Clinical utility of folate pathway genetic polymorphisms in the diagnosis of autism spectrum disorders

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Background **The rationale of the current study was to test the clinical utility of the folate pathway genetic polymorphism in predicting the risk for autism spectrum disorders (ASD) and to address the inconsistencies in the association of MTHFR C677T and hyperhomocysteinemia with ASD.**

Patients and methods **An artificial neural network (ANN) model was developed from the data of 138 autistic and 138 nonautistic children using GCPII C1561T, SHMT1 C1420T, MTHFR C677T, MTR A2756G, and MTRR A66G as the predictors of autism risk. A neuro fuzzy model was developed to explore the genetic determinants of homocysteine. Meta-analyses were carried out on 1361 ASD children and 6591 nonautistic children to explore the association of MTHFR C677T and homocysteine with the risk for ASD.**

Results **The ANN model showed 63.8% accuracy in predicting the risk of autism. Hyperhomocysteinemia was observed in autistic children (9.67 ± 4.82 vs. 6.99 ± 3.21 μmol/l). The neuro fuzzy model showed synergistic interactions between MTHFR C677T and MTRR A66G inflating homocysteine levels. The meta-analysis showed MTHFR to be a genetic risk factor for autism in both fixed-effects (odds ratio: 1.47, 95% confidence interval: 1.31–1.65) and random-effects (odds ratio: 1.57, 95% confidence interval: 1.16–2.11) models. The meta-analysis of nine studies showed hyperhomocysteinemia as a significant risk factor for autism in both fixed-effects ($P < 0.0001$) and random-effects ($P = 0.026$) models.**

Conclusion **Genetic polymorphisms of the folate pathway were moderate predictors of autism risk. MTHFR C677T and hyperhomocysteinemia have been identified as risk factors for autism worldwide. Synergistic interactions between MTHFR C677T and MTRR A66G increase homocysteine.** ***Psychiatr Genet*** **00:000–000**

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Introduction

Autism spectrum disorders (ASDs) are the pervasive developmental disorders characterized by difficulty in social interaction, verbal/nonverbal communication, and repetitive behavior. The *Diagnostic and Statistical Manual of Mental Disorders*, 5th ed. (DSM-V) grouped individuals with a well-established DSM-IV diagnosis of autistic behavior, Asperger's syndrome (AS), and pervasive developmental disorder not otherwise specified into ASD.

Metabolic studies in children with ASD showed a lower ratio of S-adenosylmethionine/S-adenosylhomocysteine (SAH) and increased oxidative stress (James *et al.*, 2004). Higher homocysteine and suboptimal B_{12} levels were observed in Romanian children with ASD (Paşca *et al.*, 2006). Hyperhomocysteinemia, lower levels of plasma folate, and B_{12} were observed in Omani autistic children (Ali *et al.*, 2011). Plasma levels of reduced glutathione, cysteine, methionine, and homocysteine were shown to be lower in American autistic children (Geier and Geier, 2006). Impaired methylation, increased oxidative stress, and gene–gene interactions between reduced folate carrier 1 (RFC1) G80A and methylene tetrahydrofolate reductase (MTHFR) C677T were found to increase the AQ4 risk for autism (James *et al.*, 2006). Folinic acid and methylcobalamin supplementation was found to be beneficial in improving the transmethylation/transsulfuration metabolites and glutathione redox status of autistic children (James *et al.*, 2009). MTHFR C677T was shown to increase the risk for autism in Indians (Mohammad *et al.*, 2009), Han Chinese (Guo *et al.*, 2012), and North Americans (Liu *et al.*, 2011). A higher frequency of MTHFR C677T polymorphism was observed in autistic children compared with children with AS and pervasive

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developmental disorder not otherwise specified (Paşca *et al.*, 2009). Null association was observed between ASD and MTHFR C677T in Brazilian (dos Santos *et al.*, 2010) and Turkey (Sener *et al.*, 2014) children. MTHFR A1298C was identified as a risk factor for autism in Koreans (Park *et al.*, 2014). Maternal RFC1 G80A, elevated levels of homocysteine, adenosine, and SAH were found to increase susceptibility to autism (James *et al.*, 2010).

Despite the inconsistencies in genetic associations of the folate pathway with autism risk, majority of the studies showed impaired remethylation of homocysteine and increased oxidative stress as the metabolic hallmarks associated with ASD. The current study aimed (i) to explore the clinical utility of folate pathway genetic polymorphisms in the diagnosis of autism using ANNs; (ii) to carry out a meta-analysis to elucidate the contribution of MTHFR C677T toward risk for ASD; and (iii) to evaluate the role of homocysteine in the etiology of ASD through meta-analysis.

Patients and methods

Recruitments of the patients

A total of 138 autistic children (120 boys and 18 girls) and 138 nonautistic children (120 boys and 25 girls) matched for age (4.4±1.7 vs. 4.4±1.6 years), sex, and ethnicity were recruited during the period of 2001–2006 for this study. All the autistic children were diagnosed on the basis of DSM-IV criteria and Autism Behavior Checklist (ABC) scoring. Parents were interviewed to collect information on sensory stimuli, relating, body and objective use, language, and social help of the children. The children with an ABC score higher than 68 were enrolled as cases and the control children had an ABC score less than 21. Other details on the assessment of language function and developmental assessment have been described elsewhere (Naushad *et al.*, 2013). Autistic children showed developmental quotient in the range of 30–80, whereas control children showed developmental quotient higher than 80. All the patients with a neurological, inflammatory, endocrine, or immune disorder were excluded from the study. A comprehensive genetic and biochemical analysis was carried out to exclude patients with fragile X syndrome, chromosomal anomalies, and inborn errors of metabolism. The study protocol was approved by the institutional ethical committee of Center for DNA Fingerprinting and Diagnostics, Hyderabad, India. Informed consent was obtained from parents before the enrollment of their children.

Genetic analyses

Whole-blood samples collected in EDTA were used to extract DNA using the standard phenol–chloroform extraction method following proteinase K digestion. PCR-RFLP analyses were carried out to detect glutamate carboxypeptidase II (GCPII) C1561T, serine hydroxymethyl transferase 1 (SHMT1) C1420T, MTHFR C677T, 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) A2756G, and 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) A66G polymorphisms as described earlier (Mohammad *et al.*, 2011).

Development of an artificial neural network model for prediction of autism risk

The data corresponding to 138 autistic children and 138 nonautistic children described earlier (Mohammad *et al.*, 2009) were used to develop an ANN model. The input variables were GCPII C1561T, SHMT1 C1420T, MTHFR C677T, MTR A2756G, and MTRR A66G.

The target variable was disease outcome computed in the form of '1' and '0' to represent the presence and absence of the disease, respectively. The data on the genetic polymorphisms were computed as 0, 1, and 2 to represent wild, heterozygous, and homozygous mutant, respectively. The rationale for inclusion of these candidate genes was their role in catalyzing the intestinal absorption of folate (GCPII), conversion of tetrahydrofolate into 5,10-methylene tetrahydrofolate (SHMT), reduction of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate (MTHFR), remethylation of homocysteine to methionine (MTR), and reductive methylation of cobalamin (MTRR). Hence, any perturbation in their activities will adversely affect the folate pathway.

A neural network pattern recognition tool was used to train the data. The data were segregated into training (70% data), validation (15% data), and testing subsets (15% data) randomly. The nodes were optimized by trial and error. The performance of the final model was assessed on the basis of receiver operating characteristic curves and confusion matrix.

Plasma homocysteine determination

Freshly collected fasting blood samples were used for the analysis of homocysteine. Tributyl-*N*-phosphine was used to reduce the thiols in plasma and trichloro acetic acid was used for deproteinization. Precolumn derivatization of all the sulfur-containing amino acids was performed using ammonium-7-fluorobezo-2-oxa-1,3-diazole 4-sulfonate. These derivatives were resolved by reverse-phase HPLC using a Hichrom C18, 250×4.6 mm column (~~Berkshire,~~ UK) as the stationary phase and a 96 : 4 ratio of 0.1 µmol/l KH_2PO_4 buffer (pH 2.1 adjusted with orthophosphoric acid) and acetonitrile were used as the mobile phase. Isocratic separation, followed by fluorescent detection at an excitation wavelength of 385 nm or an emission wavelength of 515 nm was used to obtain the peaks corresponding to sulfur-containing amino acids.

Development of the neuro fuzzy model to study the contributing factors for homocysteine

Sex, GCPII C1561T, SHMT1 C1420T, MTHFR C677T, MTR A2756G, and MTRR A66G were used as the input variables and plasma homocysteine as the output variable to develop the neuro fuzzy model. The subclustering tool was used to generate a fuzzy inference system. The training was performed using 0.0001 as target error and 3000 epochs. A total of 25 fuzzy rules were generated to explain the contributing factors for homocysteine. The testing and checking data were computed to assess the prediction accuracy.

Meta-analysis of association between MTHFR C677T and autism

All the case–control studies published in Pubmed, Medline, and Google scholar during the period of 2004–2015 following Hardy–Weinberg equilibrium were retrieved using the keywords 'MTHFR', 'polymorphism', and 'autism'. In total, eight case–control studies representing 1361 ASD children and 6591 healthy controls were used for the meta-analysis. The computational web page '*http://www.statdirect.org*' was used to carry out the meta-analysis. The total alleles (MTHFR C-allele and T-allele) and minor allele (T-allele) number in cases and controls of each study were computed.

Meta-analysis of association between homocysteine and autism

All the case–control studies published in Pubmed, Medline, and Google scholar during the period of 2004–2015 were retrieved using the keywords 'homocysteine', 'autism', and 'autism spectrum disorders'. In total, eight case–control studies were used for the meta-analysis. The computational web page '*http://www.statdirect.org*' was used to carry out the meta-analysis. SD in means of cases and controls, and SE and variance were calculated.

Fixed-effect and random-effect models were generated on the basis of the Mantel–Haenszel and the DerSimonian–Laird algorithms. Cochran's Q-test was used to evaluate the noncombinability of studies. I^2-statistics were used to identify heterogeneity in association. Egger's test was performed to assess publication bias.

Results

The ANN model of folate pathway SNPs showed 63.2, 65.2, and 65.2% accuracy in predicting the risk of autism. The overall accuracy of prediction was 63.8%. (Fig. 1) The area under the curve in receiver operating characteristic curve was 0.72 [95% confidence interval (CI): 0.65–0.76, $P = 0.002$]. This association has 90% power with a type I α-error of 0.01.

Three studies on MTHFR showed a statistically significant association of the MTHFR C677T polymorphism with the risk for ASD (Boris *et al.*, 2004; Mohammad *et al.*, 2009; Guo *et al.*, 2012). Null association was observed for this polymorphism in Koreans (Park *et al.*, 2014) and Brazilians (dos Santos *et al.*, 2010) with autism. All other studies showed a borderline association. Cumulatively, the MTHFR C677T polymorphism was found to show 1.47-fold (95% CI: 1.31–1.65, $P < 0.0001$) and 1.57-fold (95% CI: 1.16–2.11, $P = 0.003$) increased risk for ASD in fixed-effects and random-effects models, respectively (Fig. 2). No publication bias was detected in Egger's test ($P = 0.29$). The Cochran Q-value was 17.82 ($P = 0.01$), with an I^2-value of 6.1%.

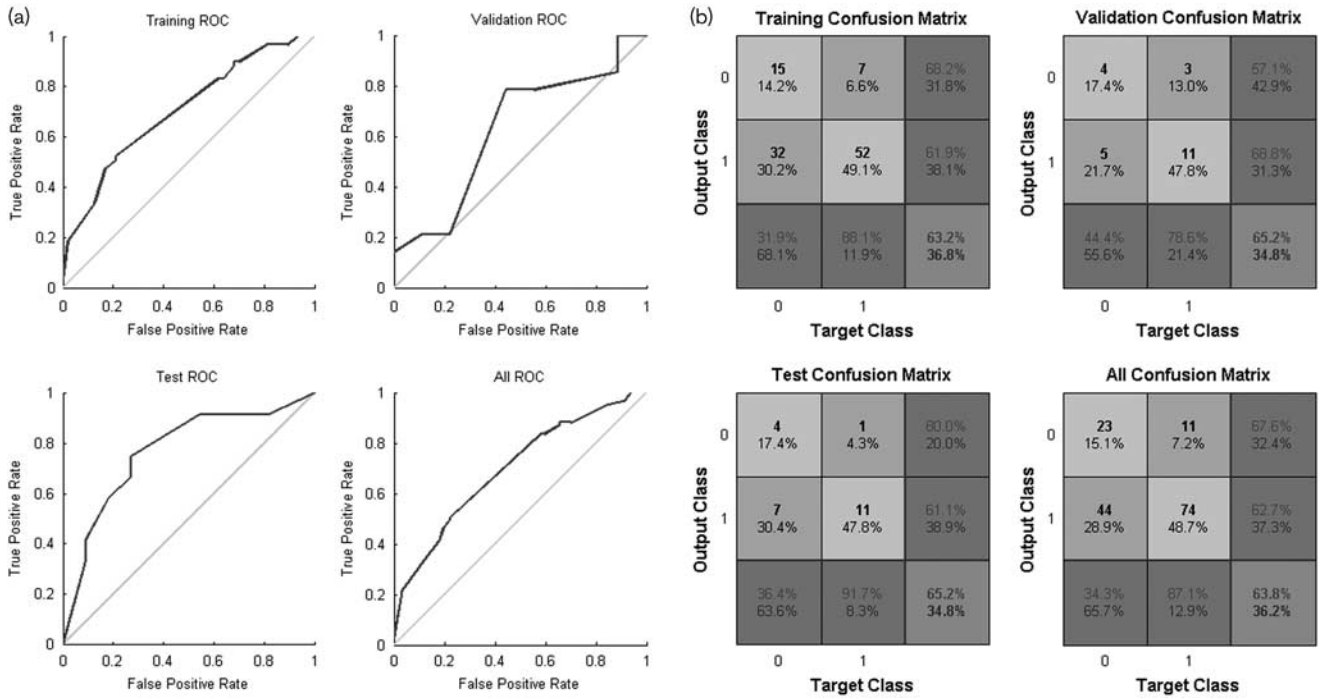
The current study cohort showed elevated homocysteine levels in autistic children (9.67 ± 4.82 vs. 6.99 ± 3.21 μmol/l). The neuro fuzzy model showed a mean absolute error of 3.55 μmol/l. This model was used to generate surface plots showing bivariate interactions between variables. These surface plots showed MTRR A66G as the most important determinant of homocysteine, followed by MTHFR C677T. SHMT1 C1420T was shown to negate the MTRR A66G-mediated homocysteine elevation (Fig. 3).

Three studies showed lower homocysteine levels in children with ASD (James *et al.*, 2006, 2009; Paşca *et al.*, 2009). Five published studies (James *et al.*, 2004; Paşca *et al.*, 2006; Ali *et al.*, 2011; Al-Farsi *et al.*, 2013; Han *et al.*, 2015) and the current study showed elevated homocysteine levels in ASD. Cumulatively, both fixed-effect ($P < 0.0001$) and random-effect ($P = 0.026$) models showed elevated levels of homocysteine in ASD. (Fig. 4) The Cochran Q-value was 354.64 ($P < 0.0001$), with an I^2-value of 97.7%.

Discussion

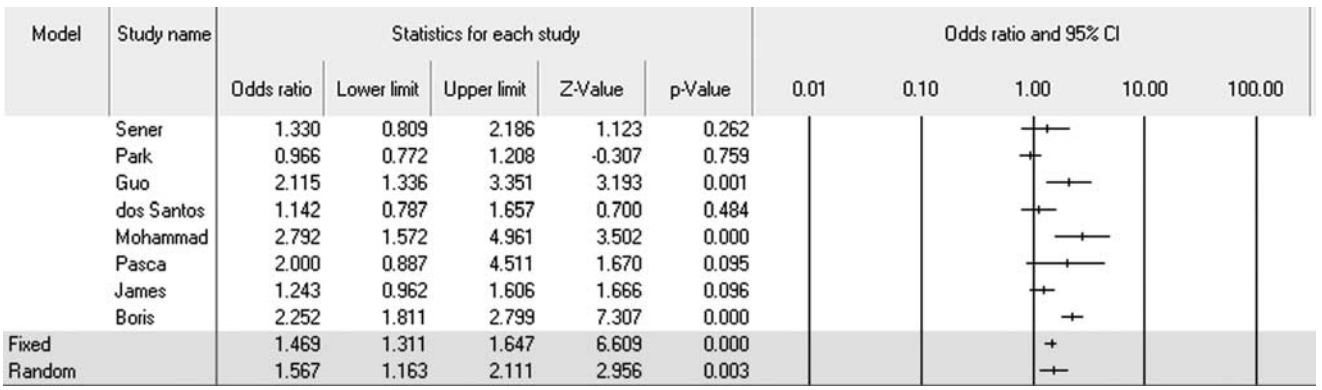
The application of artificial neural networks for the diagnosis of autism has been attempted earlier on the basis of DSM criteria using the neuro fuzzy system (Arthi and Tamilarasi, 2008), which showed 85–90% accuracy in predicting autism. The association studies on the folate pathway genetic polymorphism, with ASD risk and the metabolic signatures observed in ASD suggest the possibility of perturbations in this pathway in the pathophysiology of autism. Hence, we have tested the clinical utility of folate pathway genetic polymorphisms in predicting the risk for ASD using an ANN model. This ANN model showed 63.8% accuracy and 0.72 area under the curve in predicting the risk for ASD, suggesting a moderate risk for ASD with perturbations in the folate pathway. Furthermore, we carried out a meta-analysis of all the studies related to the association of MTHFR C677T with the risk for ASD. This polymorphism was identified as a genetic risk factor for ASD cumulatively in both fixed-effect and random-effect models. As the I^2-value for the association of MTHFR C677T with ASD was low, it can be ascertained that there is very little variation across different studies and a fixed-effects model might be appropriate. However, the I^2-value for the association

Fig. 1



The performance of an artificial neural network model in predicting autism risk. The neural network pattern recognition tool was used to predict autism risk using GCPII C1561T, SHMT1 C1420T, MTHFR C677T, MTR A2756G, and MTRR A66G polymorphisms as predictors. The model was trained using 70% of the data and the remaining data were used for validation and testing. The performance of the model was represented in the form of (a) receiver operating characteristic (ROC) curves and (b) confusion matrix. The confusion matrix indicates the following: the green color indicates true prediction (true positive and true negative), whereas the red color indicates false prediction (false positive and false negative). If target and predicted data are in agreement, that is (0,0) and (1,1), they are classified as true negative and true positive, respectively. If there is disagreement, that is (0,1) and (1,0), they are classified as false positive and false negative, respectively.

Fig. 2

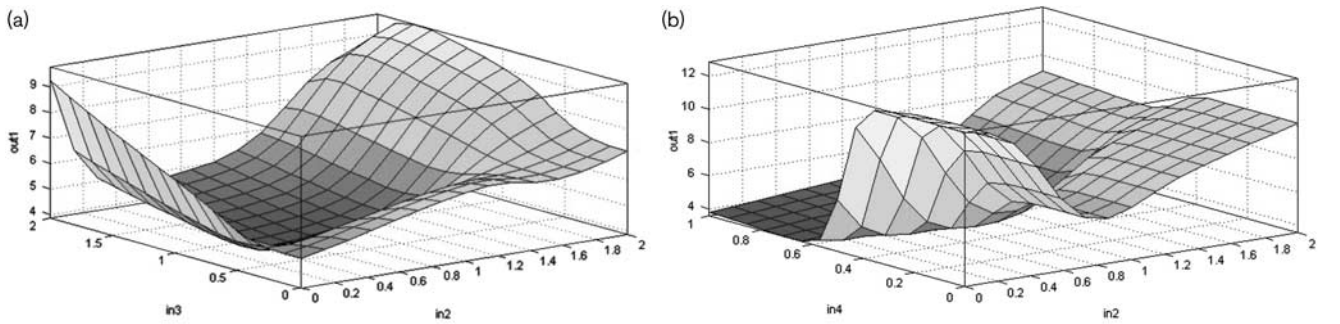


Meta-analysis of association of the MTHFR C677T polymorphism with the risk for autism spectrum disorder (ASD). A total of eight case–control studies representing 1361 ASD children and 6591 nonautistic children were used to carry out meta-analysis using a 'statdirect' module. Fixed-effects and random-effects models identified MTHFT C677T as a risk factor. CI, confidence interval.

of homocysteine with ASD is higher, suggesting that heterogeneity in association and a random-effects model might be appropriate in this scenario.

MTHFR is the major rate-limiting enzyme that dictates the flow of folate either toward synthesis or methylation of DNA by regulating the levels of 5,10-methylene tetrahydrofolate and 5-methyl tetrahydrofolate. The

Fig. 3



Predictions of a neuro fuzzy system of homocysteine. A neuro fuzzy system was developed using GCPII C1561T, SHMT1 C1420T, MTHFR C677T, MTR A2756G, and MTRR A66G as the predictors of homocysteine. This system showed (a) synergistic interactions between MTRR A66G (in2) and MTHFR C677T (in3) inflating homocysteine (out1). 0, 1, and 2 represent wild, heterozygous, and homozygous mutant genotypes of the respective polymorphisms. (b) SHMT1 C1420T (in4) polymorphism negating MTRR 66G-mediated elevation in homocysteine.

Fig. 4

Model	Study name	Std diff in means	Standard error	Variance	Lower limit	Upper limit	Z-Value	p-Value
	Pasca 2009	-1.583	0.434	0.188	-2.433	-0.732	-3.647	0.000
	James 2009	-0.131	0.221	0.049	-0.565	0.302	-0.594	0.552
	James 2006	0.561	0.165	0.027	0.237	0.884	3.397	0.001
	James 2004	-0.501	0.288	0.083	-1.065	0.062	-1.743	0.081
	Pasca 2006	1.065	0.471	0.221	0.143	1.988	2.264	0.024
	Ali 2011	3.782	0.373	0.139	3.050	4.514	10.129	0.000
	Al-Farsi	4.835	0.443	0.196	3.967	5.703	10.918	0.000
	Han 2015	0.451	0.203	0.041	0.054	0.848	2.225	0.026
	Current	0.708	0.175	0.031	0.366	1.051	4.049	0.000
Fixed		0.634	0.082	0.007	0.474	0.795	7.729	0.000
Random		0.995	0.447	0.200	0.119	1.870	2.226	0.026

Meta-analysis of nine studies to show an association of homocysteine with autism risk. The meta-analysis of homocysteine with autism was carried out to represent the SD in means of cases and controls and SE to calculate the *z*-value and the *P*-value. Both fixed-effects and random-effects models showed elevated homocysteine as a risk factor for autism. CI, confidence interval.

MTHFR C677T polymorphism induces the thermolabile variant MTHFR, which dissociates into inactive monomers from the active dimer form with loss of flavin adenine dinucleotide-binding capacity (Yamada *et al.*, 2001). Hence, this polymorphism was shown to be associated with impaired remethylation of homocysteine.

In the current study, hyperhomocysteinemia was observed in autistic children of Indian origin, which is consistent with the impaired remethylation hypothesis. The neuro fuzzy model proved this hypothesis by showing synergistic interactions between MTRR A66G and MTHFR C677T increasing homocysteine levels. The current meta-analysis of all the case–control studies supports this hypothesis by showing statistically significant elevated homocysteine levels in children with ASD. Earlier, we reported the MTRR 66GG genotype as a risk factor for autism and the AA-genotype as a protective factor (Mohammad *et al.*, 2009). No such association was observed between autism and MTRR A66G in the US population, suggesting a possible role of gene–nutrient interactions in modulating genetic susceptibility. MTRR was reported to carry reductive methylation of cobalamin to form methylcobalamin, which acts as a cofactor in the remethylation of homocysteine (Wolthers and Scrutton, 2009). The MTRR 66G-allele was reported to contribute toward elevated homocysteine levels (Laraqui *et al.*, 2006). *N*-methyl D-aspartate and group I metabotropic glutamate antagonists were shown to confer protection against homocysteine-induced neurodegeneration in rat hippocampus (Yeganeh *et al.*, 2013). This suggests that homocysteine exerts neurotoxic effects by activation of *N*-methyl D-aspartate and group 1 metabotropic glutamate receptors.

The counteracting interactions between SHMT1 C1420T and MTRR A66G could be because of induction of futile folate cycle by SHMT1, which increases folate levels in the presence of the 1420T-allele (Mendes *et al.*, 2013), thus resulting in lowering of homocysteine.

A recent randomized trial suggested the efficacy of methyl B_{12} in improving the Clinical Global Impressions Improvement score in children with ASD, which correlated with increased levels of methionine and the S-adenosylmethionine/SAH ratio (Hendren *et al.*, 2016). Two large-scale prospective studies showed a reduced risk of autistic disorder in mothers receiving prenatal folic acid supplementation (Berry, 2013; Surén *et al.*, 2013). These intervention studies corroborate our current observations, thus highlighting the role of perturbations in the folate pathway in the etiology of ASD.

In terms of clinical utility, the studied polymorphisms of the folate pathway were shown to be moderate predictors of ASD risk. In view of growing evidence on the potential benefits of folinic acid and methyl B_{12} in reducing the risk for ASD, it can be assumed that such supplementations might be beneficial for the children with MTHFR C677T and MTRR A66G polymorphisms.

The limitations of the current study were the lack of data on the folate and B_{12} status of the studied patients and the predictability of the model could have been improved by analyzing other functional polymorphisms of the folate pathway. Nevertheless, the associations observed in the current study corroborated with the existing literature and the results of meta-analyses. The major strengths of the current study were application of ANN and neuro fuzzy models to understand the role of folate pathway aberrations in autism and to address the homocysteine elevation. The addition of meta-analyses strengthens these observations further.

To summarize, folate pathway genetic variants are moderate predictors of ASD risk. They contribute toward elevated homocysteine levels, which in turn increase the risk for ASD. The meta-analyses showed MTHFR C677T and hyperhomocysteinemia as risk factors for ASD.

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Conflicts of interest

AQ9 None declared.

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