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## Assessment of malondialdehyde levels, superoxide dismutase, and catalase activity in children with autism spectrum disorders

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### ABSTRACT

**OBJECTIVE:** Blood biomarkers for diseases have recently become a topic of great interest. Many studies of Autism Spectrum Disorder (ASD) have been made to date looking for biomarkers in peripheral tissues, but no specific biomarker has yet been found. The aim of this study was to examine oxidative stress parameters including malondialdehyde (MDA) levels, superoxide dismutase (SOD), and catalase (CAT) activity and to determine both their sensitivity and specificity as biomarkers associated with oxidative stress in ASD.

**METHODS:** This study measured the plasma MDA levels, SOD, and CAT activities in erythrocyte in 52 patients with ASD (aged 3–6 years) and in 48 age- and gender-matched healthy controls. ASD severity was rated using the Childhood Autism Rating Scale (CARS).

**RESULTS:** MDA levels, SOD, and CAT activity were significantly higher in patients with ASD in comparison to the controls ( $p < .001$ ). The receiver operator characteristic curve analysis showed a high diagnostic value for MDA, SOD, and CAT. Their areas under curve (AUC) were 0.937, 1.0, and 1.0, respectively ( $p < .001$ ). A positive statistically significant correlation was determined between the total CARS score and MDA levels in ASD patients ( $r = 0.368$ ,  $p = 0.007$ ).

**CONCLUSION:** This study shows that oxidative stress is higher in children with ASD. Increased vulnerability to oxidative stress may contribute to the development of ASD. Given the high sensitivity and specificity results, it is thought that these selected oxidative stress parameters could be important as biomarkers for ASD. Future studies should focus on the sensitivity and specificity of oxidative stress biomarkers in larger ASD populations.

### ARTICLE HISTORY

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Autism spectrum disorder; biomarkers; malondialdehyde; oxidative stress; superoxide dismutase

## Introduction

According to the classification in the Diagnostic and Statistical Manual of Mental Disorders 5th Edition (DSM-V), autism spectrum disorder (ASD) is a neuro-development disorder characterized by impaired reciprocal social communication skills and social interaction, and by limited and repetitive patterns of behaviour, interests, or activities [1]. Since there has been a marked increase in the number of cases diagnosed with ASD in recent years, the need to understand the causes and underlying pathophysiology of ASD has become more urgent than ever. It has been suggested that genetic susceptibility, immunological alterations, and environmental factors might play an etiopathogenic role in ASD [2–5].

There are currently no accepted biomarkers for diagnosis and/or screening, meaning that ASD diagnosis is made only on the basis of clinical findings [6]. Young children with ASD can benefit a great deal if therapy is given early on. Such benefits include fewer ASD symptoms and corresponding maladaptive disorders, which in turn can lead to better results [7].

Such therapy is best applied early on, but this requires early diagnosis, which can be a problem if the patient only has mild to moderate ASD symptoms. This is why researchers are trying to find biomarkers to help make differential diagnoses in those cases where evaluations of behaviour are inconclusive so as to detect ASD before the symptoms begin to manifest [8,9].

The practical definition of “biomarker” is a biological measure known to be associated with a particular condition or that can vary from one group to another. Biomarkers are features that can be measured and assessed objectively in order to identify biological or pathogenic processes or determine how the body is responding to treatment. Biomarkers have great potential for improving care for psychiatric patients [10,11]. It is hoped that biomarkers might be useful in ASD cases in terms of identifying the mechanisms involved in the abnormalities seen in ASD so that it can be identified early on. Biomarkers might also help with risk assessment in addition to characterizing subgroups and predicting possible responses to therapy. Various measures have been looked at and even

suggested as biomarkers for ASD including neurophysiological and neuropsychological measures as well as neuroimaging in addition to genetic and biochemical markers [10]. Blood biomarkers for diseases have recently become a topic of great interest. Such biomarkers are relatively non-invasive for the patient and inexpensive to analyse. Early intervention with behavioural therapies might be possible with a blood-based biomarker for ASD. Many studies have been made to date examining peripheral tissues for biomarkers in ASD. These studies have looked at oxidative stress parameters, antioxidant enzyme levels, cytokines, brain-derived neurotrophic factors, and serotonin [8,10,12–16]. Nevertheless, no specific biomarker has yet been identified. Of the biomarker candidates being discussed, oxidative stress and antioxidant enzyme activity in peripheral blood are both simple to evaluate and inexpensive.

Oxidative stress is thought to play a role in the etiology of ASD [17–20]. Oxidative stress is defined as an intracellular imbalance between reactive oxygen species (ROS), produced either during aerobic metabolism or as a result of the pathological processes, and the antioxidants that are involved in defending against ROS [21]. A panel of different markers including DNA, proteins, and lipid residues, which are pathognomonic of oxidative damage of biomolecules, can be studied in order to detect oxidative stress [12,21,22]. The relationship between oxidative stress and different psychiatric diseases such as schizophrenia, bipolar disorder, major depression, ASD, and attention deficit hyperactivity disorder has been examined in studies to date [23–27]. Both the central and peripheral markers (including lipid peroxidation levels) of oxidative stress have been implicated in the pathogenesis of various psychiatric diseases. Many major psychiatric disorders may have a common underlying pathogenic mechanism as the brain is relatively more susceptible to oxidative damage [22,23]. Metabolic markers of oxidative stress such as abnormal levels of metabolites, which signify impaired methylation, and increased oxidative stress have been detected in ASD [17]. There have also been reports of changes in the levels of detoxifying agents (such as glutathione) and antioxidants in ASD cases [18]. Furthermore, a reduced glutathione redox status has been determined in ASD, and in post-mortem analyses of ASD brain tissues [28].

Malondialdehyde (MDA) is the final product of lipid peroxidation, which causes tissue damage and can, therefore, be used as an indicator of oxidative stress [21]. Previous studies have reported an increase in MDA levels in children with ASD [25,29–31]. Superoxide dismutase (SOD) and catalase (CAT) both neutralize ROS and are therefore considered to be antioxidant enzymes. Different studies have reported that SOD activity has decreased, increased, or

remained unchanged in plasma and erythrocyte in ASD cases [19,32–35]. It has been determined that CAT activity in ASD is decreased in erythrocyte, unchanged in plasma, and increased in serum [19,31,36,37].

Despite research into oxidative parameters such as MDA and antioxidant enzymes such as SOD and CAT in ASD, it has not been possible to make neurobiological inferences as there are no studies looking at the sensitivity and specificity of these parameters. Currently, there is a lack of published research on this topic in psychiatric disorders. However, the diagnostic suitability of MDA, prolidase, and CAT have been researched in major depression, bipolar disorder, and schizophrenia [24,38,39]. A recent study reported that selected biomarkers related to oxidative stress and energy metabolism are highly predictive values in children with ASD [40].

The primary aim of this study is to investigate plasma MDA levels, SOD, and CAT activity in drug-naïve patients with ASD and healthy control subjects while the second aim is to evaluate the sensitivity and specificity of selected oxidative stress parameters and whether or not they are reliable biomarkers related to oxidative stress in children with ASD.

## Methods

The study included 52 children aged 3–6 years (45 male, 7 female) who presented at the Child and Adolescent Psychiatry Clinics of Kahramanmaraş Sutcu Imam University and Muğla Sıtkı Koçman University between October 2016 and November 2017, and who had been diagnosed with ASD but were not receiving any medication. ASD was diagnosed according to the DSM-V criteria [1]. The severity of ASD symptoms was evaluated using the Childhood Autism Rating Scale (CARS) score [41]. CARS was developed to aid in the differential diagnosis of autism as opposed to other developmental disorders. The items in the scale are relating to people, imitative behaviour, emotional response, body use, object use, adaptation to change, visual response, listening response, perceptive response, fear or anxiety, verbal communication, non-verbal communication, activity level, level and consistency of intellectual relations, and general impressions. A total score of between 30 and 36.5 indicates mild-to-moderate autism whereas a range of 37–60 denotes severe autism. CARS is scored by observing the child and through interviews with the family. The validity and reliability analysis of the Turkish version of CARS were made by Incekas Gassaloğlu et al. The Cronbach's alpha value for the total CARS score was determined as 0.95. Test–retest reliability ( $r = 0.98$ ,  $p < .01$ ), and inter-rater reliability ( $r = 0.98$ ,  $p < .01$ ) were determined for the total score of the scale. The cut-off point for the scale is 29.5 [42].

Demographic data (age, gender, parents' age, and parents' education level) were recorded. There were no abnormalities in the participants' physical examinations and/or laboratory findings. Patients were excluded if there was any active infection, known allergic reaction, genetic syndrome, metabolic, endocrine or neurological disease, any diagnosis of malnutrition or obesity, current use of medication and antioxidant medication, or comorbid psychiatric disorders (except mental retardation).

The control group consisted of 48 age- and gender-matched children (37 male, 11 female), all healthy participants who had been admitted to the hospital where the study was taking place for routine check-ups. The children in the control group were given a physical and psychiatric examination and the demographic data were recorded. The control group children had no psychiatric problems, no known neurological problems, genetic syndromes, infections, metabolic or endocrine diseases, allergic reactions, and were not taking any medications or antioxidant therapy. Approval for the current study was granted by the Ethics Committee of Kahramanmaraş Sutcu Imam University Medical Faculty (No. 02, dated 12 October 2016). All the parents or legal guardians of the participating children gave written informed consent prior to inclusion in the study.

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. The Gpower version 3.1.9.2 package was used to make a post hoc power analysis of the numerical variables and the pROC library in R package was used to make a post hoc power analysis for AUCs.

## Biochemical analysis

### Oxidative stress biomarker measurements

Blood samples were collected in the morning from patients and control subjects after an overnight fast. The samples were taken from the antecubital vein in test tubes containing sodium heparin and were centrifuged at 3000 rpm for 10 minutes at 4°C. The plasma was separated, and the buffy coat was discarded by aspiration. The erythrocytes were washed four times with cold saline solution and stored at -70°C until analysis. The CAT activity in the erythrocyte was measured in samples using the Beutler method [43].

The decomposition of the substrate H<sub>2</sub>O<sub>2</sub> was monitored spectrophotometrically at 240 nm. The activity of CAT was expressed as U/g Hb. The SOD activities in erythrocyte were estimated using the method described by Fridovich [44]. SOD activity was expressed as U/g Hb. Lipid peroxidation levels in the plasma samples were expressed in MDA. Measurements were based on the Ohkawa method [45]. MDA levels were expressed as nmol/mL.

### Statistical analysis

All statistical analyses were performed using SPSS for Windows version 22.0 software (SPSS Inc., Chicago, IL, U.S.A.). Conformity to the normal distribution of the study data was evaluated using the Kolmogorov-Smirnov test. The study data were summarized using descriptive statistics (number, percentage). The chi-square test was used to compare the categorical variables of the patients and controls. The Student's *t*-test was used to compare the mean differences between two groups of normally distributed continuous variables and the Mann-Whitney *U* test was used when variables did not conform to a normal distribution. Correlations between the clinical variables were analysed using the Pearson Correlation test.

The receiver operator characteristics (ROC) curve was utilized to evaluate the accuracy of MDA, SOD, and CAT in diagnosing ASD. The ROC was plotted to determine the cut-off point. A value of  $p < .05$  was accepted as statistically significant.

## Results

The mean age was  $4.29 \pm 1.10$  years in the ASD group and  $4.64 \pm 1.12$  years in the control group. No statistically significant difference was determined between the groups in terms of age or gender ( $p > .05$ ) (Table 1). The total CARS score was  $40.44 \pm 7.44$  (min-max = 30-60) in the ASD group.

The MDA level was significantly higher in the patient group than in the controls ( $4.16 \pm 1.67$  nmol/mL;  $1.49 \pm 0.58$  nmol/mL (mean  $\pm$  sd), respectively,  $p < .001$ ) (Table 1). The ROC curve for MDA was drawn and the cut-off point from the MDA curve for diagnostic measurements was taken as 2.27 mmol/L. The area remaining under the ROC curve was significant for MDA at 0.937 ( $p < .001$ ) (Table 2). High values

**Table 1.** Characteristics and laboratory results of patients and healthy controls.

|                              | ASD group $n = 52$ | Controls group $n = 48$ | Cohen <i>d</i> | Post hoc power | <i>p</i> |
|------------------------------|--------------------|-------------------------|----------------|----------------|----------|
| Age (years)                  | $4.29 \pm 1.10$    | $4.64 \pm 1.12$         |                |                | .121     |
| Gender (male/female)         | 45/7               | 37/11                   |                |                | .219     |
| MDA (mean $\pm$ sd), nmol/ML | $4.16 \pm 1.67$    | $1.49 \pm 0.58$         | 2.16           | 100%           | <.001    |
| SOD (mean $\pm$ sd), U/g Hb  | $295.06 \pm 55.64$ | $109.26 \pm 38.57$      | 3.88           | 100%           | <.001    |
| CAT (mean $\pm$ sd), U/g Hb  | $184.77 \pm 52.50$ | $72.84 \pm 9.15$        | 2.97           | 100%           | <.001    |

Note: ASD: autism spectrum disorder; SD: standard deviation; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; Cohen's *d* bigger than 0.80 is considered as large effect size.

**Table 2.** Cut-off values of MDA, SOD, CAT, and the differentiation power of the test.

|     | AUC   | Cut-off values | <i>p</i> | Post hoc power (%) |
|-----|-------|----------------|----------|--------------------|
| MDA | 0.937 | 2.27           | <.001    | 98                 |
| SOD | 1.0   | 198.99         | <.001    | 100                |
| CAT | 1.0   | 94.94          | <.001    | 100                |

Note: AUC: area under the curve; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase.

indicated ASD. With a cut-off point of 2.27, sensitivity was 90.4% and specificity was 93.7% (Figure 1).

The SOD levels of the patients with ASD were observed to be higher than those of the controls and this difference between the groups was evaluated as statistically significant ( $295.06 \pm 55.64$  U/g Hb;  $109.26 \pm 38.57$  U/g Hb (mean  $\pm$  sd) respectively,  $p < .001$ ) (Table 1). The ROC curve for SOD was drawn and the cut-off point from the SOD curve for diagnostic measurements was taken as 198.99 U/g Hb. The area remaining under the ROC curve was significant for SOD at 1.000 ( $p < .001$ ) (Table 2). High values indicated ASD. With a cut-off point of 198.99, sensitivity was 100% and specificity was 100% (Figure 2).

The CAT levels of the patients with ASD were observed to be higher than those of the controls and this difference between the groups was evaluated as statistically significant ( $184.77 \pm 52.50$ ;  $72.84 \pm 9.15$  U/g Hb (mean  $\pm$  sd) respectively,  $p < .001$ ) (Table 1). The ROC curve for CAT was drawn and the cut-off point

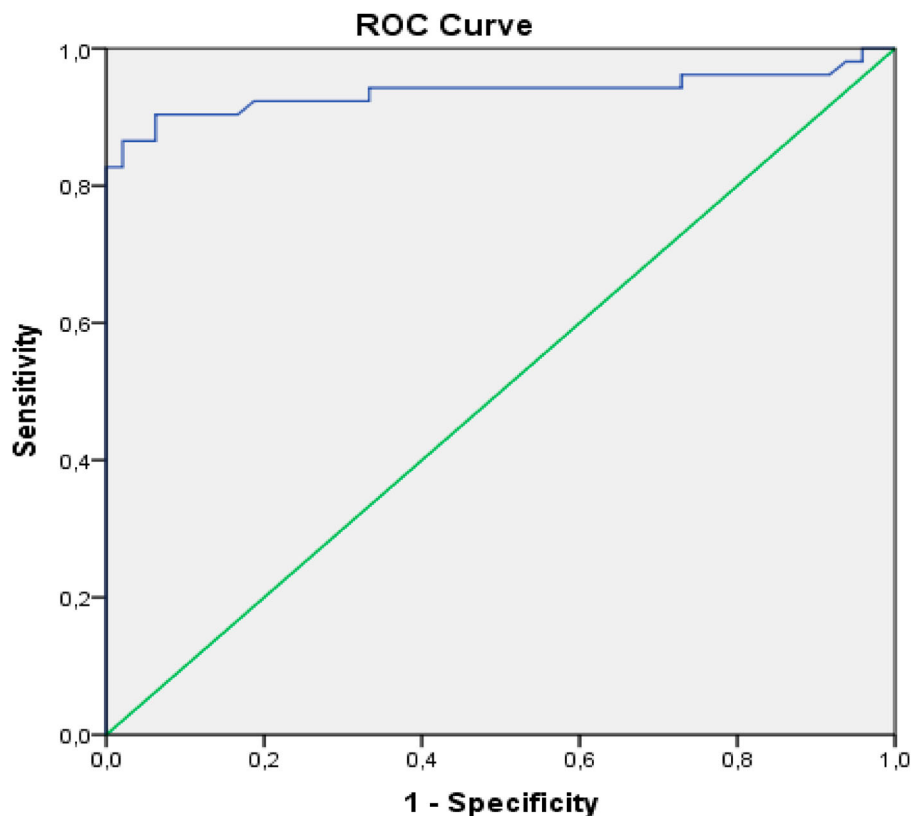
from the SOD curve for diagnostic measurements was taken as 94.94 U/g Hb. The area remaining under the ROC curve was significant for CAT at 1.000 ( $p < .001$ ) (Table 2). High values indicated ASD. With a cut-off point of 94.94, sensitivity was 100% and specificity was 100% (Figure 3).

Cohen's *d* effect size for statistically significant findings is given in Table 1 along with the post hoc power analysis results (a Cohen's *d* result larger than 0.80 is considered to be a large effect size).

According to the Pearson correlation analysis, even though a positive correlation was determined between MDA levels and ASD severity according to the total CARS scores in ASD patients, there was no correlation between SOD and CAT activities and ASD severity according to the total CARS scores ( $r = 0.368$ ,  $p = .007$ ;  $r = 0.032$ ,  $p = .822$ ;  $r = 0.067$ ,  $p = 0.636$ , respectively). There was no correlation between age and these parameters ( $p > .05$ ).

## Discussion

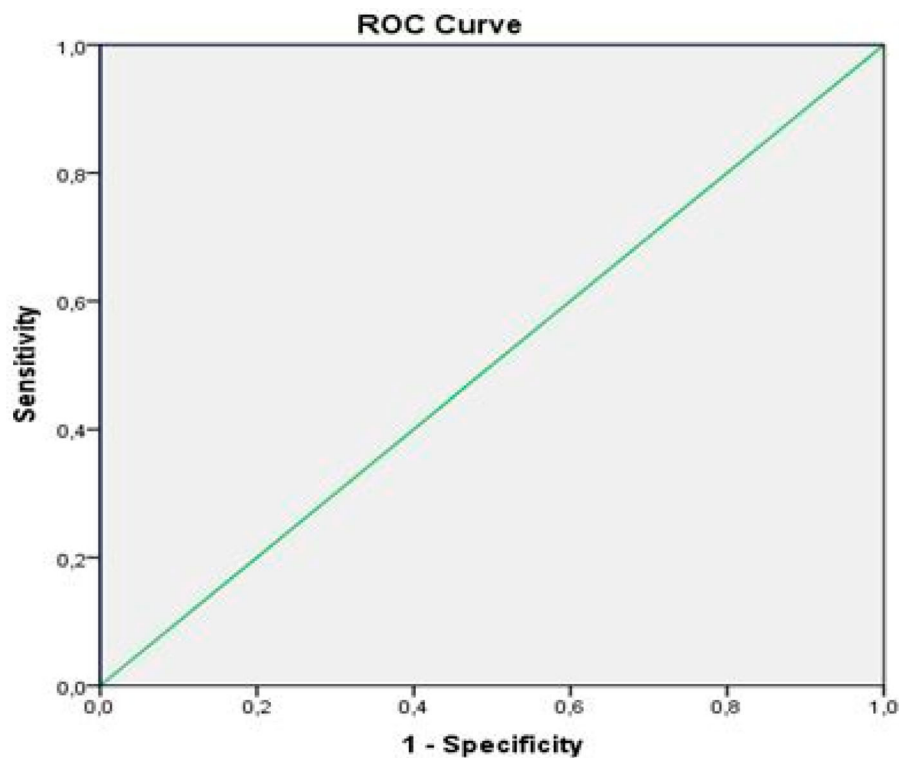
This study evaluated the MDA levels as well as SOD and CAT activity in children with ASD, and their sensitivity and specificity. The main finding of our study has indicated that there was an increase in MDA levels, SOD and CAT activity in children with ASD compared to the controls. In addition, the increased MDA level



Diagonal segments are produced by ties.

**Figure 1.** MDA ROC curve; area under the curve was determined as 0.937 for MDA with a cut-off value of 2.27.

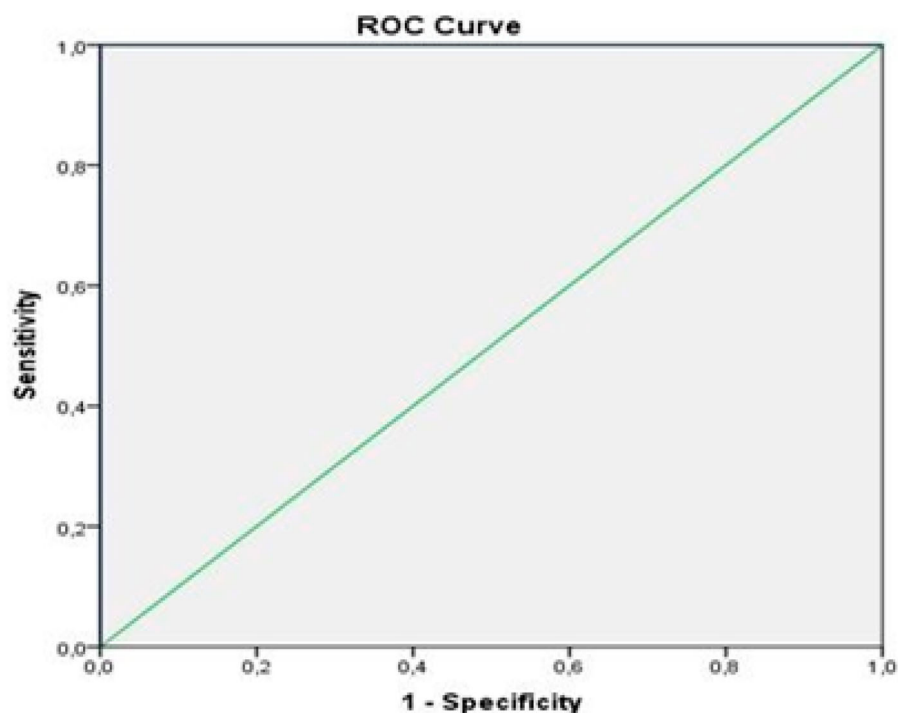




**Figure 2.** SOD ROC curve; area under the curve was determined as 1.0 for MDA with a cut-off value of 198.99.

was determined to have 90.4% sensitivity and 93.7% specificity while SOD and CAT activity were found to have 100% sensitivity and specificity according to ROC analysis. A positive correlation was determined between MDA levels and ASD severity according to the total CARS score.

In a recent meta-analysis of 29 studies involving ASD patients, levels of glutathione, glutathione peroxidase, methionine, and cysteine were seen to be lower to a statistically significant degree in ASD patients while oxidized glutathione was increased to a statistically significant degree [12]. Different studies have reported an



**Figure 3.** CAT ROC curve; area under the curve was determined as 1.0 for MDA with a cut-off value of 94.94.

increase in MDA levels in ASD children compared to healthy control groups [25,29–31]. Chauhan et al. reported higher MDA levels in children with autism while ceruloplasmin and transferrin levels were lower. Increased levels of lipid peroxidation and decreased levels of serum ceruloplasmin and transferrin indicate that ASD children have a higher level of oxidative stress, which is most probably caused by the abnormal metabolism of pro-oxidant metal ions and/or decreased antioxidant proteins [25]. In two studies made in Oman, autistic children were reported to have elevated levels of oxidative stress indicators such as NO, MDA, and protein carbonyl and reduced levels of antioxidant proteins such as ceruloplasmin and transferrin [30,46]. Post-mortem studies have also reported abnormalities in energy metabolism and evidence of oxidative stress. Examination of the cerebellum and cortical structures in autistic children has shown increased levels of lipid hydroperoxides and reduced levels of mitochondrial electron transport chain complexes [47]. The brains of autistic patients have been shown to have increased levels of lipid, protein, and DNA damage [48–50]. The increase in MDA levels in the current study supports these previous findings in the literature.

Several studies have identified changes in enzymes such as SOD and CAT in autism. While MDA levels in ASD patients are consistently increased, SOD and CAT show no such consistency. Varying results have been reported relating to the enzyme levels of SOD and CAT. SOD activity has been shown to be decreased, increased, or unchanged in plasma and erythrocyte [19,29,32–37]. Erythrocyte SOD activity was found to be higher in the ASD children than in the control group in this study. An increased SOD level is considered a compensatory response as protection against the cell damage caused by oxidative stress. The CAT enzyme is directly involved in ROS elimination. Previous studies have reported that CAT activity is reduced in erythrocytes [35,36], but unchanged in plasma and erythrocyte [19,37]. However, an increase in serum CAT activity was reported in a study by Gonzales Fraquale et al. [31]. Antioxidants excreted in urine have been found to be at a significantly lower level than normal in children with ASD, corresponding to the severity of ASD [51]. It would seem that there is a change in antioxidant status in ASD, which may then lead to several further changes in the brain. An increase in erythrocyte CAT activity was found in the current study. These higher levels of CAT activity may be caused by prior higher oxidative stress as a compensatory mechanism.

The second important finding of our study was obtained from the ROC curve analysis applied to assess the diagnostic value of increased oxidative stress parameters. The ROC curve is a tool used for analysis in which sensitivity (the chances of a positive being

correctly identified) is plotted against specificity (the chances of a negative being falsely identified as a positive) for a series of cut-off points corresponding to the entire range of values for a specific biomarker for a given disease in terms of its analytical performance [52].

Currently, there are few studies that have examined the diagnostic value of oxidants and antioxidants in psychiatric disorders. Camkurt et al. [24] reported that increased plasma MDA levels were very valuable markers for diagnosing major depression (area under curve (AUC): 1.0) [24]. In addition, it has been demonstrated that increased serum prolidase activity is a very valuable marker for diagnosing schizophrenia (AUC: 1.0) [38]. CAT was shown by Seleke et al. [39] to have a very high diagnostic value for bipolar disorder (AUC: 0.989) [39]. Currently, there are no generally accepted biomarkers for the diagnosis of ASD or for the evaluation of its severity. There have been studies which have examined oxidative stress parameters in peripheral tissues as a biomarker in ASD [9,15,37,40,53]. The results of studies conducted on the potential diagnostic value of selected biomarkers associated with oxidative stress in our study for ASD are inconclusive. One study reported that decreased serum SOD levels had a sensitivity of 84.7% and a specificity of 71.4% with an AUC of 0.811 in children with ASD [54]. Another study recently reported that increased plasma CAT levels had a sensitivity of 90.0% and a specificity of 27.6% with the AUC at 0.577, that increased plasma SOD levels had a sensitivity of 52.0% and a specificity of 85.7% with the AUC at 0.648, and that increased plasma lipid peroxides levels had a sensitivity of 90.0% and a specificity of 60.0% with the AUC at 0.794 according to ROC analysis in ASD [40]. In the current study, MDA was determined to have high sensitivity (90.4%) and specificity (93.7%) (AUC: 0.937), while SOD and CAT were determined to have very high sensitivity and specificity (100%) (AUC: 1.0). Nevertheless, since MDA has been shown to be high but conflicting results have been reported for SOD and CAT activity in previous studies of ASD children, MDA can be considered more reliable in terms of sensitivity and specificity. Another significant finding of the study was the positive correlation between the increase in MDA levels and ASD severity. Thus, it can be inferred that ASD patients with a high MDA level could have more severe ASD.

The limitations of this study include the small sample size and the cross-sectional design. MDA, SOD, and CAT were measured in the plasma and erythrocyte only, and not in the cerebral spinal fluid. It is still uncertain whether the changes seen in peripheral MDA, SOD, and CAT levels will be repeated in the central nervous system. In terms of diagnostic value, MDA, SOD, and CAT can be considered sub-optimal oxidative stress markers and further studies are required to confirm their validity as biomarkers for ASD.

## Conclusion

The results of the current study showed that oxidative stress levels were higher in the ASD patients than in the control group. With high sensitivity and specificity, it is thought that these selected oxidative stress parameters could be an important biomarker for ASD patients. However, MDA levels may be a more reliable biomarker in terms of diagnosis than SOD and CAT activity as there have been conflicting results in previous studies. Oxidative stress may have a role in the pathogenesis of autism, although the exact contribution of this pathological state has so far not been fully clarified. More studies are needed in the following areas: the early detection of oxidative stress parameters, intervention in cases with elevated levels, the sensitivity and specificity of oxidative stress biomarkers in larger ASD samples, and the reliability of MDA, SOD, and CAT as a biomarker for ASD.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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