J Autism Dev Disord
DOI 10.1007/s10803-006-0138-6

BRIEF COMMUNICATION

Brief Report: High Frequency of Biochemical Markers for Mitochondrial Dysfunction in Autism: No Association with the Mitochondrial Aspartate/Glutamate Carrier *SLC25A12* Gene

Catarina Correia · Ana M. Coutinho · Luísa Diogo · Manuela Grazina · Carla Marques · Teresa Miguel · Assunção Ataíde · Joana Almeida · Luís Borges · Catarina Oliveira · Guiomar Oliveira · Astrid M. Vicente

Abstract In the present study we confirm the previously reported high frequency of biochemical markers of mitochondrial dysfunction, namely hyperlactacidemia and increased lactate/pyruvate ratio, in a significant fraction of 210 autistic patients. We further examine the involvement of the mitochondrial aspartate/glutamate carrier gene (*SLC25A12*) in mitochondrial dysfunction associated with autism. We found no evidence of association of the *SLC25A12* gene with lactate and lactate/pyruvate distributions or with autism in 241 nuclear families with one affected individual. We conclude that while mitochondrial dysfunction may be one of the most common medical conditions associated with autism, variation at the *SLC25A12* gene does not explain the high frequency of mitochondrial dysfunction markers and is not associated with autism in this sample of autistic patients.

C. Correia · A. M. Coutinho · A. M. Vicente
Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6, 2781 Oeiras, Portugal

L. Diogo · C. Marques · J. Almeida · L. Borges · G. Oliveira
Centro de Desenvolvimento da Criança, Hospital Pediátrico de Coimbra, Coimbra, Portugal

M. Grazina · C. Oliveira
Centro de Neurociências e Biologia Celular, Coimbra, Portugal

T. Miguel · A. Ataíde
Direcção Regional de Educação da Região Centro, Coimbra, Portugal

A. M. Vicente (✉)
Instituto Nacional de Saúde Dr. Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisboa, Portugal
e-mail: astrid.vicente@insa.min-saude.pt

Keywords Autism · Mitochondrial Dysfunction · Lactate/Pyruvate Ratio · *SLC25A12* Gene · Genetic Association

Introduction

Autism is a behavioural syndrome characterized by deficits in social interaction, impaired communication and restricted and stereotyped behaviours (Folstein & Rosen-Sheidley, 2001). Although the etiology of autism is unclear, familiar aggregation of the disease and other disorders of the spectrum indicate a strong genetic contribution.

A growing number of studies, including detection of brain metabolism abnormalities and hyperlactacidemia, imply a disturbance of brain energy metabolism in autistic patients, which might be a result of mitochondrial oxidative phosphorylation dysfunction in neuronal cells (Lombard, 1998; Chugani, Sundram, Behen, Lee, & Moore, 1999; Graf et al., 2000; Pons et al., 2004). A number of cases of mitochondrial respiratory chain (MRC) disorders have been described associated with autism, including a child with autism and documented MRC complex IV deficiency by Lászlo, Horvath, Eck, and Fekete (1994), an autistic boy and his sister with Leigh syndrome with MRC complex IV defect and a mitochondrial DNA (mtDNA) G8363A mutation (Graf et al., 2000), two autistic children with 15q inverted duplication, mitochondrial hyperproliferation and respiratory complex III defect (Filipek et al., 2003) and five patients with autism and mtDNA mutations or mtDNA deletion (Pons et al., 2004). In a previous population-based study of autistic children in Portugal (Oliveira et al., 2005), we have found hyperlactacidemia, a biochemical marker of mitochondrial dysfunction, in

20.3% of 69 tested patients. Mitochondrial functional studies confirmed the diagnosis of mitochondrial disease in 5 of these patients (7.2%), a surprisingly high frequency in autism. While the mitochondrial and nuclear genomes exert a dual genetic control on the biogenesis and maintenance of mitochondrial function, in our patients mtDNA mutations were not detected, suggesting the involvement of nuclear genes encoding proteins involved in mitochondrial function (Oliveira et al., 2005).

Recently, a strong association of two polymorphisms within the *SLC25A12* gene, rs2056202 and rs2292813, with autism has been reported (Ramoz et al., 2004). The *SLC25A12* gene maps to the 2q24-q33 region, where linkage with autism has previously been found. This gene spreads over about 110 kb and is expressed primarily as 2.9 and 3.2 kb mRNA species, which encode a 678 amino-acid protein that is a calcium-dependent aspartate/glutamate mitochondrial carrier (AGC1). AGC1 plays an important role in the malate/aspartate shuttle, regulating the cytosolic redox state that controls conversion of lactate to pyruvate (Palmieri et al., 2001). We therefore examined whether, in autistic patients, this gene influences an intermediate biochemical phenotype characteristic of mitochondrial dysfunction, defined by plasma lactate levels and lactate/pyruvate ratio distribution, and hence might play a role in the etiology of autism. For this purpose we tested the association of two *SLC25A12* single nucleotide polymorphisms (SNP), rs11757 and rs2056202, with plasma lactate levels, lactate/pyruvate ratio, with autism and with autism associated with mitochondrial dysfunction, in a population sample of 241 nuclear families with one affected individual. While an association of rs2056202 with autism was reported by Ramoz et al., rs11757, located in the *SLC25A12* gene's regulatory region, was not tested in that previous study.

Methods

241 autistic nuclear families were recruited at Hospital Pediátrico de Coimbra (HP). Patients were diagnosed as previously described (Oliveira et al., 2005) and only idiopathic subjects and patients with mitochondrial disease were included in the study. Controls were healthy adult blood donors.

Plasma lactacte and pyruvate levels were measured enzymaticaly (Vassault, Bonnefont, Specola, & Saudubray, 1991). Measurement of muscle respiratory chain complex activities in isolated deltoid muscle mitochondria and mtDNA study were performed as previously described (Oliveira et al., 2005). The clinical/laboratorial classification of these patients was according to the revision by Bernier et al. (2002) of current diagnostic criteria in adults for application to paediatric age. Mental retardation and hyperlactacidemia were considered minor criteria. Enzymatic complex activity (expressed as a ratio to citrate synthase) less than 20% and between 20–30% of the normal mean in muscle were major and minor criteria, respectively. MRC disorder was considered *definite* if two major or one major plus two minor criteria were present, *probable* if one major plus one minor or at least three minor criteria were present, and *possible* if one major or two minor criteria (one of which must be laboratorial) were present.

The Ethical Committee at the HP approved the collection of data and biological specimens from patients for research purposes, and all participants signed an informed consent.

Two SNPs at the *SLC25A12* gene were tested: rs11757 in the 5′UTR region and rs2056202 in intron 3–4. PCR conditions in 12.5 μl were: 25 ng gDNA, 200μM dNTP, 2 mM $MgCl_2$, Taq polymerase, 0.4 μM primers (F: 5′-GGAAAGCCACTATGAACATG-3′;R: 5′-TATTGATTATTGCCCAGTTT-3′ for rs2056202; F: 5′-TGATAAAAGGCATAACGAGA-3′;R: 5′-TGAAGGGAAATTGTGAAAGT-3′ for rs11757), 30 cycles of 30″/94°C, 30″/46°C, 30″/72°C, followed by 10 min 72°C. PCR products for rs2056202 were digested with *HpyCH4*IV (New England Biolabs) and for rs11757 with *Mae*III (Roche) and separated on 2.5% agarose gel.

Linkage disequilibrium was tested using the family-based association test (FBAT). Haplotypes were analysed using TRANSMIT. Genotypic and allele frequencies between groups were compared using a chi-square test. Association with the distribution of plasma lactate levels and pyruvate/lactate ratio was tested with the Kruskal–Wallis nonparametric test. Linkage disequilibrium between the two markers was assessed by Lewontin's D', using the GOLD program.

Results

Plasma lactacte and pyruvate levels were measured in 210 subjects from this sample. Of 210 patients, 36 (17.2%) had hyperlactacidemia and 53 out of 192 patients (27.6%) had elevated lactate/pyruvate ratio. No association of either polymorphism or their haplotypes was found with lactate level distribution (rs11757: $H(2, N = 197) = 1.813$, $P = 0.404$; rs2056202: $H(2, N = 199) = 1.261$, $P = 0.5323$; haplotypes: $H(6, N = 192) = 3.867$, $P = 0.6947$) or with lactate/pyruvate plasma ratios (rs11757:$H(2, N = 183) = 2.725$, $P = 0.2561$; rs2056202: $H(2, N = 183) = 0.259$, $P = 0.8784$; haplotypes: $H(6, N = 177) = 3.986$, $P = 0.6786$). The four possible haplotypes were found in our population, and the two markers are located far apart in the

chromosome (71,44 kb). However, they were found to be in strong linkage disequilibrium ($D' = 0.832$).

From the group of 36 patients with hyperlactacidemia, 20 have been fully assessed for mitochondrial disease, and in seven the diagnosis has been confirmed. Allele and genotype frequencies for the two markers and their haplotypes were compared between: autistic patients with mitochondrial disease, autistic patients with no evidence of mitochondrial dysfunction (no hyperlactacidemia or high lactate/pyruvate ratios), autistic individuals with hyperlactacidemia or high lactate/pyruvate ratio, and controls. No significant differences in allele or genotype frequencies were found between any of these groups (Table 1).

In 241 nuclear families no transmission disequilibrium was found for either *SLC25A12* marker alleles (FBAT: $Z = 0.873$, $P = 0.3827$ for rs2056202; $Z = 0.75$, $P = 0.4535$ for rs11757) or haplotypes of the two (TRANSMIT: $\chi 2 = 2.875$, df = 3, $P = 0.4113$).

Discussion

Our results in this population sample suggest that mitochondrial disease may be one of the most common medical conditions associated with autism. Genetic variation at the *SLC25A12* gene, however, did not explain the high frequency of mitochondrial dysfunction markers in our patients, such as hyperlactacidemia and high lactate/pyruvate ratio. Because hyperlactacidemia and increased lactate/pyruvate ratio may be due to one of several inherited metabolic defects of gluconeogenesis, pyruvate oxidation, the Krebs cycle or the respiratory chain, it is possible that multiple genes influence the observed changes in lactate and pyruvate levels, and that genetic heterogeneity underlies mitochondrial dysfunction associated with autism. Supporting this hypothesis is the fact that, even in the limited number of patients with confirmed mitochondrial disease in our study, we find deficiencies in more than one respiratory chain complexes (Oliveira et al., 2005). The findings also do not corroborate the recently described association of autism with the *SLC25A12* gene (Ramoz et al., 2004). Again, this discrepancy may reflect the expected genetic heterogeneity in autism indicating that, if *SLC25A12* variants indeed play a role in autism, a different physiologic mechanism may be involved. It would be interesting to find out if biochemical markers of mitochondrial dysfunction are present in the population where association with *SLC25A12* has been found. The results therefore do not necessarily dismiss *SLC25A12* gene as a susceptibility factor, and analysis in additional populations will be required for a definite conclusion.

In our population, any deleterious variants of *SLC25A12* associated with autism are not frequently represented, either in the total sample or in the subgroups of autistic patients with biochemical markers of mitochondrial dysfunction or confirmed mitochondrial disease. Analysis of other nuclear genes encoding mitochondrial proteins will be required to understand the molecular basis of autism associated with mitochondrial dysfunction.

Table 1 (**A**) Allele frequencies of rs11757, rs2056202 and haplotypes in controls and in autistic patients' subgroups; (**B**) Results of the comparison of allelic frequencies between different subgroups: Subgroup 1: Autistic Patients with mitochondrial disease; Subgroup 2: Autistic Patients with normal lactate levels; Subgroup 3: Autistic Patients with hyperlactacidemia; Subgroup 4: Autistic Patients with high lactate/pyruvate ratio; Subgroup 5: Autistic Patients with normal lactate/pyruvate ratio

(A)

Population		Controls	Subgroup 1	Subgroup 2	Subgroup 3	Subgroup 4	Subgroup 5
Markers/Alleles							
rs11757	G	$N = 60$ (67%)	$N = 11$ (92%)	$N = 243$ (72%)	$N = 48$ (73%)	$N = 70$ (70%)	$N = 185$ (69.5%)
	C	$N = 30$ (33%)	$N = 1$ (8%)	$N = 95$ (28%)	$N = 18$ (27%)	$N = 30$ (30%)	$N = 81$ (30.5%)
rs2056202	C	$N = 80$ (87%)	$N = 10$ (100%)	$N = 282$ (87%)	$N = 54$ (87%)	$N = 87$ (87%)	$N = 234$ (88%)
	T	$N = 12$ (13%)	$N = 0$ (0%)	$N = 42$ (13%)	$N = 8$ (13%)	$N = 13$ (13%)	$N = 32$ (12%)
Haplotypes rs11757/ rs2056202	GC	$N = 48$ (59%)	$N = 9$ (90%)	$N = 197$ (58.6%)	$N = 36$ (62%)	$N = 54$ (57.4%)	$N = 151$ (58%)
	CC	$N = 26$ (31%)	$N = 1$ (1%)	$N = 95$ (28.3%)	$N = 15$ (26%)	$N = 28$ (29.8%)	$N = 79$ (30.4%)
	GT	$N = 8$ (10%)	$N = 0$ (0%)	$N = 43$ (12.8%)	$N = 7$ (12%)	$N = 12$ (12.8%)	$N = 29$ (11.2%)
	CT	$N = 0$ (0 %)	$N = 0$ (0%)	$N = 1$ (0.3%)	$N = 0$ (0%)	$N = 0$ (0%)	$N = 1$ (0.4%)

(B)

	Controls vs Subgroup 1	Subgroup 1 vs Subgroup 2	Subgroup 2 vs Subgroup 3	Subgroup 4 vs Subgroup 5	Subgroup 5 vs Subgroup 1
rs11757	$\chi^2 = 2.058$; $P = 0.151$	$\chi^2 = 1.391$; $P = 0.238$	$\chi^2 < 0.001$; $P = 0.991$	$\chi^2 = 0.002$; $P = 0.965$	$\chi^2 = 1.742$; $P = 0.187$
rs2056202	$\chi^2 = 0.489$; $P = 0.484$	$\chi^2 = 0.538$; $P = 0.484$	$\chi^2 = 0.037$; $P = 0.847$	$\chi^2 = 0.005$; $P = 0.942$	$\chi^2 = 0.440$; $P = 0.507$
Haplotypes	$\chi^2 = 3.831$; $P = 0.147$	$\chi^2 = 4.083$; $P = 0.339$	$\chi^2 = 0.395$; $P = 1$	$\chi^2 = 0.529$; $P = 1$	$\chi^2 = 4.16$; $P = 0.328$

Acknowledgments This work was supported by the Fundação Calouste Gulbenkian and by the Fundação para a Ciência e Tecnologia (POCTI/39636/ESP/2001). C. Correia and AMCoutinho are supported by fellowships from the Fundação para a Ciência e Tecnologia (SFRH/BD/16907/2004 and SFRH/BD/3145/2000).

References

Bernier, F. P., Boneh, A., Dennett, X., Chow, C. W., Cleary, M. A., & Thorburn, D. R. (2002). Diagnostic criteria for respiratory chain disorders in adults and children. *Neurology, 59*(9), 1406–1411.

Chugani, D. C, Sundram, B. S., Behen, M., Lee, M., & Moore, G. J. (1999). Evidence of altered energy metabolism in autistic children. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 23*, 635–641.

Filipek, A. P., Juranek, J., Smith, M., Mays, Z. L., Ramos, R. E., Bocian, M., Masser-Frye, D., Laulhere, M. T., Mdahl, C., Spence, A., & Gargus, J. J. (2003). Mitochondrial dysfunction in autistic patients with 15q inverted duplication. *Annals of Neurology, 53*, 801–804.

Folstein, S. E., & Rosen-Sheidley, B. (2001). Genetics of autism: Complex aetiology for a heterogeneous disorder. *Nature Reviews/Genetics, 2*, 943–955.

Graf, W. D., Marin-Garcia, J., Gao, H. G., Pizzo, S., Nariaux, R. K., Markusic, D., Barshop, B. A., Courchesne, E., & Haas, R. H. (2000). Autism associated with mitochondrial DNA G8363A transfer RNA (Lys) mutation. *Journal of Child Neurology, 15*, 357–361.

Laszlo, A., Horvath, E., Eck, E., Fekete, M. (1994). Serum serotonin, lactate and pyruvate levels in infantile autistic children. *Clinica Chimica Acta, 229*(1–2), 205–207.

Lombard, J. (1998). Autism: a mitochondrial disorder? *Medical Hypotheses, 50*, 497–500.

Oliveira, G., Diogo, L., Grazina, M., Garcia, P., Borges, L., Vicente, A. M., & Oliveira, C. R. (2005). Mitochondrial dysfunction in autism spectrum disorders. *Developmental Medicine and Child Neurology, 47*(3), 185–189.

Palmieri, L., Pardo, B., Lasorsa, F.M., del Arco, A., Kobayashi, K., Iijima, M., Runswick, M. J., Walker, J. E., Saheki, T., Satrustegui, J., & Palmieri, F. (2001). Citrin and aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria. *The EMBO Journal, 20*(18), 5060–5069.

Pons, R., Andreu, A. L., Checcarelli, N., Vila, M. R., Engelstad, K., Sue, C. M., Shungu, D., Haggerty, R., de Vivo, D. C., & DiMauro, S. (2004). Mitochondrial DNA abnormalities and autistic spectrum disorders. *Journal of Pediatrics, 144*, 81–85.

Ramoz, N., Reichert, J. G., Smith, C. J., Silverman, J. M., Bespalova, I. N., Davis, K. L., & Buxbaum J. D. (2004). Linkage and association of the mitochondrial aspartate/glutamate carrier *SLC25A12* gene with autism. *The American Journal of Psychiatry, 161*, 662–669.

Vassault, A., Bonnefont, J. P., Specola, N., & Saudubray, J. M. (1991). Lactate, pyruvate and ketones bodies. In F. A. Hommes, (Ed.), *Techniques in diagnostic human biochemical genetics: A laboratory manual* (pp. 285–308). New York: Wiley-Liss.