Antineuronal antibodies and 8-OHdG an indicator of cerebellar dysfunction in autism spectrum disorder: a case–control study

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ABSTRACT
Objectives: Autism spectrum disorder (ASD) is a neurodevelopmental disorder, that starts in early childhood and presents with deficits in social-communicational domains along with restricted and repetitive behaviours/interests. While genetic factors are dominant in its pathogenesis, many factors, including neurological, environmental and immunological have been identified. Furthermore, although cerebellar dysfunction in the etiology of autism has been shown in different studies, the possible causes of the dysfunction and the role of neuroinflammation among these causes have not been clarified yet. Anti-Yo, anti-Hu, anti-Ri and anti-Amphiphysin antibodies have been found to be associated with cerebellar degeneration. The aim of the present study was to compare anti-Yo, anti-Hu, anti-Ri and anti-Amphiphysin antibodies and 8-OHdG values in blood using the ELISA method between ASD patients and healthy children to demonstrate the role of neuroinflammation as a potential cause of cerebellar dysfunction and DNA damage and evaluate the relationship between Childhood Autism Rating Scale (CARS) scores in children diagnosed with ASD and these parameters.

Methods: Thirty-five consecutive children between the ages of 3 and 12 referred to the Child and Adolescent Psychiatry Outpatient Clinic of Harran University Hospital and diagnosed with ASD according to the DSM-5 diagnostic criteria were included in the study. The children did not have any chronic physical disorders and were treatment naive. Thirty-three healthy children between the ages of 3 and 12 without any physical or psychiatric disorders were included as the healthy control group. For psychiatric evaluation, a sociodemographic form and to measure the severity of autism, CARS was used. In the study, anti-Yo, anti-Hu, anti-Ri and anti-Amphiphysin antibodies and 8-OHdG values in blood were investigated using the ELISA method.

Results: Thirty-five cases with autism (62.9% males) and thirty-three healthy controls (72.7% males) were included in the present study (p = 0.385). The median age was 6.0 in the ASD group and 7.0 in the control group (p = 0.146). Among ASD patients, anti-Ri antibody positivity was detected, while no anti-Ri antibody positivity was found in the control group (p = 0.002). In the ASD group, the anti-Hu and 8-OHdG values were found to be significantly higher than those of the controls (p < 0.001, p = 0.001); no significant difference was found between the ASD and control groups with regard to the anti-Yo and anti-Amphiphysin values (p = 0.113, p = 0.275).

Conclusions: The results of the present study suggest that antibodies against cerebellum may be present among children with ASD and DNA damage may occur due to oxidative stress.

Introduction

Autism spectrum disorder (ASD), is a neurodevelopmental disorder, symptoms of which arise in early childhood. It presents clinically with deficits in socio-communicational domains and restricted-repetitive behaviours/interests [1]. Based upon community-wide surveys the most recent prevalence rates may be approximately 1/68. ASD occurs in all racial, ethnic and socioeconomic groups and is five times more common among males. Although genetic factors are preponderant in its pathogenesis, many factors such as neurological, environmental, and immunological, are implicated. The etiology is far from being completely understood, and there are no definite biological or clinical markers for diagnosing ASD [2].

The role of cerebellar pathology in the etiology of autism has been demonstrated by various studies. The selective loss of Purkinje cells in the cerebellum

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and atrophy of cerebellar lobules are among common neurological abnormalities encountered in individuals with autism [3,4]. Additionally, cerebellum is known to have varied connections to cortical and subcortical structures and to modulate cognitive, language, motor sensory, and emotional functions associated with these regions [5]. The connections between the cerebellum and the parietal lobe may play a role in motor dysfunction in ASD [6]. The cerebellum is also known to play a part in conditional reflexes, mental imagination, planning, attention, emotional behaviours, visuospatial organization, and sensory data collection. Many of these functions may also be impaired in ASD. Supporting this position, posture, balance, and motor skills, which are associated with the cerebellum, have also been demonstrated to be disturbed in some individuals with ASD [7]. Cerebellar dysfunction may also play a role in problems with gaze, eye contact and joint attention in ASD [8,9]. Even though cerebellar dysfunction has been demonstrated in autism, the probable causes of dysfunction and the role of neuroinflammation among these causes remain to be clarified.

Anti-Yo is an antibody against the antigens in the cytoplasm of Purkinje cells and indicates cerebellar degeneration; anti-Hu, anti-Ri and anti-Amphiphysin are antibodies against cellular nuclear antigens, and they become positive in paraneoplastic conditions and neuropathies [10,11]. The association of many antibodies, especially anti-Yo and anti-Hu, anti-Ri, anti-Amphiphysin, with cerebellar degeneration has been demonstrated [12,13]. In a study by Naael et al. on the mothers of children with ASD, anti-Yo and anti-Amphiphysin antibodies were found to be significantly higher than in mothers in the healthy control group. As to anti-Hu and anti-Ri antibodies, they were found at higher rates in the mothers of children with autism than control groups, but the difference was not statistically significant [14].

8-hydroxy-2-deoxyguanosine (8-OHdG), is one of the most common of 23 oxidative base damage products caused by reactive oxygen species (ROS) in DNA, and it is the one whose mutagenicity is best known. There has been an increasing number of studies on 8-OHdG in recent years, and findings indicate increased levels of 8-OHdG in Alzheimer’s, Parkinson’s, and Schizophrenia patients [15–17]. In a study on children with status epilepticus, hypoxic ischemic encephalopathy, and CNS infection, CSF 8-OHdG levels were found to be significantly higher than in controls, which has been shown to be a more accurate marker of brain damage than urinary 8-OHdG levels [18]. There is data in the literature indicating that in individuals with ASD, 8-OHdG levels increase or remain changed [19–21]. In a study by Sajdel et al. in which the cerebellum of autism patients was investigated post-mortem, 8-OHdG levels were found to be high [19]. These findings suggest that 8-OHdG may be used as a marker of cerebellar damage in individuals with autism.

The aim of the present study was to compare anti-Yo, anti-Hu, anti-Ri and anti-Amphiphysin antibodies and 8-OHdG values in blood using the ELISA method between ASD patients and healthy children to demonstrate the role of neuroinflammation as a potential cause of cerebellar dysfunction and DNA damage to evaluate the relationship between CARS scores in children diagnosed with ASD and these parameters.

**Material and methods**

**Subjects**

The study was conducted at the Harran University School of Medicine Department of Child and Adolescent Psychiatry between November 2017 and June 2018. Children diagnosed with ASD according to DSM-5 criteria in this period (n = 70) were evaluated for eligibility. Fifteen children were excluded due to regressive ASD and a further 20 were excluded due to chronic medical/ neurological disorders or use of psychopharmacological agents. In the end, Thirty-five children with ASD were enrolled in the study. Intellectual disability was allowed as a comorbidity in the ASD group. The exclusion criteria of the patient groups were as follows: the use of any drug; known neurological, genetic, or other medical diseases; the use of antioxidants; and a history of atopic eczema and/or allergies. The control group included thirty-three healthy children, who did not have any physical or psychiatric diseases and who had been referred to Harran University Hospital for vaccination or control and matched by age and sex with the patient group. Financial support for the study was provided by the Harran University Scientific Investigation Coordination Committee. The study protocol was approved by the ethics committee of Harran University, Faculty of Medicine (Date: November 16, 2017; No:09), and the study procedures were in accordance with the Declaration of Helsinki and local laws and regulations. Informed consent was obtained from the parents of children to be included in the study. The parents were also informed about the objective and benefits of the study as well as their right to be excluded at any time according to their decision.

In addition, since no prior power analysis was performed, post hoc power analysis results were given to enable the readers to grasp the context of their findings. Post-hoc power analysis demonstrated that 16.0%, 33.0%, 16.0% and 88.0% power was achieved for anti-Yo, Anti-Hu, Anti-Amphiphysin and 8-OHdG, respectively.
Data collection tools

**Sociodemographic and clinical data collection form**

A data form was prepared for the purpose of this study and filled in during the interview with the children and their parents.

**Childhood autism rating scale (CARS)**

CARS is a behavioural rating scale that has 15 subscales and was developed to distinguish children with autism from children with intellectual disability [22–24]. It was used by research reliable severity score patients with a CARS score of 30–36 were classified as mild–moderate; patients with a CARS score of 37–60 were classified as severely autistic. The adaptation of the scale to Turkish was conducted by Sucuoğlu et al. [25].

**Collection of blood samples and laboratory investigations**

A total of 5 ml of venous blood was drawn from children from the patient and control groups following at least 8 h of overnight fasting and placed in biochemistry tubes. The blood samples drawn were centrifuged without waiting at 4°C and 4000 rpm for five minutes. The serum was separated into two parts and kept in Eppendorf tubes at –80°C until analysis. In the Harran University Biochemistry Laboratory, anti-Yo (SunRed CN:201-12-0574), anti-Hu (Fine Test CN:EH2621), and 8-OHdG (Fine Test CN:EU2548) levels were measured with the ELISA (Enzyme-Linked Immunosorbent Assay) kit method.

**Statistics**

For statistical analysis, the SPSS 23.0 programme was used. Descriptive statistics, frequency, and percentage, were used as categorical variables. In the analysis of categorical variables, Pearson chi square tests were used. A Shapiro–Wilk normality test was conducted to determine whether data were normally distributed. As they were not normally distributed, nonparametric tests were used. Data were expressed with the median (IQR). In the comparison of the two groups, the Mann–Whitney U test was used. For correlation analysis, Spearman correlation analysis was performed. The receiver operator characteristics (ROC) curve was utilized to evaluate the accuracy of anti-Yo, anti-Hu and anti-Amphiphysin antibodies and 8-OHdG in diagnosing ASD. The ROC curve was plotted to determine the cut-off point. A value of \( p < 0.05 \) was accepted as statistically significant.

# Results

Thirty-five autism cases and thirty-three healthy controls were included in the present study. The boy/girl ratio was 22/13 (62.9%/37.1%) in the ASD group and 24/9 (72.7%/27.3%) in the control group (\( p = 0.385 \)). The median (IQR) age was 6 years (3) in the ASD group and 7 years (3) in the control group (\( p = 0.146 \)). No significant difference was found between the ASD and control groups with respect to the sex, age, BMI, and ages of the mothers and fathers (Table 1). Of the 35 cases in the ASD group, 25.7% \( (n = 9) \) had mild moderate autism while 74.3% \( (n = 26) \) had severe autism. Of the autism cases, 62.9% \( (n = 22) \) had no speech while, 37.1% \( (n = 13) \) could form sentences of at least two words.

Anti-Ri antibody positivity was found at the rate of 23.3% in the control group; there was no anti-Ri antibody positivity in the control group. The discrimination ratio was 22/13 (62.9%/37.1%) in the ASD group and 24/9 (72.7%/27.3%) in the control group \( (p = 0.385) \). The positive predictive values (PPVs) and negative predictive values (NPVs) for Anti-Ri antibodies were 100.0% and 56.0%, respectively.

In the ASD group, the anti-Hu and 8-OHdG values were found to be significantly higher. \( (p < 0.001) \), \( (p = 0.001) \). The anti-Amphiphysin values were found to be higher in ASD groups, but the difference was not statistically significant \( (p = 0.275) \). No significant difference was found between ASD and control groups with regard to anti-Yo values \( (p = 0.113) \) (Table 3). The ROC analysis showed that anti-Hu antibodies and 8-OHdG may be used in ASD diagnosis (for Anti-Hu, AUC = 0.781; \( p < 0.001 \), and for 8-OHdG, AUC = 0.728; \( p = 0.001 \)) (Table 4). The ROC curves of these parameters were shown in Figure 1.

### Table 1. Comparison of ASD and control groups in terms of sex, age, BMI and age of parents.

|                      | ASD group \( (n = 35) \) | Controls group \( (n = 33) \) | \( p \) *
|----------------------|--------------------------|-------------------------------|---
| Sex (boy/girl)       | 22/13 (62.9%/37.1%)      | 24/9 (72.7%/27.3%)            | 0.385*
| Age (year)           | 6 (3)                    | 7 (3)                         | 0.146**
| BMI (kg/m²)          | 15.70 (2.89)             | 15.34 (2.53)                  | 0.873**
| Age of mother (year) | 34 (10)                  | 30 (7)                        | 0.290**
| Age of father (year) | 37 (10)                  | 36 (6)                        | 0.622**

*Chi-square test, **Mann–Whitney U test, IQR: Inter-Quartile Range.

### Table 2. Distribution of groups according to anti-Ri Positivity.

| Anti-Ri | ASD group \( (n = 35) \) | Controls group \( (n = 33) \) | \( p \)* |
|---------|--------------------------|-------------------------------|---|
| Positive n (%) | 9 (25.7%) | 0 (0%) | 0.002 |
| Negative n (%) | 26 (74.3%) | 33 (100%) | |

*Chi-square test.
Cohen’s effect sizes are provided in Table 1. No significant difference was found between cases with mild-moderate autism and those who had severe autism with respect to anti-Yo, anti-Hu, anti-Amphiphysin and 8-OHdG values ($p > 0.05$). No significant correlation was found between the CARS scores of the patients and laboratory parameters.

### Table 3: The comparison of ASD with control groups in terms of anti-Yo, anti-Hu, anti-Amphiphysin and 8-OHdG values.

|                   | ASD group ($n = 35$) | Controls group ($n = 33$) | Cohen's d | $p^*$  |
|-------------------|----------------------|---------------------------|-----------|-------|
| Anti-Yo (ng/ml)   | 1.98 (1.63)          | 2.33 (1.53)               | -0.157    | 0.113 |
|                   |                      |                           |           |       |
| Anti-Hu (ng/ml)   | 1.64 (0.27)          | 1.56 (0.07)               | 0.298     | <0.001|
|                   |                      |                           | $Z =$-1.454 |       |
| Anti-Amphiphysin  (pg/ml) | 47.57 (40.35)   | 45.06 (45.98)          | 0.162     | 0.275 |
| 8-OHdG (ng/ ml)   | 34.02 (12.63)        | 26.7 (6.11)               | 0.697     | <0.001|
|                   |                      |                           | $Z =$-1.092 |       |

*Mann-Whitney U test, IQR: Inter-Quartile Range.

### Table 4. The ROC curves of anti-Yo, anti-Hu, anti-Amphiphysin and 8-OHdG for the prediction of ASD.

|                  | AUC | Cut-off values | Sensitivity | Specificity | $p$  |
|------------------|-----|----------------|-------------|-------------|------|
| Anti-Yo          | 0.388 | 2.20          | 43 %        | 39 %        | 0.113 |
| Anti-Hu          | 0.781 | 1.60          | 71 %        | 76 %        | <0.001|
| Anti-Amphiphysin | 0.577 | 45.34         | 60 %        | 52 %        | 0.275 |
| 8-OHdG           | 0.272 | 32.11         | 66 %        | 85 %        | 0.001 |

AUC: area under the curve.

Discussion

In the present study, 35 children between the ages of 3 and 12 diagnosed with ASD were compared with 33 healthy children in the same age range in terms of anti-Yo, anti-Hu, anti-Ri, anti-Amphiphysin, and 8-OHdG levels, and the relationship between these parameters and CARS scores was evaluated in children with ASD. To our knowledge, the present study is the first study to investigate the relation between ASD and anti-Yo, anti-Hu, anti-Ri, and anti-Amphiphysin values in autistic children and to evaluate the effect of these antibodies on 8-OHdG levels, which is an indicator of DNA damage.

Antibodies, targeting neuronal epitopes, were first recognized in paraneoplastic neurological disorders, cerebellar degeneration or encephalitis patients. Some neuron-specific antibodies were strongly combined with cancer cells and target antigens were expressed by neurons and cancer cells, which led to these antibodies collectively being termed “paraneoplastic” or “onconeuronal.” Although these antibodies are associated especially with tumours (most commonly small cell lung, breast, and ovarian tumours), they were also detected in patients with neurological syndromes without a known cause or sometimes in healthy individuals [26]. Paraneoplastic antineuronal antibodies have various targets. They target both nuclear and cytoplasmic protein antigens, such as anti-Yo and, anti-Hu, or intracellular synaptic proteins, such as anti-Amphiphysin [11]. Many antibodies have been found to be associated with cerebellar degeneration, especially anti-Yo, anti-Hu, and anti-Amphiphysin
antibodies [12,13]. The relationship between these antibodies, whose association with the cerebellum has been demonstrated, in autism has recently begun to be investigated. In the literature, a recent study by Naeel et al. on the mothers of children with autism found that they had significantly higher levels of anti-Yo and anti-Amphiphysin antibodies than the control group. Anti-Hu and anti-Ri antibodies were also found at higher levels in the mothers of children with autism, but the difference with control groups was not statistically significant [14]. In our study, no significant difference was found between the ASD and control groups in terms of anti-Yo and anti-Amphiphysin values. However, the anti-Hu value was found to be significantly higher in the ASD group. In addition, while anti-Ri positivity was shown in 25.7% of children with ASD, it was not seen in any children in the healthy control group, resulting in a significant difference. To our knowledge, no previous study in the literature has evaluated anti-Yo, anti-Hu, anti-Ri, and anti-Amphiphysin antibodies in children diagnosed with autism. However, in many studies, in individuals diagnosed with autism, different autoantibodies against varying regions of the CNS have been identified (i.e. serotonin receptors, neuron axon filament protein, myelin basic protein, cerebellar neurofilaments, nerve growth factor, and autoantibodies against alpha-2 adrenergic connection sites) [6,27–31]. The mechanical role of these antibodies in ASD and whether they are pathogenic or develop secondary to neuronal damage are not clear. The selective loss of Purkinje cells in the cerebellum and atrophy of cerebellar lobules are among common neurological abnormalities in individuals with autism [32]. In addition, in at study by Vargas et al., it is suggested that there is an active and chronic neuroinflammatory process in the cerebellum of children with autism [33]. In the present study, anti-Hu and anti-Ri antibodies, which are associated with cerebellar degeneration, were found at significantly higher levels in ASD patients. In view of the information in the literature, the results of our study support the role of cerebellar dysfunction in autism.

Oxidative stress in ASD may arise due to a lack of balance between ROS produced by endogenous/exogenous oxidants and antioxidants. Rodent models and clinical studies suggest that the cerebellum of patients with ASD may display elevated oxidant products and reduced antioxidants [34,35]. The accumulation of ROS leads to functional alterations and chemical modifications in DNA, RNA, protein, lipid, and carbohydrate groups, resulting in cellular dysfunction. 8-OHdG is the most common of 23 oxidative base damage products caused by ROS in DNA, and its mutagenicity is well known. 8-OHdG is also a marker of mitochondrial dysfunction and impaired metabolism [36]. In the literature, increased oxidative stress and oxidative DNA damage levels were reported in patients with ASD and animal models. In a study by Shpyleva et al., cerebellar oxidative DNA damage was shown both in postmortem examinations of individuals with autism and in mouse models of autism [35]. Similarly, in a study by Napoli et al., mitochondrial DNA damage was reported at higher rates in children with autism [37]. In a study by Ming et al., the urinary levels of 8-OHdG were found to be higher in autistic patients, but the difference with healthy controls was not found to be significant [21]. In a study by Sajdel et al., 8-OHdG levels were found to be high in a postmortem examination of the cerebellum in autism cases [19]. In a study in which 68 children diagnosed with ASD were compared with 54 healthy controls, 8-OHdG levels in peripheral lymphocyte DNA were found to be significantly higher in the ASD group [20]. However, in other studies no significant difference was found between autism and healthy control groups in terms of urinary 8-OHdG levels [38,39]. In the present study, when 8-OHdG values were compared between the ASD and control groups, the 8-OHdG value was found to be significantly higher in ASD patients.

The ROC curves and area under the curve (AUC) are commonly used as diagnostic markers. According to Hosmer and colleagues, an AUC between 0.7 and 0.8 suggests acceptable discriminatory power and our results suggest that anti-Hu antibodies and 8-OHdG may prove acceptable markers for ASD diagnosis [40].

In addition, it has been shown in various studies that as the severity of autism increased, oxidative stress also increased and the antioxidant system became weaker [41,42]. In our study, no significant difference was found between mild/moderate and severe autism cases with respect to anti-Yo, anti-Hu, anti-Amphiphysin and 8-OHdG values.

The study population was relatively small for asserting that the blood parameters can be used as diagnostic biomarkers for ASD, and those markers were obtained peripherally, so their reliability for central nervous system disorders is questionable. Moreover, allowing the comorbid diagnosis of ID may have affected our results. On the other hand, the groups were matched by age and gender and the effects of chronic medical disorders and previous treatments were controlled. These are the strengths of our study.

Conclusion

It has been demonstrated in many studies that immune system abnormalities are involved in the pathogenesis of autism, some specific antibodies against the brain have been detected, antioxidant system function is inadequate, and DNA damage occurs because of oxidative stress. However, whether this condition is one of the factors underlying the etiology of the disease or a consequence of pathophysiological mechanisms
of autism remains debatable. The results of the present study reveal that some antibodies are produced against the cerebellum of children with ASD (anti-Hu, anti-Ri) and that increased 8-OHdG levels may be an indicator of oxidative DNA damage in the cerebellum. However, further studies are needed to determine the value of these parameters as biomarkers of ASD.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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