Oxysterol Metabolism Balance as a Candidate Biomarker in Autism Spectrum Disorder

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Objective: The aim of this study was to investigate the role of cholesterol metabolism disorders in the etiopathogenesis of Autism Spectrum Disorder (ASD) through the analysis of central and peripheral oxysterol levels (24-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol). These compounds, found in the cholesterol excretion pathways, are considered potential biomarkers for diagnosing and monitoring various neuropsychiatric disorders.

Materials and Methods: This study included 42 children diagnosed with ASD, aged between 1 and 6 years, who had no additional psychiatric or medical illnesses other than cognitive delay/intellectual disability and were not on medication, along with 38 age-matched typically developing children. After comprehensive mental health assessments, the symptom severity in children with ASD was evaluated using the Childhood Autism Rating Scale, Autism Behavior Checklist, and Repetitive Behavior Scale-Revised Form. After the clinical evaluation, peripheral blood samples were obtained from all children. Oxysterol levels were assessed using liquid chromatography coupled with tandem mass spectrometry.

Results: In the ASD group, levels of 24-hydroxycholesterol and 25-hydroxycholesterol were significantly higher compared to the control group, while 27-R-hydroxycholesterol levels were lower. The ratio of 24-hydroxycholesterol (µg/L) to 27-hydroxycholesterol (µg/L) was notably higher in the autism group. The receiver operating characteristic (ROC) analysis indicated that this ratio was statistically significant and could discriminate between ASD and non-ASD diagnoses with “acceptable discrimination potential.”

Conclusion: Our findings suggest that alterations in oxysterol levels, commonly associated with neurodegenerative processes, can also be observed in ASD and may serve as a potential candidate biomarker for the disorder.

Keywords: Autism spectrum disorder, biomarker, oxysterol, 24-hydroxycholesterol, 27-hydroxycholesterol.
INTRODUCTION

Cholesterol is a crucial component of myelin, which constitutes 70% of brain content and neuronal cell membranes. It has also been shown to contribute to synaptogenesis, axonal plasticity, neuroprotection, and the proliferation of glial cells. Cholesterol is not uniformly distributed across biological membranes; it tends to accumulate in lipid rafts along with other lipid molecules. These lipid rafts act as hubs for cellular signaling, play a role in regulating synaptic plasticity, and are implicated in various neurological disorders, including Alzheimer’s, Parkinson’s, and Huntington’s disease. There is a substantial overlap between the signaling factors or neural circuits believed to be associated with Autism Spectrum Disorder (ASD) and the synaptic proteins linked to lipid rafts. Additionally, research suggests a functional connection between lipid rafts and synaptic dysfunctions in autism. As such, it is believed that disrupted cholesterol metabolism may play a role in the etiology of ASD by impairing lipid raft functions.

There is increasing evidence that disruptions in cholesterol metabolism are common in neuropsychiatric diseases, which has led to a focus on oxysterols. These compounds are formed through the enzymatic oxidation of cholesterol by CYP450 enzymes or non-enzymatically by autoxidation. Oxysterols act as regulators in various biological processes, and although present in minimal amounts in mammalian tissues, can increase significantly under various pathological conditions. Consequently, oxysterols, which are generated in the cholesterol excretion pathways, are speculated to serve as biomarkers. Changes in oxysterol concentrations have been observed in several neuropsychiatric conditions that exhibit similarities to ASD, especially in terms of synaptic dysfunction.

To our knowledge, only one study documented in the literature has investigated the correlation between oxysterol levels and ASD. This study suggested that 24-hydroxycholesterol (24-HC), a type of oxysterol, could serve as a diagnostic tool. The final product and a key indicator of brain cholesterol metabolism, is crucial for regulating brain cholesterol levels. It is hypothesized that 24-HC may induce alterations in brain function through cytotoxicity, oxidative stress, apoptosis, and synaptic irregularities, all implicated in the development of ASD. Another oxysterol, 27-hydroxycholesterol (27-HC), linked to neurodegenerative mechanisms, originates from sources outside the brain and enters brain tissues by crossing the blood-brain barrier. Unlike 24-HC, the concentration of 27-HC progressively increases from white matter to gray matter. Within human nerve cells, the intake of 27-HC, estimated at 5 milligrams per day, has been shown to reduce beta-amyloid production and regulate the expression levels of ATP Binding Cassette Transporter G1 (ABCG1), and Apolipoprotein E (APOE). Several studies have highlighted fluctuations in the blood levels of 27-HC in various neurodegenerative diseases. Furthermore, some studies underscore the importance of maintaining a balance between 24-HC and 27-HC for optimal neuronal function. Additionally, the disruption of this equilibrium has been identified as a critical factor contributing to amyloidogenesis and the progression of neurodegeneration. 25-hydroxycholesterol (25-HC), another oxysterol involved in peripheral cholesterol metabolism, shares a close molecular structure with 24-HC and 27-HC. Precise quantification of other oxysterols requires elution. Although the literature on diseases and pathological mechanisms associated with oxysterols, which are involved in many metabolic pathways, is growing rapidly, more research is still needed.

The objective of this study is to evaluate the levels of the most crucial oxysterols in the cholesterol pathway—24-HC, 27-HC, and 25-HC—along with clinical parameters in children newly diagnosed with autism, and to compare these profiles with those of a non-autistic group. Additionally, the study aims to compare the balance between 24-HC, representing the primary product of brain oxysterol metabolism, and 27-HC, representing peripheral oxysterol metabolism, between the groups. It also investigates the hypothesis that oxysterols could serve as biomarkers in ASD.

MATERIALS AND METHODS

Participants

The study received approval from the Ethics Committee for Non-Interventional Clinical Research at Ankara University Faculty of Medicine on May 13, 2019 (decision no: 09-712-19). We analyzed children between the ages of 12 and 60 months who were referred to our Infant Mental Health Unit from June 2019 to May 2020. Of these, 42 children diagnosed with ASD based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria, and with no comorbid psychiatric diagnoses except for cognitive developmental delays/mental disability, were randomly selected and included in the autism group. Additionally, 38 children aged 12-72 months, who agreed to participate in the study after being informed about it and who did not meet any psychiatric diagnosis according to DSM-5 criteria, were randomly selected among the children who applied to the clinic or the relatives of the applicant families. They were age-matched with the ASD group and included in the study as the non-autistic group. The exclusion criteria for both groups included the use of any psychotropic medication, the presence of any chronic medical condition, and/or parents experiencing physical or mental issues that would hinder their participation in survey interviews. The study was conducted cross-sectionally.
Procedure
The children first underwent a diagnostic psychiatric evaluation according to DSM-5 criteria, conducted by a child psychiatrist. For those in the autism group, the Childhood Autism Rating Scale was completed. Following the diagnostic examination, a socio-demographic data form was used to collect comprehensive demographic and medical histories, and height and weight measurements of the children were taken. Parents of children in the autism group then completed the Autism Behavior Checklist and the Repetitive Behavior Scale. The developmental levels of infants and toddlers were assessed using the Ankara Developmental Screening Inventory (ADSI), administered by a clinical psychologist, with parental reports serving as the primary source of data. After the clinical assessments, peripheral blood samples were collected in the morning from both groups following an 8-hour fasting period for biochemical analyses.

Clinical Assessment Tools
Sociodemographic Questionnaire
The sociodemographic data form, completed by the researcher during interviews with the parents, included a series of items such as details about pregnancy and birth, developmental milestones, and ASD diagnosis.

Ankara Developmental Screening Inventory (ADSI)
The ADSI is a parent-reported assessment tool comprising 154 items, administered by a physician to evaluate the developmental status of children within the age range of 0 to 6 years. It evaluates various domains including language-cognitive abilities, social interaction capabilities, fine and gross motor skills, and self-care proficiency. The ADSI is deemed culturally appropriate, validated, and reliable for measuring development.19

Childhood Autism Rating Scale (CARS)
CARS is a behavioral rating scale extensively used in clinical practice to differentiate autism from other neurodevelopmental disorders.20 The validity and reliability of the Turkish version of the scale have been established, with a cut-off score for diagnosing autism set at 29.5.21

The Autism Behavior Checklist (ABC)
ABC is a scale designed to assess symptom domains, symptom severity, and clinical progression in children with autism.22 The validity and reliability of the Turkish version of the scale have been confirmed, and the cut-off score for autism diagnosis has been set at 39.23

The Repetitive Behavior Scale - Revised (RBS-R)
RBS-R assesses repetitive behaviors and their severity in children.24 The increase in scores from the scale, for which the validity and reliability of the Turkish version have been validated, indicates an increase in the severity of repetitive behaviors.25

Oxysterol Level Analysis
Oxysterol level analysis was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Thermo Access Max Tandem Mass Spectrometer, following the method developed by Sugimoto et al.26 The samples prepared for analysis included the addition of 10 µL of phosphate-buffered saline (PBS) buffer, 2 mL of sodium hydroxide, and 5 mL of methanol, and were incubated at 50 °C for one hour for saponification. Post-saponification, the samples underwent hexane treatment, followed by preparation for extraction and analysis. Calibrators, controls, and internal standards were prepared using acetonitrile. The calibrators, controls, and samples were analyzed with LC-MS/MS, and chromatographic separation was performed for 25-HC, 24-HC, and 27-HC. Detected oxysterols were isolated and quantified through mass spectrometry.

Statistical Analysis
Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) Statistics 22.0, with the significance threshold set at p=0.05. Descriptive statistics were presented as means and standard deviations for continuous variables, and as frequencies and percentages for categorical variables, depending on data distribution. Categorical data were analyzed using the Chi-square test, while continuous data were analyzed with the Mann-Whitney U test, based on group size and normality assumptions. The diagnostic accuracy of the 24-HC/27R-HC ratio was assessed using receiver operating characteristic (ROC) curve analysis. The area under the curve, along with sensitivity and specificity values, were calculated to evaluate the results of the ROC curve analysis and to determine diagnostic efficiency.

RESULTS
Sociodemographic Characteristics
The mean age of the children with ASD was 3.55±1.11 years, compared to 3.50±1.03 years in the non-autistic group (p=0.828). The ASD group comprised 47.6% (n=20) girls and 52.4% (n=22) boys, while the non-autistic group consisted of an equal distribution of 50% (n=19) girls and 50% (n=19) boys (p=0.832). The mean Body Mass Index (BMI) for children with ASD was 17.68±2.02, versus 17.8±2.44 in the control group (p=0.711). Among the children with ASD, 85.7% (n=36) lived in nuclear families, 9.5% (n=4) in extended families, and 4.8% (n=2) with a single parent due to divorce. In the control group,
81.6% (n=31) lived in nuclear families, 2.6% (n=1) in extended families, and 15.8% (n=6) with a single parent due to divorce (p=0.199). The groups showed similar characteristics in terms of parents' mean age and education level (p>0.05). Table 1 presents the sociodemographic characteristics of the groups.

### Clinical Characteristics of the ASD Group

In the evaluation of children with ASD concerning the level of autism, 26.2% (n=11) exhibited low autism, 52.4% (n=22) mid-level autism, and 21.4% (n=9) severe autism. Additionally, 83.3% (n=35) of these children's diagnoses were associated with cognitive developmental delays. Scale scores revealed that the mean total score on the CARS was 38±5.65. For the ABC, the mean total score was 47.3±20.37, with mean scores on the sensory subscale at 8.52±4.09, relating behaviors at 9.71±4.24, body and object use at 9.61±5.07, social and self-help at 9.21±4.92, and language at 10.23±5.66. The mean total score for the RBS-R was 21.11±14.21. Table 2 presents the clinical characteristics and scale scores of the ASD group.

### Oxysterol Analyses

Table 3 compares oxysterol levels between groups. No significant differences were observed in the levels of 27-S-HC and 24-S-HC between the groups. However, the levels of 25-HC and 24-HC were significantly higher in the autism group compared to the control group, while 27-R-HC levels were significantly lower in the autism group. The 24-HC (µg/L)/27R-HC (µg/L) ratio also showed a notable difference between the groups. No statistically significant correlation was found between autism severity levels and oxysterol levels in the ASD group (p>0.05).

We investigated the cutoff value of the 24-HC/27R-HC ratio, which may be a determinant for ASD, using ROC analysis. The ROC analysis was conducted to assess the predictive capability of the 24-HC/27R-HC ratio for autism. With a cutoff value of 1.64, the area under the curve was determined to be 0.79 (95% confidence interval (CI): 0.689–0.894, p<0.001), exhibiting a sensitivity of 72% and a specificity of 73%. The positive and negative predictive values for the 24-HC/27R-HC ratio of 1.64 were 73.2% and 73%, respectively (Fig. 1).
DISCUSSION

Several studies have compared cholesterol levels in children with autism to those in healthy children.27-29 While some studies have yielded conflicting results, the prevailing consensus suggests that dysfunctions in cholesterol metabolism are often evident in individuals with autism.30 Numerous factors, such as age, gender, and diet, introduce confounding variables that diminish the utility of cholesterol levels as biomarkers. Consequently, the absence of distinct indicators for early detection prompts exploration into metabolic byproducts of the cholesterol cycle as potential candidates for biomarkers.31

In this context, increasing evidence in recent years has underscored the importance of disorders in neuronal development, synaptic formation, and cholesterol metabolism in autism, making oxysterols a significant focus in the etiopathogenesis of the condition.13 In our study, both central and peripheral parameters of oxysterol metabolism, crucial for neuronal development, were evaluated in ASD and non-autistic groups. When examining the groups based on oxysterol levels, 25-HC and 24-HC levels were significantly higher in the autism group compared to the control group, while 27-R-HC levels were low. The 24-HC (µg/L) to 27R-HC (µg/L) ratio was significantly higher in the autism group. ROC analysis showed that this ratio was statistically significant and could discriminate an ASD diagnosis with “acceptable discrimination potential.”

To our knowledge, only one study in the existing literature examines the correlation between oxysterol levels and autism. In their research, Grayaa et al.12 conducted a comparison between 36 autistic children and 38 typically developing children. Their findings suggested that ASD correlates with changes in the levels of circulating oxysterols. The researchers have suggested that 24-HC, specifically, stands as an independent risk factor for the condition and could potentially serve as a diagnostic aid. Unlike our research, no significant differences related to the disease were observed in 27-HC and other oxysterol levels. In our study, 21.4% of the autism group was classified as having severe autism, compared to the much higher rate of 75.6% reported by Grayaa et al.12 for children with severe autism. Given that the exact role of oxysterol levels in the etiology of autism remains partially understood, this disparity is considered the primary variable that could influence the findings. Furthermore, oxysterols are known to exist in the bloodstream at extremely low concentrations.9 Therefore, studies with larger sample sizes are necessary to consistently identify the expected alterations in ASD. Although the research findings do not coincide, they collectively indicate a disruption in neuronal system cholesterol homeostasis. Disturbances in cholesterol balance could potentially lead to functional changes in the central nervous

<table>
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<tr>
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<th>ASD (n=42) Median (Min–Max)</th>
<th>NA (n=38) Median (Min–Max)</th>
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<tr>
<td>25 HC (µg/L)</td>
<td>66.31 (8.86–191.36)</td>
<td>48.52 (0.52–113.96)</td>
<td>0.046&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>24S HC (µg/L)</td>
<td>66.15 (9.91–162.57)</td>
<td>46.57 (11.6–185.33)</td>
<td>0.108&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 HC (µg/L)</td>
<td>68.11 (11.41–211.77)</td>
<td>45.62 (16.07–111.33)</td>
<td>0.031&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>27R HC (µg/L)</td>
<td>27.22 (6.95–121.43)</td>
<td>48.34 (7.66–187.36)</td>
<td>0.009&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>27S HC (µg/L)</td>
<td>24.93 (2.89–110.76)</td>
<td>40.62 (8.62–185.87)</td>
<td>0.105&lt;sup&gt;a&lt;/sup&gt;</td>
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| 24 HC (µg/L)/27R HC (µg/L) | 2.51 (0.17–9.34) | 1.27 (0.24–4.69) | <0.001<sup>a</sup>

<sup>a: Mann-Whitney U Test; ASD: Autism spectrum disorder; NA: Non-autistic.</sup>
system through various mechanisms, including oxidative stress, cytotoxicity, synaptic dysfunction, and apoptosis, all recognized contributors to the pathophysiology of ASD. It is also known to affect membrane permeability and the integrity of lipid rafts, which play a significant role in the pathogenesis of many neuropsychiatric diseases. Lipid rafts, crucial for cellular signal transmission, have been implicated in regulating synaptic plasticity and are linked to neurodegenerative conditions such as Alzheimer’s, Parkinson’s, and Huntington’s disease. Additionally, there is considerable overlap between signaling molecules, lipid rafts, and associated synaptic proteins believed to be involved in ASD pathogenesis. In summary, cholesterol, a major component of lipid rafts in ASD, is thought to impair the function of lipid rafts with dysregulation in the production-destruction homeostasis, establishing a functional link between this condition and synaptic dysfunction.

To date, 27-HC and 24-HC are the oxysterols most implicated in neurodegenerative processes. Research on brain oxysterol levels has highlighted the critical role of the balance between 27-HC and 24-HC in maintaining neuronal functions. Significantly increased 27-HC/24-HC ratios in certain brain regions, resulting from disruptions in the brain’s cholesterol production and breakdown/excretion cycle, have been associated with neurodegenerative processes in some studies. Given this information, it can be suggested that the low peripheral 27-R-HC levels observed in our study may be associated with an increased accumulation of this compound in the brain. This could potentially underlie the increased volume of gray matter structures observed in ASD, although this hypothesis requires validation through postmortem and cellular studies. Moreover, studies have shown that 27-HC, entering the brain through the blood-brain barrier after peripheral production, decreases amyloid-beta peptide production. This inhibition further promotes the buildup of amyloid precursor protein and sustains an anabolic state. Earlier research on Autism Spectrum Disorder has demonstrated that elevated levels of amyloid precursor protein lead to an augmentation in intracranial neuronal growth, along with increases in neuronal size and density, fostering the expansion of gray matter.

Limitations of the Study
Since a homogeneous patient group was selected for our study, our findings may primarily pertain to autism spectrum disorder. However, it is important to further evaluate our hypothesis regarding the disruption of oxysterol metabolism in ASD within the broader context of neurodevelopmental processes and prognosis. This should be pursued through follow-up studies involving a larger sample size. Additionally, our study did not include a biochemical analysis of parents. Conducting this assessment in future research could provide important data for excluding familial cholesterol disorders.

CONCLUSION
Our study findings revealed that autistic children in our cohort had significantly higher levels of 24-HC (µg/L) and 25-HC (µg/L), but lower levels of 27-HC compared to typically developing children. Furthermore, the ratio of 24-HC (µg/L) to 27R-HC (µg/L) shows promise as a potential biomarker for autism. Although our study makes important contributions to the literature, community-based case-control studies are necessary to confirm and generalize the results. Ongoing studies are exploring the relationship between alterations in oxysterol metabolism observed in ASD and cellular-level mechanisms such as membrane lipid disorders, inflammation, abnormal immune system activation, and oxidative stress. Molecular studies in brain tissue will enhance our understanding in this field. We hope that our study, which evaluates both peripheral and central oxysterol levels and their balance in ASD, will provide valuable insights for future research in this area.

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