

# Comparison of serum B12, folate and homocysteine concentrations in children with autism spectrum disorder or attention deficit hyperactivity disorder and healthy controls

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**Objective:** We aimed to investigate the serum concentrations of vitamin B12, folate and homocysteine in children diagnosed with attention deficit hyperactivity disorder (ADHD) or autism spectrum disorder (ASD) and healthy controls.

**Materials and methods:** Serum vitamin B12, folate and homocysteine concentrations were measured in 118 children (48 children diagnosed with ADHD, 35 children diagnosed with ASD and 35 healthy controls). Symptom severity in the ADHD and ASD groups was evaluated by the Childhood Autism Rating Scale and Turgay-DSM-IV-Based Screening and Assessment Scale for Disruptive Behavior Disorders. Multivariate analysis of covariance was used to evaluate the effects of diagnosis and gender on biochemical parameters.

**Results:** The ADHD and ASD groups and the healthy controls differed significantly regarding vitamin B12 and homocysteine concentrations, but not folate levels. Patients with ASD had the lowest vitamin B12 and the highest homocysteine levels. Vitamin B12 levels correlated negatively with hyperactivity and/or impulsivity and oppositionality symptoms in children with ADHD. There were no relationships between psychometric evaluations and laboratory measurements in children with ASD. Gender did not affect vitamin concentrations.

**Conclusion:** Previous studies found that vitamin B12 was reduced while homocysteine was elevated among patients with ADHD and ASDs. Our results also support those reported previously. Oppositionality and hyperactivity and/or impulsivity may be related to vitamin B12 and homocysteine levels in children with ADHD. Further studies are required to define the role of these parameters and effects on the etiology and clinical manifestations of ASD and ADHD.

**Keywords:** vitamin B12, folate, homocysteine, attention deficit hyperactivity disorder, autism spectrum disorder

## Introduction

Although both autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) are highly heritable neurodevelopmental disorders, approximately 70.0–80.0% of the phenotypic variance of each disorder could be explained by genetic factors.<sup>1</sup> An increase in the prevalence of ASD and ADHD has stimulated research on etiology of these neurodevelopmental disorders, including environmental factors such as vitamins which have important roles in central nervous system (CNS) functioning.<sup>2,3</sup>

Vitamin B12 and folate play important roles in the development, differentiation, and functioning of the CNS.<sup>4</sup> They are involved in the methionine-homocysteine

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pathway, which is responsible for the methyl groups required for DNA and protein synthesis.<sup>4,5</sup>

In case of deficiencies, there is a decrease in re-methylation of homocysteine, which leads to an increase in homocysteine level.<sup>6</sup> Reflecting their importance in CNS metabolism, previous studies reported that deficiencies in vitamin B12 and folate levels, as well as changes in homocysteine, might be related with depression, mood disorders, psychotic disorders, an obsessive-compulsive disorder.<sup>7-9</sup> Recently, some studies have reported deficiencies in the levels of vitamins B12 and folate (vitamin B9) in patients diagnosed with ASD and ADHD as well as beneficial effects of those vitamins for some of the associated ASD and ADHD symptoms.<sup>10-12</sup> Also in some other studies, altered levels of homocysteine were found to be associated with clinical symptoms of ASDs and ADHD-associated behavioral problems, respectively.<sup>13,14</sup>

As far as we are aware, no study from Turkey evaluated levels of vitamin B12, folate and homocysteine all together in children with ADHD and ASD. Therefore, in this study, we aimed to investigate the serum levels of vitamin B12, vitamin B9 (folate) and homocysteine in children diagnosed with ADHD and ASD and compare those findings to healthy controls. Also, we aimed to investigate if the serum levels of B12, folate and homocysteine correlate with ASD and ADHD symptom severity.

## Materials and methods

### Study design

This single center, case-control study was conducted at the Düzce University Medical Faculty, Department of Child and Adolescent Psychiatry. The study was conducted in accordance with the Declaration of Helsinki (International Committee of Medical Journal Editors, 1989) and IRB approval for this study was acquired from local ethics committee (Date: 03.05.2016, Number: 107).

### Participants

Patients who were admitted to the Department of Child and Adolescent Psychiatry for the first time and who met inclusion criteria (diagnosis of ADHD or ASD) were enrolled in the study. The healthy control group was composed of children who applied to the Department of Pediatrics within the specified time frame with temporary health conditions (upper respiratory tract infection, etc.) and have neither chronic medical conditions nor psychiatric history. Patient and control groups consisted of

medication-free children and also have no chronic disorders and vitamin supply intake before the study. Written Informed consent forms were procured from the families of both patients and control groups.

## Procedures

### Psychiatric evaluation and diagnosis

Diagnosis of ADHD (n=48) was based on the Schedule for Affective Disorders and Schizophrenia for School Age Children – Present and Lifetime Version (K-SADS-PL), which was developed based on DSM-IV diagnostic criteria.

### Scales used in psychiatric evaluation

Schedule for Affective Disorders and Schizophrenia for School Age Children – Present and Lifetime Version (K-SADS-PL)

A semi-structured interview form developed by Kaufman et al was used to determine past and present psychopathologies of children and adolescents according to DSM-IV diagnostic criteria.<sup>15</sup> Because the original version of K-SADS-PL does not include a module for the assessment of ASDs, the diagnosis of ASD was made based on the DSM-5 diagnostic criteria. KSADS-PL was applied to both children or adolescents and their primary caregiver.

### Childhood Autism Rating Scale (CARS)

CARS is a 15-point behavioral rating scale developed to separate non-autistic children with intellectual disabilities from children with autistic symptoms. It is especially effective on separating autistic children from children with moderate intellectual disability. It also enables us to determine the clinical severity of autism as mild-moderate and moderate-serious. Each item is graded with a half degree scoring between 1 and 4. The possible total score ranges from 15 at a minimum to 60 at maximum. According to the scoring, children with scores between 15 and 29.5 do not show autistic symptoms. Children with scores between 30 and 36.5 have a clinically mild-moderate autism and children between 37 and 60 have severe autism.<sup>16</sup>

### The Turgay DSM-IV Disruptive Behavior Disorders Rating Scale

T-DSM-IV-S was used for symptom severity in this study.<sup>17</sup> The items in the scale are identical to the list of symptoms described in the DSM-IV criteria for ADHD (inattention: 9 items, hyperactivity-impulsivity: 9 items), oppositional defiant disorder (8 items) and conduct disorder (15 items). The T-DSM-IV-S was developed by Turgay (1994) and translated and adapted in Turkish by Ercan et al (2001).<sup>18</sup> The

symptoms are scored by assigning a severity estimate for each symptom on a 4-point Likert-type scale (namely, 0= not at all; 1= just a little; 2= much; and 3= very much). Ratings of “much” and “very much” for each item were considered positive, as done in previous studies.

### Preparation of colorimetric analysis

After informed consent was procured, 3.0 mL of blood were taken from the antecubital veins of the non-dominant hand while sitting. Samples were centrifuged at 3000 rpm at +4.0 °C for 10 mins with EDTA tubes; then, the supernatant section was taken up with a separate Eppendorf tube and stored at – 80.0°C until assay time.

### Measurement of vitamin B9 (folate) and vitamin B12 levels

Serum folate and vitamin B12 were analyzed on Epoch Analyzer™ spectrophotometric system (Biotek Diagnostics Inc.) according to manufacturer protocol. The measurements of parameters were made with the Human V B12 Elisa Kit (Cat: E-EL-0010) and Human VB9 Elisa Kit (Cat: E-EL-0009) (Elabscience, USA). Standardization of assay principles was diluted according to the stock standard solution contained in the kit and patient sample values were calculated according to standard concentration at the ending.

Before analysis, samples and kits were brought to room temperature. A quantity of 100 µ of working reagent, washing solution and standard curves were prepared from the stock buffer. A quantity of 90 µL of detection reagent was added to the whole standard, sample and blank wells for incubation. The plate was incubated 37°C for 4 hrs. Then, each well was washed for three times and substrate solution was added. A quantity of 50 µL of stopping solution was added at the appropriate incubation time. After 15 mins incubation in dark, the contents were removed and run through the microplate reader with measurements at 450 nm wavelength.

The ELISA was based on the competitive binding enzyme immunoassay technique. The concentrations of folate and vitamin B12 were measured by comparing the O.D. of the standard curve. The sample data were linearized by log concentrations 0–2000 ng/mL with the odd absorbance nm value determined by progression analysis. Coefficients of variation for folate and vitamin B12 levels were 5.0% for both.

### Measurement of homocysteine

Homocysteine levels of experimental and control group samples were tested by Human ELISA kit (Elabscience,

Cat: 201611). For quantification, 100 µL patient serum and 100 µL anti-homocysteine antibody are added to a plate coated with a homocysteine conjugate. Patient samples and kit conjugate were incubated 3 hrs at 37°C in the dark. This assay protocol differs from others (ie, folate and vitamin B12) because the sample and conjugate compete for antibody binding, which generates a reverse curve. Samples with high homocysteine levels will bind to most of the antibody, which gets washed away and results in a low O.D., while samples with low homocysteine levels will leave more antibody available to bind to the conjugate, producing a high signal. Binding plates were read in 570 nm. Evaluation of the results was carried out according to the kit protocol after completed stages. The coefficient of variation for homocysteine levels was 3.7%.

### Statistical analyses

A priori power calculation was conducted using the results of previous studies as well as recent reviews.<sup>19–25</sup> Overall effect sizes for changes in levels of vitamin B12, folate and homocysteine in ASD and ADHD were small to medium. We calculated that 144 participants would be required to conduct a MANCOVA at 90.0% power at a *p* level of 0.05 for three groups and three response variables and controlling for the effects of age and gender.<sup>26</sup> However, we could enroll 118 children within the specified time frame leading to 85.0% power.

SPSS 22.0 software (IBM Inc.) was used in analyses. Descriptive statistics (means and standard deviations for normally distributed variables, medians and inter-quartile ranges for non-normal variables) were used to summarize data. Assumptions of normality were tested with Kolmogorov–Smirnov test. Nominal variables between groups were compared with chi-square test. Yates and Fisher’s corrections were used as needed. Due to outliers and non-normality, Pillai’s trace was used for MANCOVA. Bonferroni correction was used in post-hoc analyses. Bivariate correlations were analyzed with Spearman’s rho. *P* was set at 0.05 (two-tailed).

## Results

### Sociodemographic variables

One hundred and eighteen children (ADHD, n=48; ASD, n=35; controls, n=35) were enrolled. The majority of the sample (81.4%) were male. Rates of male children in control, ADHD and ASD groups were 100.0%, 68.8% and 80.0%; respectively. The groups differed significantly

regarding gender (chi-square=46.4,  $df=2$ ,  $p=0.000$ ) with the control group being significantly different from children with ADHD and ASD.

The ages of children were not distributed normally ( $p=0.000$ ), and the median age of the whole sample was 8.0 (IQR=3.0) years. Median ages and inter-quartile ranges for controls, ADHD and ASD children were 9.0 (IQR=3.9), 8.6 (IQR=2.8) and 6.0 (IQR =3.0) years, respectively. The groups differed significantly regarding age (chi-square=45.7,  $df=2$ ,  $p=0.000$ ). Post-hoc comparisons revealed that median ages of controls ( $Z=-5.6$ ,  $p=0.000$ ) and children with ADHD ( $Z=-6.2$ ,  $p=0.000$ ) were similar between themselves and significantly higher than those with ASDs (Bonferroni correction, Mann–Whitney U test).

## Vitamin B12, folate (folic acid) and homocysteine levels

None of the measurements were distributed normally in samples ( $p=0.000$ , 0.001 and 0.001 for vitamin B12, folate and homocysteine, respectively). Neither covariance matrices (Box's  $M=71.9$ ,  $p=0.000$ ) nor error variances were equal (Levene's test,  $p<0.05$  for all). Laboratory values according to diagnostic groups are summarized in Table 1.

The effects of age ( $F(3, 111)=0.6$ ,  $p=0.614$ , partial  $\eta^2=0.02$ ) and gender ( $F(3, 111)=1.6$ ,  $p=0.202$ , partial  $\eta^2=0.04$ ) were not significant. However, diagnosis affected the vitamin levels significantly ( $F(6, 224)=22.8$ ,  $p=0.000$ , partial  $\eta^2=0.379$ ). Univariate analyses revealed that diagnostic status affected vitamin B12 ( $F=106.7$ ,  $p=0.000$ , partial  $\eta^2=0.654$ ) and homocysteine levels ( $F=28.3$ ,  $p=0.000$ , partial  $\eta^2=0.333$ ) but did not affect folate ( $F=1.3$ ,  $p=0.288$ , partial  $\eta^2=0.022$ ). In pair-wise comparisons, controls had the highest vitamin B12 levels

**Table 1** Laboratory evaluations of subjects according to diagnostic groups

	Controls (n=35)	ADHD (n=48)	ASD (n=35)
Vitamin B12(pg/ mL)	1611 (357)	929 (774)	268 (407)
Folate (ng/ml)	1319 (155)	1229 (701)	1315 (302)
Homocysteine ( $\mu\text{mol/L}$ )	1297 (487)	1582 (238)	1912 (598)

**Note:** Numerical values in the table are shown in nm to clearly indicate the level change between the groups and are presented as median (IQR).

**Abbreviations:** ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder.

followed by children with ADHD ( $p=0.000$ , Bonferroni). Children with ASD had the lowest levels of vitamin B12. Lastly, children with ASD had the highest levels of homocysteine, followed by children with ADHD ( $p=0.000$ , Bonferroni). Controls had the lowest levels of homocysteine.

## Psychometric evaluations

Symptoms of inattention, hyperactivity/impulsivity, oppositionality and conduct disorder were evaluated in children with ADHD using DSM-IV-Based Screening and Assessment Scale for Disruptive Behavior Disorders. Median scores and inter-quartile ranges for the corresponding subscales were 19.0 (IQR=7.0), 13.0 (IQR=9.0), 7.0 (IQR=9.8) and 2.0 (IQR=5.8), respectively.

Spearman's correlation analyses were conducted to evaluate relationships between symptom severity and laboratory measurements in patients with ADHD. Vitamin B 12 levels and hyperactivity/impulsivity ( $\text{Rho}=-0.36$ ,  $p=0.01$ ) and oppositionality ( $\text{Rho}=-0.36$ ,  $p=0.01$ ) scores displayed low to medium negative correlations. No other relationships could be discerned. As a result, vitamin B12 levels could explain 13.0% of the variance in hyperactivity/impulsivity and oppositionality scores of patients with ADHD.

Symptoms of children with ASDs were evaluated with CARS. Median score of CARS in this group was 37.5 (IQR=12.5). In correlation analyses, no relationships between CARS total and item scores and laboratory evaluations could be discerned.

## Discussion

In this single-center, cross-sectional, case-control study, we found that patients with ADHD and ASDs and healthy control children differed significantly regarding vitamin B12 and homocysteine. Patients with ASDs had the lowest vitamin B12 and the highest homocysteine levels. We did not find a significant difference in serum folate levels both in ASD and ADHD group compared to control group. Gender and age of children had no significant effect on vitamin levels.

Our study results support the studies showing low serum vitamin B12 and folate levels in ASD patients.<sup>27,28</sup> A recent study conducted in Turkey found lower vitamin B12 serum levels in children diagnosed with ASD compared to controls.<sup>29</sup> Similarly, another study from Oman reported lower serum folate and vitamin B12 levels in children

diagnosed with ASD.<sup>28</sup> As for ADHD, Unal et al. reported that Turkish children with ADHD had significantly lower vitamin B12 levels and that those levels negatively correlated with psychosomatic symptoms and learning problems as reported by teachers.<sup>23</sup> This finding was supported and extended to folate in another study by Altun et al.<sup>30</sup>

Vitamin B12 status in the brain may play an important role in methylation-dependent processes and low serum vitamin B12 levels in ASD patients were interpreted to reflect increased levels of oxidative stress and impaired DNA methylation which can be an important factor in the pathophysiology of ASD.<sup>31,32</sup> DNA methylation, an epigenetic regulatory system is known to be important in during pre- and post-natal brain development and alterations in DNA methylation have been shown in patients with ASDs.<sup>32,33</sup> In line with these study results, DNA hypomethylation which affect the development of the CNS may be a possible explanation for the relation between vitamin B12 deficiency and autism.<sup>8</sup> Lower levels of serum B12 were also found in ADHD group compared to control group. Vitamin B12 is required for a unique process known as dopamine-stimulated phospholipid methylation (PLM) performed by D4 receptor in CNS which is mostly associated with ADHD.<sup>34</sup> PLM activity – differs by D4 gene variants – plays an important role in attention and neuronal synchronization and is known to be disturbed in children with both ASDs and ADHD.<sup>35</sup> Although cross-sectional nature of our study precludes speculations on causality and may not reflect micro-nutrient levels in the fetal period, deficits in vitamin B12 and folate along with reduced methionine synthase activity might be posited to contribute to ADHD and ASD pathogenesis. Further, longitudinal studies exploring this issue are needed.

Patients with ASDs and ADHD in our sample were also found to have higher serum homocysteine levels compared to control group. Reduced vitamin B12 levels may increase the level of homocysteine. Homocysteine is also known to be a powerful excitotoxin, and its metabolic products may cause neuronal damage and disrupt the synthesis of proteins and neurotransmitters which are important for the structural integrity of the brain. As well as high levels of homocysteine were found to play a role in the etiology of various psychopathologies, recent clinical studies also reported higher serum and urine homocysteine levels in children with ASDs when compared to healthy controls.<sup>27,36,37</sup> As for ADHD, some studies report elevated levels of homocysteine while others report reduced levels.<sup>13,25,30</sup> The differing results may be due to

enrollment of children with different comorbidities, diagnosed with separate ADHD subtypes and from variable age ranges. Our results support and extend those findings in showing reduced vitamin B12 and elevated homocysteine levels in patients with ADHD and ASDs.

As another finding in our study, oppositionality and hyperactivity/impulsivity symptoms in ADHD group were related to vitamin B12 and homocysteine levels which partially supported Saha et al's results.<sup>13</sup> Some preliminary studies suggest that vitamin and micronutrient supplements may reduce emotional lability, aggression and oppositional behaviors in children with ADHD and ASDs.<sup>38–40</sup>

Our results should be evaluated within the context of their limitations. Due to the cross-sectional nature of our study direction of causality could not be ascertained. Children with ASDs often display avoidance/restriction of certain foods due to sensory reactivity and resistance to novelty. This may also cause deficiencies of vitamins in children with ASDs. To control for this possibility, we also evaluated correlations with CARS sensory reactivity item scores but did not apply checklists to parents (ie, ABC, etc.). Also, in the planning stage of our study, we aimed to conduct separate univariate analyses of variance controlling for type I errors as per the Garipardic study but chose to conduct a multivariate analysis of covariance to control for effects of gender and age.<sup>19</sup> Our data violated some of the assumptions of MANCOVA but we tried to control for this by using robust measures (ie, Pillai's trace). Also, our findings may be affected by timing of measurements and may not reflect CNS levels of micronutrients in earlier development. Regardless of those limitations, our study is among the first ones to evaluate vitamin B12, vitamin B9 (folate) and homocysteine levels in children with ASDs and ADHD and compare the results with controls.

Our study results show low B12 and high homocysteine levels are detected in children diagnosed with ASD and ADHD compared to healthy controls. Children with ADHD or ASD often show emotional and behavioral problems. Deficiencies in micronutrients like B12 may play a role in the etiopathogenesis and clinical symptoms of these neurodevelopmental disorders. However, these parameters should be analyzed in a wider population to clarify the effect on the etiology of ADHD and ASD.

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## Disclosure

The authors alone are responsible for the content and writing of the paper and they report no conflicts of interest in this work.

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