by increases in hydrogen peroxide production,[17] this rate was evaluated in lymphocytic mitochondria from all participants (eMethods has further details).

A mitochondrion harbors 2 to 10 copies of mtDNA.[18] This copy number can be modulated according to the energy needs of the cell with changing physiological conditions, but not necessarily coupled with mitochondrial proliferation.[19] For instance, increases in mtDNA copy number or mtDNA overreplication (without increases in the oxidative phosphorylation capacity) are observed in cells in response to oxidative stress.[20] The mtDNA copy number was evaluated by the ratio of mtDNA to nuclear DNA. Essentially, 3 mitochondrial genes (cytochrome *b* [*CYTB*; GenBank NC_012920 gi: 4519], NADH dehydrogenase 1 [*ND1*; GenBank NC_012920 gi:4535], and NADH dehydrogenase 4 [*ND4*; GenBank NC_012920 gi:4538]) evaluated by quantitative polymerase chain reaction were normalized by a single-copy

nuclear gene (either pyruvate kinase or amyloid β A4 precursor protein) in lymphocytes and granulocytes to assess whether the changes in mtDNA copy number were cell-specific.

Overreplication was defined as mean values of the ratio of mtDNA to nuclear DNA that were significantly higher and different from the mean control values. The majority of mtDNA deletions involve the major arc of the mitochondrial genome between the origin of the heavy strand replication (nucleotides 110-441) and the origin of the light strand replication (nucleotides 5721-5798). In the majority of patients with single and multiple deletions, the *ND4* and/or *CYTB* genes are deleted, whereas the *ND1* gene is rarely deleted[21]; therefore, we evaluated the gene copy number ratios of *ND4* to *ND1* and *CYTB* to *ND1* with dual-labeled probes to detect mtDNA deletions. Deletions of mtDNA were considered if the mean ratios of *ND4* to *ND1* or *CYTB* to *ND1* were significantly lower than the mean ratios of the controls. All other experimental details are included in eMethods.

An a priori analysis to compute the required sample size based on the difference between 2 independent means (input parameters: $\alpha = .05$; power = 0.95; effect size = 0.50; allocation ratio: $n_2/n_1 = 1$) was performed by using the G* power software version 3.0.10. Three different scenarios were used to compute the sample size based on NADH oxidase, succinate oxidase, and PDHC activities (eMethods). To obtain a power level higher than 0.992, 3 to 8 individuals per group were required. Given the limitations of the power analyses when using small sample sizes, we chose to recruit 10 individuals per group. Experiments were run in triplicate and repeated at least 2 times in independent experiments unless noted otherwise. Summary statistics are expressed as mean (95% confidence intervals [CIs]), whereas individual data are expressed as mean (SD). The data were evaluated using the 2-tailed *t* test using StatSimple version 2.0.5 (Nidus Technologies, Toronto, Ontario, Canada), and a *P* value of .05 or less was

considered statistically significant. The 99% CIs in the tables were used to define a reference range for the individual data rather than for statistical comparisons.

RESULTS

Demographic and clinical data for the larger CHARGE study population were comparable with those for the subset used in this study, except that the 10 cases with autism were somewhat higher functioning than the cases with autism from the original CHARGE study sample; therefore, the case-control differences in cognitive and adaptive scores were somewhat attenuated (**TABLE 1**). Cases and controls had similar age, sex, and ethnicity, although controls were more likely to be nonwhite. Controls had typical development, which by definition meant they scored better on cognitive and adaptive tests.

Mitochondrial-dependent oxygen consumption was impaired in peripheral blood lymphocytes from children with autism compared with control children. The NADH oxidase activities (normalized to citrate synthase) in samples from children with autism and controls were 4.4 (95% CI, 2.8-6.0) and 12 (95% CI, 8-16), respectively (*P* = .001; **TABLE 2**). However, the values for succinate oxidase, cytochrome *c* oxidase, and adenosine triphosphatase activities in children with autism were not different from the control values (Table 2). Using the control values to define a reference range (set at 99% CI) for the individual data, low NADH oxidase activity was the most common deficiency (8 of 10), followed by succinate oxidase (6 of 10), adenosine triphosphatase or complex V (4 of 10), and complex IV or cytochrome *c* oxidase (3 of 10) (Table 2).

To ascertain the sites of the mitochondrial defects reflected as low oxygen uptake of respiratory substrates, activity of each individual complex was assessed (eTable 1), which revealed other electron transport chain deficiencies (Table 2 and eTable 2). Based on polarographic and enzymatic assays, the most common deficiency was found at

Table 1. Demographic and Clinical Characteristics of CHARGE Study Participants and CHARGE Subset

	CHARGE Study				CHARGE Subset				P Value
	Autism (n = 364)		Control (n = 289)[a]		Autism (n = 10)		Control (n = 10)		
Maternal education, No. (%)									
<Bachelor's degree	198	(54.6)	137	(47.4)	4	(40.0)	1	(10.0)	
≥Bachelor's degree	165	(45.4)	152	(52.6)	6	(60.0)	9	(90.0)	.02[b]
Child's sex, No. (%)									
Male	316	(86.8)	234	(81.0)	9	(90.0)	9	(90.0)	
Female	48	(13.2)	55	(19.0)	1	(10.0)	1	(10.0)	
Child's birth year, No. (%)									
1998-2001	183	(50.3)	68	(23.5)	4	(40.0)	0		
2002-2005	171	(47.0)	217	(75.1)	6	(60.0)	10	(100.0)	
2006-2007	10	(2.8)	4	(1.4)	0		0		
Child's ethnicity and race, No. (%)									
White, non-Hispanic	183	(50.3)	146	(50.5)	9	(90.0)	4	(40.0)	.03[c]
Nonwhite, non-Hispanic	75	(20.6)	56	(19.4)	0		4	(40.0)	
Hispanic	106	(29.1)	87	(30.1)	1	(10.0)	2	(20.0)	
	No.	Mean (SD)	No.	Mean (SD)	No.	Mean (SD)	No.	Mean (SD)	
Age[d]									
Maternal, y	364	34.9 (5.5)	289	34.6 (5.8)	10	37.1 (3.3)	10	37.5 (5.0)	
Paternal, y	356	37.5 (6.5)	286	37.1 (7.0)	10	40.1 (3.1)	10	41.1 (6.9)	.03[c]
Child, mo	364	43.9 (9.5)	289	42.3 (9.5)	10	45.0 (7.7)	10	46.0 (5.8)	
ADI-R[e]									
Social interaction (scores: 0-30)	364	19 (5)			10	17 (5)			
Verbal communication (scores: 0-26)	149	16 (4)			7	15 (2)			
Nonverbal communication (scores: 0-14)	215	11 (2)			3	11 (1)			
Stereotyped behaviors (scores: 0-12)	364	6 (2)			10	7 (1)			
ADOS[e]									
Module 1									
Communication (scores: 0-10)	258	6 (1)			3	5 (1)			
Social interaction (scores: 0-14)	258	11 (2)			3	9 (2)			
Module 2									
Communication (scores: 0-10)	104	6 (1)			7	6 (1)			
Social interaction (scores: 0-14)	104	9 (2)			7	9 (1)			
Module 3									
Communication (scores: 0-8)	2	2 (<1)			0				
Social interaction (scores: 0-14)	2	9 (1)			0				
Mullen Scales of Early Learning[f]									
Visual reception (scores: 20-80)	361	28 (13)	289	56 (11)	10	37 (17)	10	60 (8)	.03[c]
Fine motor (scores: 20-80)	360	27 (11)	289	53 (13)	10	35 (18)	10	53 (16)	.005[c]
Receptive language (scores: 20-80)	361	26 (11)	289	52 (11)	10	31 (13)	10	55 (14)	
Expressive language (scores: 20-80)	361	26 (10)	289	52 (11)	10	31 (10)	10	52 (13)	
Composite (scores: 49-155)	360	59 (17)	289	106 (18)	10	71 (21)	10	110 (20)	.03[c]
SCQ (scores: 0-39)[e]			283	3 (3)			10	3 (3)	
Vineland Adaptive Behavior Scales (scores: 20-160)[f]									
Communication	360	66 (18)	288	105 (13)	10	82 (22)	10	107 (14)	.002[c]
Daily living skills	360	65 (14)	288	100 (15)	10	75 (14)	10	94 (14)	.02[c]
Socialization	360	67 (13)	288	104 (13)	10	76 (14)	10	99 (10)	.02[c]
Motor skills	360	76 (19)	288	106 (15)	10	85 (17)	10	105 (17)	
Composite	360	64 (14)	288	105 (15)	10	76 (18)	10	101 (16)	.01[c]

Abbreviations: ADI-R, Autism Diagnostic Inventory-Revised; ADOS, Autism Diagnostic Observation Schedule; CHARGE, Childhood Autism Risks From Genetics and Environment; SCQ, Social Communication Questionnaire.
[a] Includes 1 control patient who scored 112 total on the Mullen Scales of Early Development for whom the Vineland Adaptive Behavior Scales were not completed.
[b] Comparison is between control patients in the CHARGE study and control patients in the CHARGE subset.
[c] Comparison is between patients with autism in the CHARGE study and patients with autism in the CHARGE subset.
[d] At entry into the CHARGE study.
[e] Higher score represents greater impairment.
[f] Higher score represents better function.

the level of complex I (6 of 10), mainly in combination with complex V (4 of 10), followed by complex IV or complex III only (1 of 10 each).

No statistically significant differences were found in the mean values for glycemia or lactate level between cases and controls. However, mean plasma pyruvate level was significantly higher in children with autism (0.23 mM; 95% CI, 0.15-0.31 mM) compared with controls (0.08 mM; 95% CI, 0.04-0.12 mM) ($P=.02$), and as a consequence, the lactate-to-pyruvate ratio was also significantly lower (6 [95% CI, 4-8] and 12 [95% CI, 8-16], respectively; $P=.002$). Two of 10 children with autism had high lactate levels and 8 had high pyruvate levels. In children with autism, the mean PDHC activity was 1.0 (95% CI, 0.6-1.4) nmol $\times$ [min $\times$ mg of protein]$^{-1}$ compared with 2.3 (95% CI, 1.7-2.9) nmol $\times$ [min $\times$ mg of protein]$^{-1}$ in control children ($P=.01$; Table 2).

The rates of hydrogen peroxide production in lymphocytic mitochondria from children with autism were higher compared with controls at both complex I (0.15 [95% CI, 0.05-0.25] nmol of hydrogen peroxide $\times$ [min $\times$ mg of protein]$^{-1}$ vs 0.07 [95% CI, 0.03-0.11] nmol of hydrogen peroxide $\times$ [min $\times$ mg of protein]$^{-1}$, respectively; $P=.03$) and complex III (0.34 [95% CI, 0.26-0.42] nmol of hydrogen peroxide $\times$ [min $\times$ mg of protein]$^{-1}$ vs 0.16 [95% CI, 0.12-0.20] nmol of hydrogen peroxide $\times$ [min $\times$ mg of protein]$^{-1}$; $P=.02$). Thus, lymphocytic mitochondria in autism not only had a lower oxidative phosphorylation capacity, but also contributed to the overall increased cellular oxidative stress.

The mean mtDNA copy number in lymphocytes (measured as ratio of mtDNA to nuclear DNA) was not significantly different comparing cases

with controls, leading to a higher mean copy number of 239 (TABLE 3). However, 5 of 10 children with autism presented mtDNA overreplication leading to a higher mean value of 239 (95% CI, 217-239) compared with 179 (95% CI, 165-193) for control children ($P=10^{-4}$).

In the controls, the ratio of mtDNA to nuclear DNA in lymphocytes was evaluated in isolated granulocytes to assess whether the changes observed in lymphocytes were cell-specific. In the controls, the ratio of mtDNA to nuclear DNA in lymphocytes was 179 (95% CI, 165-193), which was 497% of mean granulocyte values (36 [95% CI, 1-73]; Table 3) (eTable 3 at http://www.jama.com), supporting the higher contribution to the total energy requirement of mitochondrial oxidative phosphorylation relative to glycolysis in lymphocytes compared with granulocytes.[8] A higher mean mtDNA copy number was obtained in granulocytes from chil-

Table 2. Mitochondrial Activities in Lymphocytes[a]

	Mean (SD)[b]					
	Oxidase			Adenosine Triphosphatase (n = 10)	Affected Complexes (n = 10)[d]	Pyruvate Dehydrogenase Complex Activity (n = 8), mean (SD)[b]
	NADH (n = 10)	Succinate (n = 10)[c]	Cytochrome c (n = 10)			
Autism, patient No.						
1	2.6 (0.3)[e]	2.6 (0.3)[e]	5.6 (0.4)	79 (9)	III	2.2 (0.3)
2	5.5 (0.6)[e]	6.4 (0.8)	8.5 (0.7)	71 (8)	I	0.7 (0.1)[e]
3	3.0 (0.3)[e]	2.8 (0.3)[e]	11.6 (0.9)	45 (5)[e]	I, V	0.9 (0.1)[e]
4	4.6 (0.5)[e]	8 (1)	9.2 (0.7)	22 (2)[e]	I, V	NA
5	6.0 (0.6)[e]	3.8 (0.5)[e]	2.9 (0.2)[e]	22 (2)[e]	I, III, IV, V	0.37 (0.05)[e]
6	0.9 (0.1)[e]	2.1 (0.3)[e]	0.34 (0.05)[e]	407 (45)[f]	IV	0.8 (0.1)[e]
7	5.2 (0.5)[e]	2.2 (0.3)[e]	13 (1)	35 (4)[e]	I, V	NA
8	7.3 (0.7)	10 (1)[f]	8.3 (0.7)	97 (10)	None	0.5 (0.1)[e]
9	8.6 (0.9)	10 (1)[f]	30 (2)[f]	264 (30)[f]	None	0.6 (0.1)[e]
10	0.5 (0.1)[e]	1.2 (0.1)[e]	0.8 (0.1)[e]	68 (9)	I, III, IV	1.8 (0.2)
Patients with low activity, No. (%)	8 (80)	6 (60)	3 (30)	4 (40)	8 (80)	6 (75)
	Mean Activities (95% CI)					
Autistic	4.4 (2.8-6.0)[g]	5 (3-7)	9 (3-15)	111 (33-189)		1.0 (0.6-1.4)[h]
Control (n = 10)	12 (8-16)	7 (5-9)	9 (5-13)	147 (102-192)		2.3 (1.7-2.9)
Reference 99% CI[i]	7-17	4.4-9.6	3.3-14.9	68-225		1.4-3.2

Abbreviations: CI, confidence interval; NA, data not available; NADH, reduced nicotinamide adenine dinucleotide.
[a] All activities have been normalized to citrate synthase and multiplied by 100 except for pyruvate dehydrogenase complex activity, which is expressed as nmol $\times$ (min $\times$ mg of protein)$^{-1}$.
[b] Unless otherwise indicated.
[c] Only 1 set of data is shown because these values were not significantly different from those obtained with α-glycerophosphate.
[d] Established by evaluating the activity of each segment of the electron transport chain and then comparing them with the 99% CIs obtained with control values (eTable 2 at http://www.jama.com).
[e] Values were outside the lowest limit of the 99% CI.
[f] Values were above the highest limit of the 99% CI.
[g] $P=.001$ for comparison with controls.
[h] $P=.01$ for comparison with controls.
[i] Established with control values using the formula: CI at the .01 probability level=mean $\pm$ ($t_{0.01} \times$ standard error of the mean).

Table 3. Mitochondrial DNA (mtDNA) Copy Number and mtDNA Deletions in Lymphocytes[a]

	Ratio, Mean (SD)		
	mtDNA to nDNA[b]	ND4 to ND1	CYTB to ND1
Autism, patient No.			
1	160 (2)	0.94	0.97
2	267 (8)[c]	1.05	1.12
3	165 (3)	0.97	0.93
4	216 (6)[c]	0.95	0.84[c]
5	177 (1)	0.99	1.01
6	209 (3)[c]	0.98	0.86
7	250 (1)[c]	1.00	1.01
8	167 (3)	0.95	0.93
9	251 (18)[c]	0.86	0.76[c]
10	166 (2)	1.04	0.99
	Mean (95% CI)		
Autistic (n = 10)	186 (161-211)	0.97 (0.93-1.01)	0.94 (0.88-1.00)
Control (n = 10)	179 (165-193)	0.99 (0.81-1.17)	0.99 (0.89-1.09)
	Affected No./Total (%)		
Autistic	5/10 (50)	0/10	2/10 (20)
Control	2/10 (20)	0/10	0/10

Abbreviations: CI, confidence interval; CYTB, cytochrome b; ND1, reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase 1; ND4, NADH dehydrogenase 4; nDNA, nuclear DNA; PK, pyruvate kinase.
[a] The mtDNA copy number was evaluated by the ratio of mtDNA to nDNA, whereas the mtDNA deletions were based on the mitochondrial gene ratios of ND4 and CYTB to ND1.
[b] Calculated as the average of the 3 gene ratios (ND1 to PK, ND4 to PK, and CYTB to PK).
[c] Values were significantly different from control values and either above the mean ratio for mtDNA to nDNA ($P = 10^{-4}$) or below the mean for mitochondrial gene ratios ($P = .01$).

dren with autism vs controls (54 [95% CI, 24-87] vs 36 [95% CI, 1-73]; $P = .02$), indicating that the mtDNA overreplication was not cell-dependent (eTable 3).

Two of 10 children with autism presented deletions at the segment of cytochrome b (ratio of cytochrome b to ND1: 0.80 [95% CI, 0.68-0.92] vs 0.99 [95% CI, 0.93-1.05] for controls; $P = .01$), whereas there were no deletions at the segment encoded by ND4 (Table 3). All children with autism that had mtDNA deletions also had mtDNA overreplication.

COMMENT

Defective or abnormal lymphocytic mitochondria in children with autism were observed in this exploratory study as determined by the following parameters: (1) low PDHC activity accompanied by low lactate-to-pyruvate ratios, (2) impaired complex I alone or in combination with other complexes (mainly complex V), (3) enhanced mitochondrial rate of hydrogen peroxide production, and (4) mtDNA overreplication and/or deletions.

The high prevalence of mitochondrial dysfunction observed in this preliminary study performed with children presenting with full syndrome autism may or may not indicate an etiological role. Whether the mitochondrial dysfunction in children with autism is primary or secondary to an as-yet unknown event remains the subject of future work; however, mitochondrial dysfunction could greatly amplify and propagate brain dysfunction, such as that found in autism, given that the highest levels of mtDNA abnormalities are observed in postmitotic tissues with high energy demands (eg, brain).[22]

The higher levels of plasma pyruvate (and in some cases accompanied by higher levels of lactate) found in children with autism is consistent with the higher levels of alanine (the transamination product of pyruvate) and lactic acid reported by others.[5,23] Because plasma pyruvate and lactate-to-pyruvate ratios suggest PDHC deficiency,[24] we evaluated PDHC activity in children with autism and found it was

almost half of that found in the control children. Defects in PDHC lead to inadequate removal of pyruvate and lactate, resulting in insufficient energy production. Hence, PDHC deficiency could contribute to brain dysfunction in autism. Our experimental conditions could not provide information on whether the defect was primary (genetic) or secondary (oxidative modifications). The major cause of PDHC deficiency is mutations in the X-linked gene coding for the PDHA1 subunit[25]; thus, a defect in this gene could play a role in the 4:1 ratio of males to females with autism.[26] Other PDHC deficiencies are ascribed to oxidative modifications or dietary or hormonal imbalances.[24] Oxidative modifications of PDHC, and the ensuing inactivation elicited by oxidative or nitrative stress, seem consistent with the higher rate of hydrogen peroxide production and the low complex V activity found in children with autism, considering the high sensitivity of this complex to nitration and oxidation of key amino acid residues.[27]

The lactate-to-pyruvate ratio reflects the redox state of the cytosolic compartment,[28] such that a lactate-to-pyruvate ratio of 12 (as in controls) indicates a ratio of oxidized NADH to reduced NADH of 750:1 and a lactate-to-pyruvate ratio of 6 (as in autism) indicates a ratio of oxidized NADH to reduced NADH of 1500:1. This more oxidized cytosolic redox state in autism could favor anaerobic glycolysis over oxidative phosphorylation as a source of adenosine triphosphate.[28] Although skeletal muscle can tolerate this shift in metabolism, consequences for brain function could be devastating due to its heavy reliance on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes.[7,29]

The relative activities of the respiratory chain complexes are consistently conserved in functional mitochondria, allowing a tight balance between respiratory chain activities with various substrates while minimizing hydrogen peroxide production. Some complex activities were found above the control interval in 2 cases (Table 2 and

eTable 2 at http://www.jama.com). This might represent an attempt to compensate for the low PDHC activities (or other mitochondrial dysfunction), as has been observed in other mitochondrial disorders.[30]

Only 1 child with autism in this study fulfilled the diagnostic criteria for a definite mitochondrial respiratory chain disorder[16] (10% or 1 of 10 children). This percentage is similar to that reported by others (7.2%) despite using a different experimental design (skeletal muscle biopsies, high lactate-to-pyruvate ratio screening, participants aged 7-9 years with ASD[4]). The minimum prevalence for respiratory chain disorders with onset at any age[31] is 13 per 100 000 population, implying an expected incidence of a definite mitochondrial respiratory chain disorder in children with ASD (assuming that these 2 clinical conditions are unrelated or independent) of 0.013%, significantly lower than 7.2% or 10%. Thus, the observed incidence in children with autism could be 550 to 770 times higher than that of the general population. Alternatively, the reported population prevalence of mitochondrial disorders in children could be severely underestimated because the diverse clinical presentation[32] constitutes a challenge for clinicians to diagnose.

Differences in mtDNA parameters between control children and those with autism could stem from either higher oxidative stress[33] or inadequate removal of these harmful species.[34] The increased reactive oxygen species production observed in this exploratory study is consistent with the higher ratio of oxidized NADH to reduced glutathione in lymphoblastoid cells and mitochondria from children with ASD,[33] supporting the concept that these cells from children with autism present higher oxidative stress. Increased reactive oxygen species production induced by dysfunctional mitochondria could elicit chronic oxidative stress that enhances mtDNA replication, and possibly mtDNA repair. The higher mtDNA copy number could represent a compensatory mechanism to increase the number of wild-type mtDNA templates and maintain normal levels of mitochondrial transcripts.[35] Alternatively, these mtDNA abnormalities could result from defective replication and/or repair of mtDNA of primary (genetic[36]) or secondary (oxidative damage to single base pairs inflicted by free radicals[37]) origins. Collectively, these results suggest that cumulative damage and oxidative stress over time may (through reduced capacity to generate functional mitochondria) influence the onset or severity of autism and its comorbid symptoms.

This study has some limitations. First, the number of individuals in which mitochondrial activities were assessed was relatively small, although the sample size (based on statistical power for discovery of effects) was adequate. Nevertheless, caution should be exercised with regard to the generalization of findings in a larger population. Second, the possibility of type I errors should be considered. Of the analytes reported in Table 2, 8 of 10 participants were significantly outside the 99% CI of the mean for controls for NADH oxidase, as were 8 of 10 for succinate oxidase, 6 of 8 for PDHC activity, 6 of 10 for adenosine triphosphatase, and 4 of 10 for cytochrome *c* oxidase. Such findings are highly unlikely to occur by chance alone, although the interpretation of findings with both higher and lower activities is more complex than when values are consistently in one direction. Similarly, for the mtDNA analyses, 5 of 10 participants showed significantly higher copy numbers than controls, although the ratio of *CYTB* to *ND1* was significantly reduced in 2 of 10 participants. Ten group comparisons were made in this study; of these comparisons, 6 were significant at the α level of .05 and 2 were significant at the α level of .005 (ie, using the conservative Bonferroni correction).

Third, the cases in this substudy were somewhat higher functioning than those from the original study, lessening the difference with controls. We can speculate that this could have attenuated any differences relative to the original population, but further work in a larger sample would be needed to determine the association, if any, between cognitive and adaptive function and the results on mitochondrial dysfunction reported herein. This is important because typical development (the control group) is defined herein not just as absence of an ASD, but also absence of deficits in cognitive and adaptive skills. Fourth, the differences observed between our cases and controls could represent confounding effects due to possible associations between mitochondrial dysfunction and race, which also differed by case-control status. Fifth, none of the children in this study had been previously diagnosed with a genetic syndrome or had any indications of genetic syndromes as determined by developmental pediatricians at the Medical Investigations of Neurodevelopmental Disorders Institute. Nevertheless, defects (other than deletions) in genes (other than those tested) could have been present in these samples as recently reported in another study of ASD.[38]

Sixth, inferences about a cause and effect association between mitochondrial dysfunction and typical autism cannot be made in a cross-sectional study. Several factors influence expression of mitochondrial respiratory insufficiencies in both the affected and general populations (ie, nuclear genetic backgrounds,[39] mtDNA heteroplasmy in different tissues,[40] different energy thresholds within a given tissue or organ,[41] and environmental factors[42]). Nevertheless, our exploratory study suggests that mitochondrial defects in children with autism may be more common than in controls.

CONCLUSIONS

In this preliminary study, evidence of mitochondrial dysfunction was observed in children presenting with full syndrome autism. More research is needed to understand the molecular causes of the mitochondrial dysfunction and how this and other neurometabolic defects may contribute to autism or related phenotypes.[43]

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