

One carbon metabolism disturbances and the C677T MTHFR gene polymorphism in children with autism spectrum disorders

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Received: June 3, 2008; Accepted: August 1, 2008

Abstract

Autism spectrum disorders (ASDs), which include the prototypic autistic disorder (AD), Asperger's syndrome (AS) and pervasive developmental disorders not otherwise specified (PDD-NOS), are complex neurodevelopmental conditions of unknown aetiology. The current study investigated the metabolites in the methionine cycle, the transsulphuration pathway, folate, vitamin B₁₂ and the C677T polymorphism of the MTHFR gene in three groups of children diagnosed with AD ($n = 15$), AS ($n = 5$) and PDD-NOS ($n = 19$) and their age- and sex-matched controls ($n = 25$). No metabolic disturbances were seen in the AS patients, while in the AD and PDD-NOS groups, lower plasma levels of methionine ($P = 0.01$ and $P = 0.03$, respectively) and α -aminobutyrate were observed ($P = 0.01$ and $P = 0.001$, respectively). Only in the AD group, plasma cysteine ($P = 0.02$) and total blood glutathione ($P = 0.02$) were found to be reduced. Although there was a trend towards lower levels of serine, glycine, N, N-dimethylglycine in AD patients, the plasma levels of these metabolites as well as the levels of homocysteine and cystathionine were not statistically different in any of the ASDs groups. The serum levels of vitamin B₁₂ and folate were in the normal range. The results of the MTHFR gene analysis showed a normal distribution of the C677T polymorphism in children with ASDs, but the frequency of the 677T allele was slightly more prevalent in AD patients. Our study indicates a possible role for the alterations in one carbon metabolism in the pathophysiology of ASDs and provides, for the first time, preliminary evidence for metabolic and genetic differences between clinical subtypes of ASDs.

Keywords: autism • methionine cycle • transsulphuration • vitamins • MTHFR

Introduction

Autism spectrum disorders (ASDs) form a heterogeneous group of neurodevelopmental disorders defined behaviourally by three core disturbances: marked deficits in interpersonal social interaction, disrupted verbal and non-verbal communication, and restricted repetitive and stereotyped patterns of behaviour and interests [1, 2]. The ASD phenotype includes the classical or typical autistic

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disorder (AD), Asperger syndrome (AS) characterized by no general delay in language or cognitive development and pervasive developmental disorders not otherwise specified (PDD-NOS), which is a milder condition that includes some, but not all, of the symptoms associated with classic autism. Once considered a rare clinical entity, autism is now considered common, with the most recent prevalence estimate around 1 in 150 [3, 4]. In the scientific literature, there is varying support for a wide spectrum of hypotheses regarding the causes of autism: from studies showing that genes play a greater role in the risk for autism than in any other common neuropsychiatric disorder [5] to studies implicating disruptive environmental factors during neurodevelopment in genetically susceptible individuals [6]. Nevertheless, it is becoming increasingly obvious that a single cause or unifying theory is unlikely to account for what is now better referred to as 'the autisms' [7, 8]. What is both intriguing and frustrating for researchers investigating the pathophysiological basis of this neurodevelopmental group of disorders is that no specific biomarker for autism has been identified yet in order to improve the reliability of behavioural diagnosis.

While inborn errors of metabolism can probably account for less than 5% of autistic individuals [9], it has recently been suggested that disturbances in one carbon metabolism (Fig. 1) are a frequently encountered feature of autism [10–19]. The metabolic disturbances have been suggested to contribute to the increased oxidative stress and impaired redox homeostasis that have been documented in autism [20], but also to a diminished capacity for methylation, with consequences on gene expression, neurotransmitter synthesis and, potentially, on neuronal synchronization [16].

The current study further broadens the metabolic and genetic dataset on the group of ASD patients by assessing one carbon metabolites in plasma, folate and vitamin B₁₂ in serum, and the C677T polymorphism in the MTHFR gene in three groups of children diagnosed with one of the ASD clinical subtypes (*i.e.* AD, PDD-NOS and AS) as compared to healthy age- and sex-matched controls. Our data suggest that the severity of these impairments vary across the clinical spectrum.

Materials and methods

Participants

The subjects enrolled in this study were 39 children with ASD. Each patient was examined by an experienced child neuropsychiatrist and assigned a diagnosis of AD ($n = 15$; 13 males, 2 females; mean age: 5.10 ± 0.45 years), PDD-NOS ($n = 19$; 13 males, 6 females; mean age: 8.83 ± 0.84 years) or AS ($n = 5$; 5 males; mean age: 9.23 ± 1.82 years) based on the criteria defined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IVR) [2]. Patients with childhood disintegrative disorder or rare syndromes displaying autistic features were excluded from participation. Patients in each of the three ASDs subgroups were matched by age and sex with healthy children from a pool of 25 individuals, as follows: 13 controls for the AD group, 22 controls for the PDD-NOS group and 8 controls for the AS group (see Table 1). For the genetic

analysis, additional subjects were recruited for genotyping for the control group for a total of 80 apparently healthy subjects. All participants in this study were Caucasians of Romanian origin. None of the children included in this study had taken vitamin supplements (containing folic acid, vitamin B₁₂ or vitamin B₆) in the last 6 months. Comparison subjects were drawn from the same geographical area as our patient groups, aiming to recover the same demographics of the region from where the patients were recruited. All controls were somatically and behaviourally healthy, had no past or present history of psychiatric disorder and none of them had ever taken medications for psychiatric conditions. Informed consent was obtained and the research protocol was in agreement with the Declaration of Helsinki of the World Medical Association.

Blood samples

Blood specimens were obtained after overnight fast. Samples (~6 ml in total) were withdrawn from a cubital vein into blood tubes (with heparin, EDTA or anticoagulant gel) and immediately stored on ice at 4°C. Plasma (for measuring metabolites) and serum (for measuring vitamins) were separated by centrifugation at 3000 rpm for 10 min and stored at -20°C until analysis. Samples of whole blood were also stored at -20°C for measuring total blood glutathione and isolating genomic DNA.

Plasma metabolites

Total homocysteine, methionine, total cysteine, cystathionine, serine, glycine, α -aminobutyrate, N-methylglycine and N, N-dimethylglycine were determined in plasma by stable isotope dilution capillary gas chromatography/mass spectrometry (GS-MS), as previously described by Stabler *et al.* [21, 22].

Serum vitamin B₁₂ and folate

Serum B₁₂ and folate concentrations were measured using a Roche Elecsys 2010 immunoassay analyser by an electrochemiluminescence immunoassay (ECLIA) using the Elecsys Vitamin B₁₂ reagent kit and the Elecsys Folate reagent kit (Roche Diagnostics GmbH, Mannheim, Germany). The assays were performed according to the supplier's recommendation.

Whole blood total glutathione

Total blood glutathione (tGSH) was measured in whole blood by isocratic RP-HPLC with precolumn derivatization and fluorimetric detection using the specific Chromsystems kit (Instruments and Chemical GmbH, München, Germany). The assay was performed according to the supplier's recommendation.

Genetic analysis

Genomic DNA was isolated from whole peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and was stored at -20°C until further analysis. The genetic polymorphism was determined by using the LightCycler Real-Time PCR technology based on fluorescence resonance energy

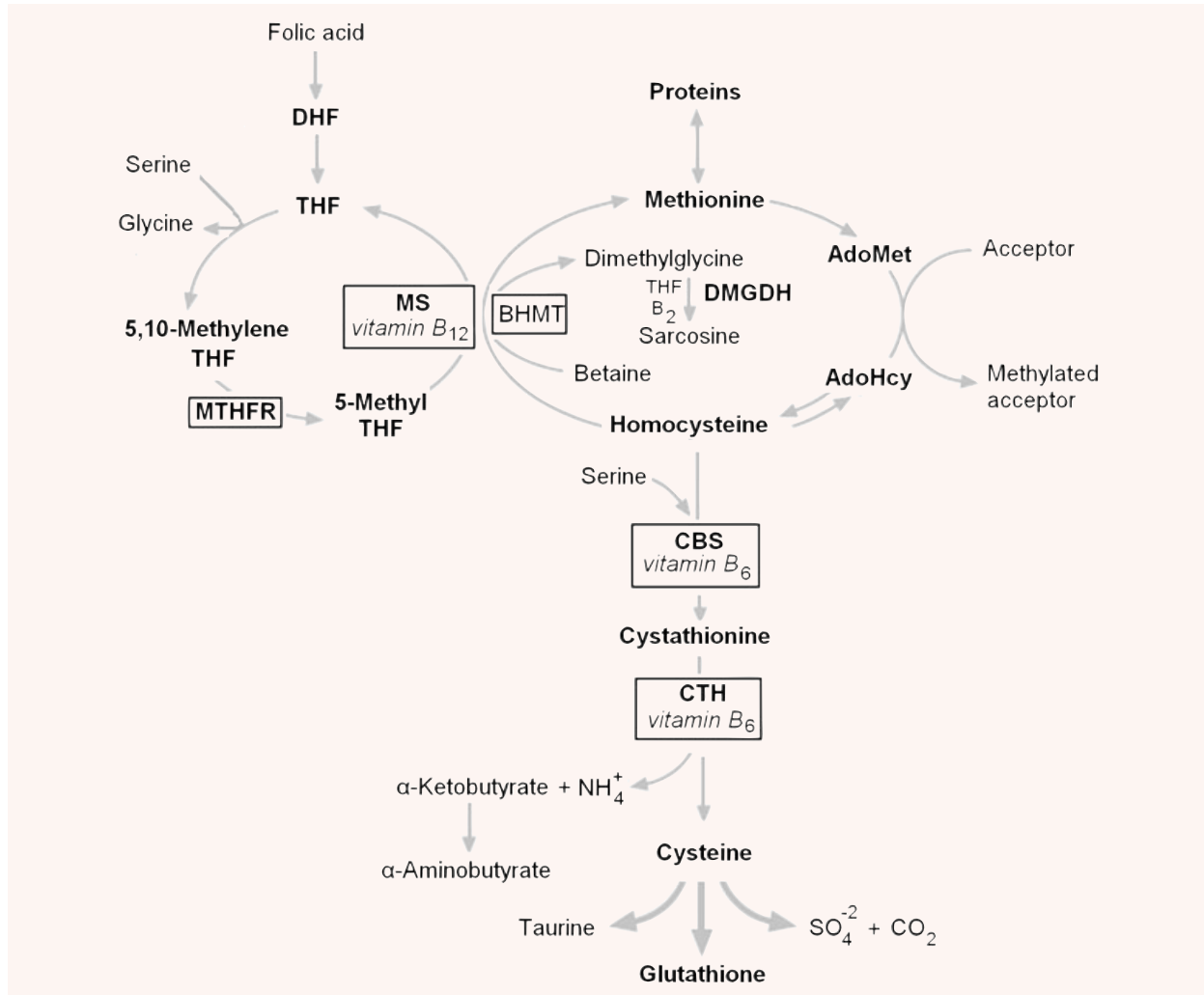


Fig. 1 Overview of the one carbon metabolism pathway. Abbreviations: S-adenosylhomocysteine (AdoHcy); S-adenosylmethionine (AdoMet); betaine homocysteine methyltransferase (BHMT); cystathionine β -synthase (CBS); cystathionine γ -lyase (CHT); dihydrofolate (DHF); dimethylglycine dehydrogenase (DMGDH); methionine synthase (MS), methylenetetrahydrofolate reductase (MTHFR); tetrahydrofolate (THF).

transfer. MTHFR C677T polymorphism analysis was performed according to the method described by Aslanidis *et al.* [23], using the LightCycler™ high-speed thermal cycler (Hoffmann-LaRoche, Basel, Switzerland) and the LightCycler™ DNA Master HybProbe kit (Roche Molecular Biochemicals, Mannheim, Germany). Melting curve analysis allowed identification of the MTHFR 677 polymorphism according to the following melting point temperatures: 63.1°C for the C allele and 55.2°C for the T allele.

Statistical analysis

The data are presented as means \pm standard error of the mean (S.E.M.). The normal distribution of continuous data was checked using the Kolmogorov-Smirnov test. For normally distributed variables, Student's

t-test was used for comparisons between the three ASD groups and their age- and sex-matched controls. When normality was not present and equal variance could not be assumed, the Mann-Whitney Rank-Sum Test was applied. Additionally, the effect size (Cohen's *d* based on sample size) for variables with a significant group mean difference was computed. Spearman's rho test was used to evaluate the correlation between different metabolites. For the polymorphism under study, genotypic and allelic frequencies were calculated. The distribution of genotypes in all groups was tested for deviation from the Hardy-Weinberg equilibrium. The Pearson's chi-square (χ^2) test and Fisher's exact test were applied to assess differences in the genotype and allelic (respectively) distribution between groups of patients and controls. A *P*-value of less than 0.05 was considered statistically significant. The SPSS software was used for performing the analysis.

Table 1 Characteristics of groups and biochemical results in patients with ASD and control subjects

	AD	Controls AD	P	PDD-NOS	Controls PDD-NOS	P	AS	Controls AS	P
Number of subjects	15	13		19	22		5	8	
Age (years)	5.10 ± 0.45	5.89 ± 0.61	0.31	8.83 ± 0.84	9.05 ± 0.91	0.96	9.23 ± 1.82	10.22 ± 1.05	0.65
Sex (male, %)	13 (86.7%)	8 (61.5%)	0.19	13 (68.4%)	13 (56.5%)	0.56	5 (100%)	8 (100%)	
Methionine (μM)*	20.69 ± 1.87	27.49 ± 2.01	0.01	23.62 ± 1.09	27.51 ± 1.27	0.03	22.70 ± 3.22	27.07 ± 1.38	0.26
α-aminobutyric acid (μM)*	11.14 ± 1.17	15.73 ± 1.37	0.01	10.73 ± 0.83	14.62 ± 0.74	0.001	21.72 ± 5.49	14.40 ± 1.37	0.26
Homocysteine (μM)*	5.19 ± 0.45	5.86 ± 0.39	0.27	6.49 ± 0.36	6.88 ± 0.52	0.54	5.90 ± 0.85	6.72 ± 0.43	0.36
Cysteine (μM)*	181.06 ± 6.42	204.76 ± 7.16	0.02	206.10 ± 5.93	205.95 ± 6.81	0.98	215.00 ± 15.81	209.50 ± 8.93	0.75
Cystathionine (nM)*	162.53 ± 16.56	160.84 ± 11.88	0.93	140.63 ± 9.30	149.00 ± 7.89	0.49	118.40 ± 13.33	155.25 ± 14.26	0.09
Serine (μM)*	99.46 ± 3.50	125.23 ± 13.12	0.08	113.31 ± 5.24	114.60 ± 8.15	0.90	96.20 ± 6.40	124.75 ± 17.35	0.16
Glycine (μM)*	184.20 ± 12.05	217.23 ± 14.55	0.09	207.94 ± 10.31	209.73 ± 9.02	0.89	188.60 ± 13.59	224.00 ± 17.61	0.14
Methylglycine (μM)*	5.59 ± 0.64	5.37 ± 0.96	0.84	4.16 ± 0.27	5.78 ± 0.62	0.08	4.85 ± 0.27	4.49 ± 0.90	0.77
Dimethylglycine (μM)*	4.76 ± 0.21	6.62 ± 1.25	0.17	5.48 ± 0.39	5.77 ± 0.72	0.73	6.48 ± 1.02	7.62 ± 1.92	0.67
Total Glutathione (μM) [#]	161.16 ± 10.68	242.67 ± 32.94	0.02	209.96 ± 21.71	213.32 ± 21.01	0.91	147.31 ± 33.21	171.29 ± 32.77	0.62
Vitamin B12 (pg/ml)**	747.09 ± 94.26	724.50 ± 140.51	0.89	608.50 ± 67.82	833.02 ± 127.34	0.13	536.04 ± 87.63	542.47 ± 175.57	0.98
Folate (ng/ml)**	10.45 ± 1.49	8.13 ± 0.68	0.17	7.98 ± 0.75	8.63 ± 0.68	0.53	13.51 ± 2.06	8.17 ± 0.91	0.06

Data are presented as mean ± standard error of the mean (S.E.M.);

*plasma, # whole blood, ** serum.

Results

Metabolic analyses

The results from metabolite measurements in plasma and serum are listed in Table 1 and graphically represented in Fig. 2. In both the AD and PDD-NOS groups plasma levels of methionine (20.69 ± 1.87 μM *versus* 27.49 ± 2.01 μM, $P = 0.01$, Cohen's $d = 0.91$, and 23.62 ±

1.09 μM *versus* 27.51 ± 1.27 μM, $P = 0.03$, Cohen's $d = 0.69$, respectively) and α-aminobutyric acid (11.14 ± 1.17 μM *versus* 15.73 ± 1.37 μM, $P = 0.01$ and 10.73 ± 0.83 μM *versus* 14.62 ± 0.74 μM, $P = 0.001$, respectively) were lower than in their age- and sex-matched controls, whereas plasma total homocysteine was in the normal range. It is worth noting that the decrease in methionine levels observed in AD was more severe than in PDD-NOS patients (~25% decrease *versus* ~14% decrease). Only in the AD group, plasma cysteine levels (181.06 ± 6.42 μM *versus*

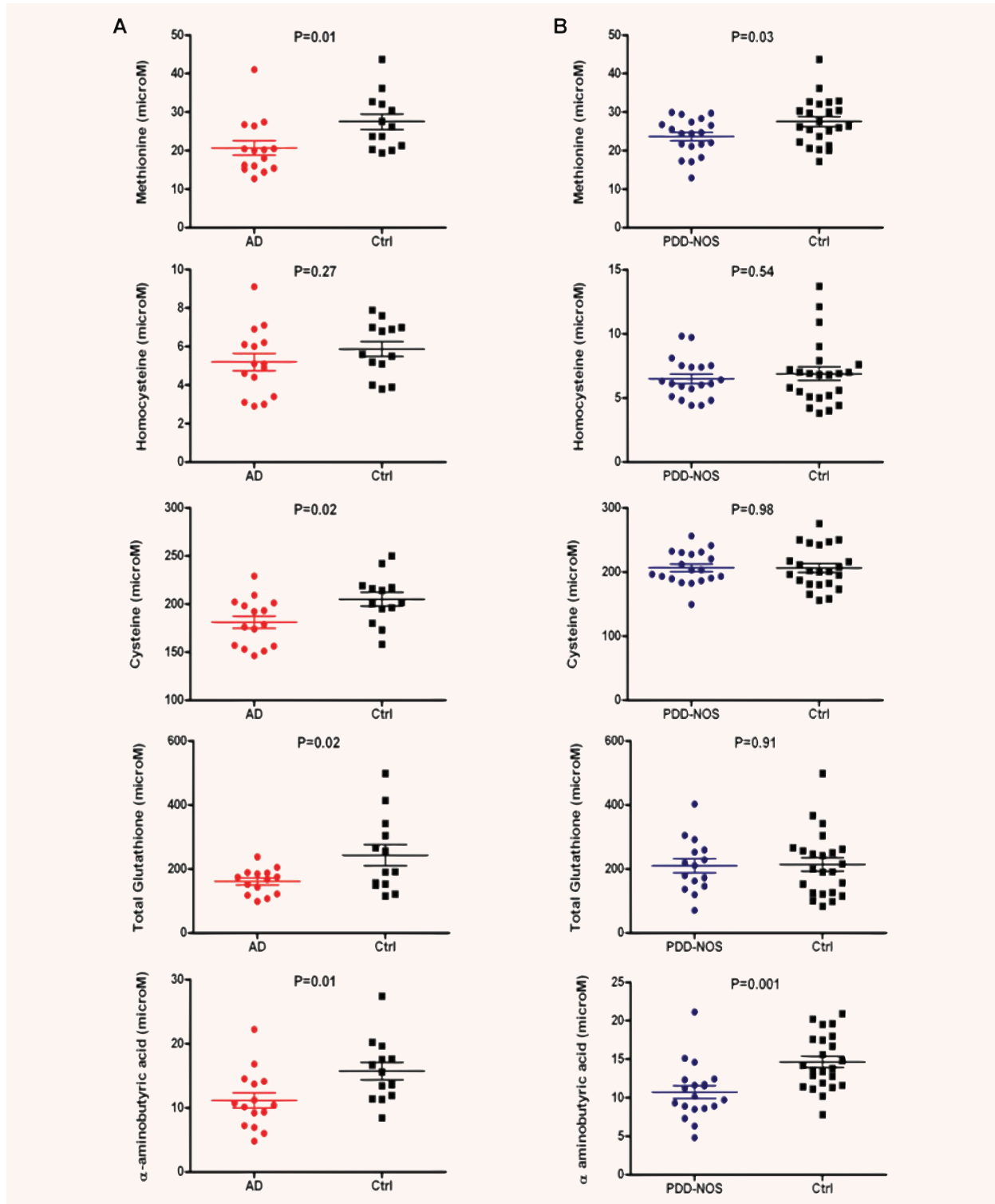


Fig. 2 Levels of methionine, homocysteine, cysteine, total glutathione and α -aminobutyrate in children with AD (A) and PDD-NOS (B) compared to controls.

Table 2 The MTHFR C677T polymorphism in patients with ASD and control subjects

	AD	Controls AD	<i>P</i>	PDD-NOS	Controls PDD-NOS	<i>P</i>	AS	Controls AS	<i>P</i>
Number of subjects	15	80		19	80		5	80	
MTHFR C677T									
CC	6 (40.0%)	46 (57.5%)	0.23	12	46 (57.5%)	0.88	3 (60%)	46 (57.5%)	0.81
CT	6 (40.0%)	28 (35.0%)		6	28 (35.0%)		2 (40%)	28 (35.0%)	
TT	3 (20.0%)	6 (7.5%)		1	6 (7.5%)		0 (0%)	6 (7.5%)	
C/T	0.60/0.40	0.75/0.25	0.09	0.79/0.21	0.75/0.25	0.60	0.80/0.20	0.75/0.25	0.72

204.76 ± 7.16 μM, $P = 0.02$; Cohen's $d = 0.91$) and total blood glutathione (161.16 ± 10.68 μM versus 242.67 ± 32.94 μM, $P = 0.02$; Cohen's $d = 0.89$) were found to be lower. In contrast, the plasma levels of other amino acids, such as cystathionine, serine, glycine, N-methylglycine and N, N-dimethylglycine did not differ significantly from those of normal controls in any of the ASD groups, although there was a trend towards lower levels of serine, glycine and N, N-dimethylglycine in the AD patients (Table 1). The levels of serum vitamin B₁₂ were in the normal range of values in all ASDs groups; only 3 children with AD had levels below 250 pg/ml, but all subjects with a PDD-NOS diagnosis had levels above this suboptimal value. Serum folate levels were also in the normal range, except for mildly higher levels in AS which reached a borderline statistical significance (13.51 ± 2.06 ng/ml versus 8.17 ± 0.91 ng/ml, $P = 0.06$). However, in the context of the small group size, this apparent difference in B₁₂ levels needs to be treated with caution.

We also examined the correlations between metabolite levels in the three groups of ASDs patients. In AD patients we found a positive correlation between tHcy levels and cysteine ($\rho = 0.54$, $P = 0.04$), cystathionine ($\rho = 0.56$, $P = 0.03$) and total blood glutathione ($\rho = 0.48$, $P = 0.08$); methionine was negatively correlated with cysteine, but statistical significance was not reached ($\rho = -0.30$, $P = 0.27$). In the AD group, we also observed a negative correlation between levels of N, N-dimethylglycine and cysteine ($\rho = -0.55$, $P = 0.03$) and a positive correlation between N-methylglycine and serum folate ($\rho = 0.44$, $P = 0.09$). Only in the PDD-NOS group a strong positive correlation was noticed between methionine and α-aminobutyric acid ($\rho = 0.66$, $P = 0.002$).

Genotyping results

The MTHFR C677T genotype data, summarized in Table 2, were obtained from 80 healthy controls and 39 patients with an ASD diagnosis (AD: $n = 15$; PDD-NOS: $n = 19$; AS: $n = 5$). The geno-

type distributions were all within the Hardy–Weinberg equilibrium. Although, at the moment, there are no reliable data on the MTHFR C677T polymorphic distribution in the general Romanian population, the frequency observed in our control group (CC, 57.5%; CT, 35.0%; TT, 7.5%; C/T, 0.75/0.25) is similar to that reported for other Central European populations [24]. The polymorphic T allele frequency of MTHFR was 0.31 in the ASD group (0.40 in AD, 0.21 in PDD-NOS and 0.20 in AS) as compared with 0.25 in the healthy controls. There was no statistical difference in the distribution of the genotypes ($\chi^2 = 0.30$, $P = 0.85$) or alleles ($\chi^2 = 0.27$, $P = 0.60$) between ASD patients overall and controls. The genotype and allele frequency was also similar in the subgroup of patients with PDD-NOS when compared with subjects in the general population ($\chi^2 = 0.12$, $P = 0.73$ and $\chi^2 = 26$, $P = 0.61$, respectively). However, the T allele was slightly more prevalent in the AD group than in controls (C/T, 0.40 versus C/T, 0.25), although the difference reached only borderline statistical significance ($\chi^2 = 0.85$, $P = 0.09$).

Discussion

One of the current obstacles in autism research is the clinical heterogeneity of the disorder, which is worsened by the absence of any reliable biological marker. The prototypical AD is diagnosed on the basis of the triad of behavioural impairments (*i.e.* social interaction, communication and restrained interests) before 3 years of age. Conversely, patients with the AS clinical subtype display apparently normal language and cognitive development, while the PDD-NOS category or atypical autism comprises cases that have many, but not all, of the defining features of AD or AS. Often, the severity or extent of the symptomatology in PDD-NOS patients is less severe, or the presentation is otherwise atypical, with a level of functioning that scores between that of the children with AD and that of the children with AS [25]. The degree to which ASD conditions, which share the common feature of dysfunctional reciprocal

social interaction, are independent developmental disorders is not known. Nonetheless, it is becoming increasingly recognized that no single explanation accounts for all autism(s) and multiple factors (genetic, epigenetic and environmental) are involved in shaping the phenotype [6–8, 26].

During the last two decades, several studies have indicated disruptions in one carbon metabolism (Fig. 1) accompanying different neurological and psychiatric disorders [27, 28]. The implications for these alterations in the methionine cycle and/or the transsulphuration pathway in neuropsychiatric conditions can be pathogenetically translated to disruptions in the methylation status, alterations in gene expression and a decreased ability to counteract neuronal oxidative stress. Recently, autism has also been linked to such metabolic perturbations, as reported in studies investigating plasma metabolites [10–13, 18, 19] or in immune cells [17] in ASD patients.

In the present study, we investigated one carbon metabolism in children with ASD, this being the first study with an emphasis on possible differences in the metabolic profiles between the clinical subtypes (*i.e.* AD, PDD-NOS and AS). Our results confirmed the presence of disturbances in these metabolic pathways, except for patients with the AS clinical subtype, who exhibited normal levels of metabolites. However, we found differences in the severity of the metabolite profiles across the spectrum of autistic disorders, with AD patients exhibiting disturbances in both the methionine cycle and the transsulphuration pathway concomitant with a slightly more prevalent T allele frequency for the MTHFR C677T polymorphism, while patients with the less severe form of the disease (*i.e.* PDD-NOS) presenting only with disturbances in the methionine cycle. In addition, since the levels of both folate and vitamin B₁₂ were within the normal range, we excluded vitamin deficiencies as a cause for these impairments. Therefore, more sensitive vitamin deficiencies markers are needed in future studies, taking also into consideration the fact that extracellular vitamin levels (*i.e.* in the serum) do not necessarily extrapolate to intracellular vitamin status. From these metabolic and genetic profiles it can be concluded that no single cause can be incriminated, rather, multifactorial interactions between environmental, including dietary, and genetic factors are likely to be culpable.

In the AD group and to a lesser extent in the PDD-NOS group, we found low levels of plasma methionine despite the fact that all children were on an unrestricted diet. Low methionine has been consistently reported in autism by other groups [10, 11, 13], and it could be the result of low dietary intake. In addition, in both AD and PDD-NOS, we observed low levels of α -aminobutyrate, a metabolite of α -ketobutyrate. This is consistent with a low methionine input with consequent low flux through the transsulphuration pathway. Repetitive behaviours and insistence on sameness can have a deleterious impact on the patient's ability to feed effectively and receive adequate nutrition. Children with AD and PDD-NOS have been reported to exhibit atypical feeding behaviour, with a highly restricted range of taste- and/or texture-based food choices [29]. Arnold *et al.* showed that children with autism on an unre-

stricted diet are significantly more likely to have deficiencies in essential amino acids, including methionine, which is consistent with poor protein nutrition [30]. Nonetheless, children with ASD from a recent study had high protein content in their diet, although they also had a high rate of reported gastrointestinal symptoms without an apparent medical cause [31].

In children with AD, the trend towards lower serine, glycine and N, N-dimethylglycine that accompanies lower methionine suggests that the remethylation pathway is perturbed. The observation that the thermolabile 677T allele of the MTHFR gene, which is associated with a decrease in enzymatic activity to 50–60% in homozygotes [32], is slightly more prevalent in the typical autism group, suggests that this polymorphism might contribute to the aforementioned metabolic disturbance. Two other studies indicated that the MTHFR C677T polymorphism is associated to a greater [15] or lesser degree [11] with ASD. Because of the small patient sample, we could not examine the influence of the MTHFR gene polymorphism on the metabolic profile. Future studies should further investigate the relative contribution of the less active variant of the MTHFR gene across the autism spectrum. The remethylation pathway, catalysed by 5-methyltetrahydrofolate–homocysteine S-methyltransferase or methionine synthase, requires vitamin B₁₂ and folate (methyltetrahydrofolate) as coenzyme and as cosubstrate (methyl donor), respectively. The levels of serum vitamin B₁₂ and folate were normal in children with AD, and apparently vitamin deficiencies are not the cause for the alterations in the methionine cycle. This does not necessarily rule out a cerebral deficiency of folate, which has been described in autistic patients [14] or an intracellular deficiency, since the RFC C80A variant of reduced folate carrier has been showed to be more frequent in autistics [11]. Moreover, preliminary data showed that the 19 bp-deletion polymorphism in dihydrofolate reductase (DHFR) is associated with autism, despite normal folate, vitamin B₁₂ and homocysteine levels, and it seems likely that this polymorphism interacts with the MTHFR and RFC single nucleotide polymorphisms to further modify the risk [18]. In addition, other studies have indicated a high prevalence of iron deficiency in AD and AS and that this is associated with falsely increased to normal folic acid levels [33]. In our study we did not evaluate the levels of S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy), but the available data in the literature consistently suggest an impaired transmethylation capacity in autistic patients (*i.e.* low AdoMet/AdoHcy ratio) [10, 11].

Interestingly, we did not observe higher levels of plasma homocysteine in children with AD, in contrast to our previous study in which hyperhomocysteinemia was present in children with ASD, but this was accompanied by suboptimal levels of vitamin B₁₂ [12]. Suh *et al.* recently demonstrated high intracellular homocysteine in leukocytes from autistic children, despite normal plasma levels [17]. It remains to be explored how much the blood metabolic profiles in autism reflect the actual intracellular metabolic dynamics, especially since there have been recent reports showing altered transport of amino acids across the cellular membrane of cultured fibroblasts from autistic patients [34].

What is particularly novel about our results is the fact that only children with typical autism (*i.e.* AD), but not PDD-NOS, have impairments in the transsulphuration pathway. More specifically, only children with AD had lower levels of plasma cysteine and total blood glutathione. Interestingly, the levels of cystathionine were within the normal range. Low levels of cysteine and glutathione accompanied by higher levels of cystathionine have also been described in the ASD group investigated by James *et al.* [11], and it could indicate a defect at the level of cystathionine gamma-lyase (CTH), a pyridoxal 5'-phosphate dependent enzyme that converts cystathionine to cysteine in the transsulphuration pathway. Another recent investigation reported that leukocytes from autistic children exhibit decreased intracellular cysteine and total glutathione, and unlike the findings in plasma, show a significant (80%) increase in intracellular homocysteine [17]. While in several studies children with ASD exhibited higher levels of vitamin B₆ than the control subjects [35–37], the activity of pyridoxal kinase and the levels of pyridoxal 5'-phosphate had been measured and found to be generally much lower than levels in control subjects [37]. It has also been suggested that the reduced flux through transsulphuration in autism, with a consequent diminished glutathione pool, could also be due to the high levels of androgens described in these patients [13, 38] since testosterone modulates the activity of CBS [39, 40]. The low levels of cysteine and glutathione, the decrease in the ratio of reduced to oxidized glutathione in plasma and the decreased ability to form sulphated metabolites in autistic patients [41] suggest that antioxidant defences are compromised in these patients [20]. Glutathione is also very important to heavy metal detoxification [42], and the variations in glutathione abnormalities across the ASD suggests the possibility that it might be functionally significant since it was shown that mercury-associated urinary porphyrin levels increase with increasing severity of ASDs [43–45]. Finally, it is important to discuss the limitations of our study. First, the number of subjects was relatively small and, unfortunately, no quantitative standardized scale for autism has been applied. Second, being a retrospective study it does not provide information about the dynamics of metabolite changes associated with different stages of development and/or in response to different dietary supplements (*i.e.* betaine, folic acid, vitamin B₆, vitamin B₁₂). By the same token, an interesting aspect of this study is that it represents the first inves-

tigation of one carbon metabolism in children with different subtypes of ASD who are free of vitamin supplements.

In conclusion, our results indicate disturbances in both the methionine cycle and the transsulphuration pathway in children with AD, which are most likely multifactorial in origin. Interestingly, children with milder clinical forms of ASD display milder metabolic perturbations. To date, only a few studies have specifically reported biological correlates of different clinical subtypes in the autistic spectrum (*e.g.* [46, 47]). The methylation deficit resulting from an altered methionine cycle supports the previously suggested contribution of epigenetic mechanisms to the pathogenesis of autism [26]. Aside from this 'metabolic reprogramming' of gene expression, dysfunctionalities in the synthesis of neurotransmitters and phospholipids would also result [28, 48]. Genomic imprinting is the classic example of regulation of gene expression *via* epigenetic modifications that leads to parent of origin-specific gene expression. From this perspective, the disturbances in the methionine cycle and the ensuing methylation deficit in AD patients support the imprinting hypothesis for the development of autism, which emphasizes the imbalances in brain development due to enhanced effects of paternally expressed imprinted genes [49]. In addition, disturbances in the methionine cycle have been hypothesized to contribute to alterations in neuronal synchronization in autism [16, 50]. On the other hand, the low levels of glutathione, resulting from the decreased flow through transsulphuration in AD, is indicative of an imbalance in the redox homeostasis, and links oxidative stress with membrane lipid abnormalities, inflammation and aberrant immune response that have been observed in autism [20].

Acknowledgements

This work was supported by grants from the National Institutes of Health (DK64959 to R.B.), funding from the Jonty Foundation (to R.B.) and by a CEEX grant (83/2006) received from the Romanian Academy of Medical Sciences (to M.D.) The authors acknowledge the assays done in the Hematology Division laboratories of Robert Allen and Sally Stabler at the University of Colorado Health Sciences Center in Denver, Colorado.

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