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The Role of Sertoli Cell Hormones in Male Preponderance Observed in Autism Spectrum Disorder

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ABSTRACT

Introduction: There is a significant, but poorly understood, male preponderance in prevalence of autism spectrum disorder (ASD). The aim of this study was to examine the relationship between male preponderance in ASD and Inhibin B (InhB) and Anti-Müllerian hormone (AMH) levels and the 2D/4D finger ratio associated with fetal androgen exposure.

Methods: 42 patients with ASD and 42 neurotypical controls between the ages of 5 and 10 were included. ASD diagnosis and severity were determined using K-SADS PL (Kiddie-SADS – Present and Life Time) Version 2016 and the Childhood Autism Rating Scale (CARS). Serum InhB and AMH were measured. The 2D/4D finger length ratio was also calculated for hand anthropometric measurements.

Results: Serum InhB levels were higher in children diagnosed with ASD compared to the neurotypical controls (p=0.003). Serum AMH levels were similar in both groups. Positive correlation was determined between AMH and CARS scores (r=0.315, p=0.05). 2D/4D finger ratios in the ASD group were significantly lower than in the control group (p<0.001).

Conclusion: The study findings suggest that InhB, AMH, and fetal testosterone may be associated with male preponderance in ASD. More research is now required for a better understanding of this subject.

Keywords: Anti-müllerian hormone, autism spectrum disorder, inhibin B, male preponderance

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by difficulty in social communication, limited areas of interest, and repetitive behaviors (1). About 1 in 36 children has been identified with ASD according to estimates from CDC's Autism and Developmental Disabilities Monitoring (ADDM) Network (2). Epidemiological studies have reported that ASD is more common in boys than girls, with a ratio between 2:1 and 5:1 (3). Although the results of studies on male preponderance in ASD are consistent, the etiology of this preponderance has not been clarified yet. Research on this subject has increased in recent years (4,5). Studies regarding male preponderance in ASD have focused on genetic factors. The genes associated with ASD are largely autosomal and have no marked relationship with sexual dimorphism (6). This also suggests the involvement of other components in addition to genetic factors. One of these may be gender-related hormonal factors. In recent years, there has been an increasing interest in the relationship between male preponderance of ASD and gender-related hormonal factors. The hypothesis of "Extreme Male Brain" suggested that exposure to fetal testosterone is associated with early male preponderance in ASD. Fetal testosterone plays an important role in the early stages of brain development by reacting with neurotransmitters and neuropeptides (7–11). Although several studies have not directly addressed fetal testosterone levels in the early developmental period, the 2D/4D ratio calculated by dividing the length of the second finger by the length of the fourth is accepted as a retrospective marker of fetal testosterone levels in the literature (12,13).

Highlights

- **Inhibin-B level was higher in male children with ASD compared to controls.**
- **The 2D/4D finger length ratio was lower in male children diagnosed with ASD compared to controls.**
- **Sertoli cell hormones might be associated with male preponderance in ASD.**

Most of the brain development occurs during the postnatal period when testosterone levels are at their lowest. Since postnatal brain sexual dimorphism cannot be explained by exposure to fetal testosterone, it is thought that other factors may play a role (14). The Sertoli cell hormones Inhibin B (InhB) and Anti-Müllerian hormone (AMH) are particularly noteworthy in that context. These hormones are members of the TGF-B super family involved in every stage of brain development and play an important role in sex differentiation (15). AMH receptors are present in most neurons and contribute to sex bias in the brain (16). A rat study found that AMH was necessary for normal social development in males. The findings of that animal study provide a rationale for investigating levels of AMH in children with disorders of socialization, such as ASD, with a male preponderance (17). The receptors modulated by InhB are widely

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expressed in the brain and have several effects on neurons (18). However, very few studies have investigated Sertoli cell hormones' effects on the brain, and even fewer have examined that the relationship between male preponderance of ASD and InhB and AMH. To determine the effects of Sertoli cell hormones, Pankhurst et al (2012) compared serum levels of InhB and AMH between children with ASD and neurotypical controls. The authors emphasized the need for further studies on the subject (16). The present study was intended to address these research gaps.

Testosterone in the fetal period and sertoli cell hormones in the postnatal period are hormonal factors that have been suggested to have effects on brain development. In this study, the researchers made effort to evaluate the relationship between these hormonal factors and ASD in a temporally integrated manner. The primary aim of this study was to examine whether serum levels of InhB and AMH and the 2D/4D ratio, which is a retrospective marker of fetal testosterone, in children with ASD differ from neurotypical control subjects. The secondary aim was also to investigate the relationship between serum levels of sertoli cell hormones and ASD symptoms.

METHODS

Sampling

The study was conducted between February and September 2021 at the Istanbul University, Istanbul Faculty of Medicine, Department of Child and Adolescent Mental Health and Diseases. A sample size of 86 (allocation 1:1) was needed to reach 80% power to examine a difference in serum AMH levels between children with ASD and controls, using a two-group independent t-test with a 0.05 two-sided significance level based upon results from Bilbay Kaynar's thesis (19). In light of this recommendation, the research was conducted with 84 volunteer participants. The case group consisted of 42 volunteer boys between the ages of 6 and 10 who have referred to our clinic and were diagnosed with ASD based on DSM-5 diagnostic criteria and Schedule for Affective Disorders and Schizophrenia for School Aged Children (6–18 Years) Kiddie-SADS – Present and Life Time Version 2016 (KSAD-S – PL) semi-structured interviews. Forty-two volunteer boys of similar ages with no diagnoses of ASD presenting for routine health checks were included in the control group. ASD was excluded in the control group on the basis of DSM-5 diagnostic criteria and KSAD-S interviews.

Inclusion criteria for the case and control groups were 1) age between 6 and 10, 2) male gender, 3) adequate Turkish language skills on the part of the parents, and 4) voluntary agreement to participation. Exclusion criteria for both groups were 1) any diagnosed metabolic, genetic, or progressive neurological disease, 2) any diagnosed visual or auditory disability, 3) a history of infection in the previous month, 4) history of long-term drug use due to a history any other chronic medical disease, and 5) failure to complete the tests and evaluations or withdrawal from the study. Individuals with any neurodevelopmental disorder diagnosed based on KSAD-S interviews were also excluded from the case group.

Study Flow Chart

Sixty-eight individuals between the ages of 6 and 10 diagnosed with ASD following DSM-5 based diagnostic interviews were identified from the cases referred to our clinic. Patients consenting to take part following provision of preliminary information about the study were included in the analysis. Six cases were excluded due to genetic diseases, 11 due to inability to perform 2D/4D finger length measurement, and nine due to inability to perform blood collection. Forty-two cases from the 68 meeting the inclusion and exclusion criteria were enrolled in the study. The parents were informed about the study, and verbal consent was obtained from children and written and verbal consent was obtained from the parents. Kiddie-SADS – Present and Life Time Version 2016 and CARS were applied by the first author.

Sixty-three children of similar ages who have referred to the hospital for routine health checks were included in the control group. Attention deficit hyperactivity disorder was determined in seven children and specific learning disorder in four at initial assessment at DSM 5-based diagnostic interview, and these were excluded from the study. Cases with no comorbid neurodevelopmental disease at initial evaluation were informed about the study and enrolled. Subsequently, ADHD was diagnosed using KDSAD-S in two participants from the control group, and while 2D/4D finger measurement could not be performed in three cases, and blood could not be collected in four. These individuals were also excluded from the study, which continued with 42 members of the control group. This study was approved by the Istanbul University, Faculty of Medicine Clinical Research Ethics Committee (Case Number 2021/45, Date: 08/01/2021) and all procedures were conducted in accordance with the principles of the Declaration of Helsinki.

Scales Used in the Research

Kiddie-SADS – Present and Life Time Version 2016 (KSAD-S – PL) (Schedule for Affective Disorders and Schizophrenia for School Aged Children (6–18 Years))

This semi-structured interview is employed to evaluate psychiatric symptoms in children and adolescents and to determine previous and current psychopathologies. The Schedule for Affective Disorders and Schizophrenia was first developed by Chambers et al. (1985), and was subsequently modified as "Present and Life Time Version" based on DSM III and DSM IV diagnostic criteria by Kaufman et al. (20,21). The reliability and validity of the Turkish language adaptation of the final version updated based on DSM-5 diagnostic criteria were confirmed by Ünal et al. (22).

Childhood Autism Rating Scale (CARS)

Childhood autism rating scale was developed by Schopler et al. for the differential diagnosis of autism from other developmental disorders. The Turkish reliability and validity studies were performed by Gassaloğlu et al. Childhood autism rating scale consists of 15 items, each scored between 1 to 4. The lowest possible score is 15 and the highest possible score is 60. The cut-off value of the Turkish language version was determined as 30. The Cronbach alpha coefficient of the Turkish language version was calculated as 0.95. The Cronbach α coefficient of the CARS in the present study was 0.84 (23).

Sample Collection, Storage, and Serum Level Analysis

Venous blood specimens collected from the children in the ASD and control groups between 08:00 and 10:00 after 12-h fasting were placed into dry tubes. Routine biochemical parameters were measured using a Cobas e602 device for AMH and a Cobas 8000 autoanalyzer (Roche Diagnostics, Mannheim, Germany) for albumin. Measurements were conducted in the Istanbul University Medical Faculty Central Laboratory. InhB measurement was performed using ELISA (Bioassay Technology Laboratory, Shanghai, China) at the Istanbul University Medical Faculty Medical Biochemistry Department. The range of the InhB ELISA kit was 7.81–500 pg/mL, and its sensitivity was 4.69 pg/mL.

Hand Anthropometric Measurements

Several studies have reported that the right hand is more representative of the effect of prenatal androgen. Right finger length was therefore measured in the present study (24–26). Finger lengths were measured directly from the palmar surface using calipers. The tensed fingers were placed on a flat, hard surface. Adduction was measured from the 2nd to the 5th fingers, while the thumb was measured from the palmar aspect in partial extension. The right 2nd and 4th fingers were measured. For

the 2nd finger, the distance between the mid-point of the proximal line dividing the base of the finger from the palm and the tip of the finger was measured. For the 4th finger, the distance between the mid-point of the proximal line dividing the base of the finger from the palm and the tip of the finger was measured (13). In order to calculate the ratio of the 2nd finger to the 4th, the formula 'length of the 2nd finger/length of the 4th finger' (2D: 4D) was employed. Hand anthropometric measurements were performed using STAINLESS brand digital vernier calipers sensitive to 0.01 mm.

Statistical Analysis

Data analysis was performed on IBM Statistical Package for Social Sciences (SPSS) program for Windows version 21.0 software. Descriptive statistics were presented as percentage, arithmetic mean, and standard deviation. Distribution of data was assessed using the Kolmogorov-Smirnov test. Normally distributed data were analyzed using Student's t test, while the Mann-Whitney U test was used for non-normally distributed data. Partial correlation analysis was employed to determine correlations. Pearson's chi-square test (χ^2) and Fisher's Exact test were applied to evaluate relationships between qualitative data. Multivariate analysis of covariance (MANCOVA) was planned to avoid Type II errors that might arise as a result of the comparison of multiple variables and to take into account potential confounding factors. Multivariate analysis of covariance test revealed a significant difference between the groups, and one-way analysis of covariance (ANCOVA) was applied to the dependent variables on an individual basis. Before the application of MANCOVA and ANCOVA, non-normally distributed variables were converted to normal distribution using logarithmic transformations. Serum log-InhB and AMH levels were compared between the case and control groups using MANCOVA test. P values <0.05 were regarded as statistically significant.

RESULTS

The study population consisted of 84 participants: 42 patients diagnosed with ASD and 42 neurotypical controls. Mean ages were similar between the two groups, with 8.22±1.18 years in the case group and 8.53±1.20 in the control group (t=1.211, p=0.229). Mean body mass index (BMI) was 17.23±3.78 in the case group and 18.33±3.17 in the control group. The two groups were also similar in terms of BMI (t=1.425, p=0.158). Both maternal (z=-3.544, p<0.001) and paternal (z=-3.325, p<0.001) education levels were significantly higher in the control group compared to the case group. The sociodemographic characteristics of the case and control groups are summarized in Table 1.

The mean total CARS score of the case group was 38.53±5.37. On the basis of their CARS scores, 18 cases were assessed as mild-moderate, and 24 as severe. Evaluation with KSAD-S – PL revealed at least one accompanying psychopathology in 81% (n=34) of the case group. Attention deficit hyperactivity disorder was present in 27 cases, at least one anxiety disorder in 13, enuresis in three, oppositional defiant disorder in two, bipolar disorder in two, tic disorder in two, and encopresis in one. At least one mental disorder was present in 40.5%(n=17) of the control group based on KSAD-S – PL, at least one anxiety disorder being present in 14 cases, enuresis in four, and obsessive compulsive disorder in one. The diagnostic characteristics of the case and control groups based on KSAD-S – PL are summarized in Table 2.

Serum InhB and AMH values were compared between the case and control groups using MANCOVA test, age, fathers' age and BMI as covariants. Multivariate analysis of covariance test identified significant differences between the groups (Pillai's Trace V=0.109, F (2.77)=4.700, p=0.012, ηp^2=0.109). At ANCOVA analysis, the same factors were adopted as covariants in order to ascertain which dependent variables represented the differences between the groups. While statistically significant differences were determined between the groups' InhB levels (F (1.78)=9.514, p=0.003), their AMH levels were similar (F (1.78)=0.035, p=0.853). The case and control group serum InhB and AMH levels are summarized in Table 3.

Right hand 2D/4D finger length ratios were significantly lower in the case group compared to the control group (z=-3.515, p<0.001). Left-hand dominance was more common in the case group, although the difference between the two groups was not statistically significant (x^2=2.029 p=0.154). Case and control group 2D/4D finger length ratios and hand preferences are summarized in Table 4.

Partial correlation analysis was performed in order to determine relationships between CARS scores and InhB and AMH levels and the

Table 1. Sociodemographic characteristics of the case and control groups

Variable	Case Group (n=42)	Control Group (n=42)	p value
Age, years, Mean ± SD	8.22±1.18	8.53±1.20	0.229[a]
BMI (kg/m^2), Mean ± SD	17.23±3.78	18.33±3.17	0.158[a]
Maternal age	37.10±5.85	37.69±5.85	0.643[a]
Paternal age	40.95±6.73	41.43±5.42	0.722[a]
Mean duration of education among mothers	7.90±4.43	11.69±5.47	0.001[b]
Mean duration of education among fathers	9.73±4.19	12.71±4.00	0.001[b]
Family income below minimum wage (n)	57.1% (24)	2.4% (1)	0.001[c]
Smoking during pregnancy	14.3% (6)	11.9% (5)	0.746[d]
Alcohol/substance use during pregnancy	0%	0%	NS
Prematurity	19.04% (8)	2.4% (1)	0.024[c]
SGA	9.5% (4)	11.9% (5)	0.500[c]

a: Independent sample t-test; b: Mann-Whitney U test; c: Fisher's exact test; d: Pearson's chi-square test
BMI: Body mass index; SD: Standard deviation; SGA: Small for gestational age.

Table 2. A comparison of KSAD-S-PL diagnoses in the case and control groups

	Case Group (n: 42)	Control Group (n: 42)	p
Depression	0	0	
Bipolar disorder	2(4.8%)	0	0.494[a]
Psychosis	0	0	
Panic disorder	0	0	
Separation anxiety	3(7.1%)	3(7.1%)	NS
Social phobia	0	5(11.9%)	**0.055[a]**
Specific phobia	12(28.6%)	10(23.8%)	0.620[b]
Generalized anxiety disorder	0	3(7.1%)	0.241[a]
OCD	0	1(2.4%)	NS
Enuresis	3(7.1%)	4(9.5%)	NS
Encopresis	1 (2.4%)	0	NS
Anorexia	0	0	NS
Bulimia	0	0	NS
ADHD	27(64.3%)	0	**0.001[a]**
Oppositional defiant disorder	2 (4.8%)	0	0.494
Behavioral disorder	0	0	
PTSD	0	0	
Tic disorder	2 (4%.8)	0	0.494

a: Chi-square test; b: Pearson's chi-square.
ADHD: Attention deficit hyperactivity disorder; OCD: Obsessive compulsive disorder; PTSD: Post-traumatic stress disorder.

Table 3. Comparison of inhibin-B and anti-Müllerian hormone levels between the case and control groups

Variable	Case Group# (n=42)	Control Group (n=42)	ANCOVA[a]				
			t/z	P	F	p	ηp^2
Inhibin-B	302.64±231.24	215.03±176.61	-2.081	0.037[a,*]	9.514	**0.003**	0.109
AMH	96.17±47.62	95.104±27.83	-0.125	0.901[b]	0.035	0.853	<0.001

Covariants: BMI, Age-years, Father's age.
a: Mann-Whitney U test; b: Student t test.
*Logarithmic transformation was performed prior to ANCOVA.

Table 4. Comparison of right-left 2D/4D and hand preferences in the case and control groups

	ASD (n: 42)	Control (n: 42)		
	Mean ± SD	Mean ± SD	Z	p value[a]
2D/4D ratio	0.95±0.046	0.98±0.025	-3.515	**<0.001**
	n (%)	n (%)	x	p value[b]
Right-Handed	32 (76.2)	37 (88.1)	2.029	**0.154**
Left-Handed	10 (23.8)	5 (11.9)		

a: Mann-Whitney U test; b: Pearson's chi-square; SD: Standard deviation.

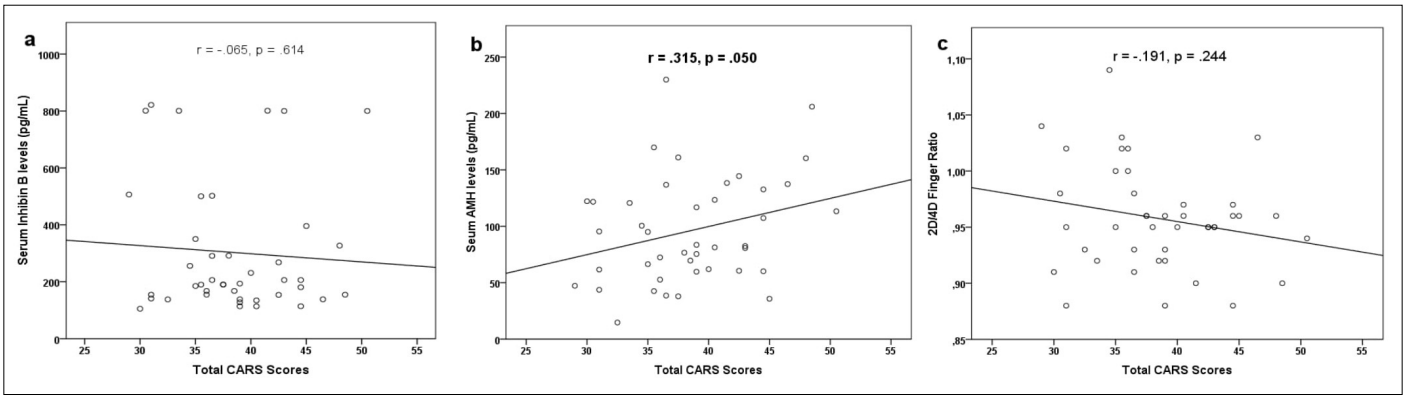

Figure 1. The correlation scatter plot for Childhood Autism Rating Scale (CARS) scores and InhB and AMH levels and the 2D/4D finger ratio in the case group.

2D/4D finger ratio in the case group. While no significant correlation was observed between CARS scores and serum InhB levels (r=-0.089, p=0.575), or the 2D/4D finger ratio (r=-0.184, p=0.243), there was a significant relationship between CARS scores and AMH levels (r=0.315, p=0.050). Correlations between CARS scores and serum biomarkers in the case group are summarized in Figure 1.

DISCUSSION

This study examined InhB, AMH, and the 2D/4D finger length ratio with a marker of exposure to fetal testosterone, thought to be potentially associated with male preponderance in ASD. The relationship between these parameters and severity of ASD symptoms was also investigated. One important finding of this study is that InhB levels were higher in the cases diagnosed with ASD than in the neurotypical control subjects. A literature review revealed that few studies have evaluated the association between ASD and InhB. Pankhurst et al. examined serum InhB levels in children diagnosed with ASD and neurotypical controls (16). Serum InhB levels were higher in the children with ASD than in the control group, although the difference was not statistically significant. When evaluating the study findings, it will be useful to remember the relatively low number of cases in the control group. Bilbay Kaynar et al. also reported higher InhB levels in the another neurodevelopmental disorder, SLD (19). To date, there are few studies that have investigated the association between InhB and male preponderance in ASD. There may be two reasons for the current situation. First, InhB is found at higher levels in boys and may be causing male preponderance in ASD with its androgenic effect. Second, in light of the possible role played by InhB in protection of neurons against brain injury acting as a neuronal rescue factor, compensatory higher InhB levels secondary to ASD-induced brain injury may be the reason of male preponderance (27).

The other finding of the study is that AMH levels did not differ between case and control groups. Morgan et al. noted that AMH is one of the important hormones potentially associated with male preponderance (15). To the best of our knowledge, very few studies have evaluated AMH levels in children with ASD. No significant relationship between AMH and ASD was determined in the present study. This finding is consistent with Pankhurst et al. (16). Our review of research investigating the relationship between AMH and male preponderance in neurodevelopmental disorders other than ASD revealed two studies involving ADHD and SLD. Gökçen et al. compared AMH levels between children with ADHD and healthy controls (28). No significant difference was found between the two groups. Similarly, Bilbay et al. evaluated the association between AMH and male preponderance in SLD (19). Those authors reported similar levels of AMH in cases diagnosed with SLD and neurotypical cases. The difference between the results for InhB and AMH in the present study may derive from their different effects on the SMAD pathway, despite both molecules being members of the same TGF B family. InhB and AMH primarily exhibit their effects through two intracellular pathways, SMAD 1.5.8 and SMAD 2.3. The effects of the two hormones on autistic symptoms and on the activation of the SMAD 1.5.8 pathway differ (16). The TGFb super family has a regulatory effect in almost all stages of brain development. Since TGFb also exhibits its effects via that SMAD pathways, we think that the SMAD pathways may be legitimate markers in the pathogenesis of ASD.

Another finding of the present study is that while a positive correlation was observed between autism symptoms and AMH, no significant correlation was found with InhB levels. In contrast to our findings, while Pankhurst et al. reported no significant correlation between AMH and social interaction and communication skills, they determined positive correlation between InhB and social interaction and communication skills (16). In interpreting the results of the two studies, methodological differences such as the tools employed for symptom assessment and sample numbers should be considered. The observation of positive correlation between AMH and ASD symptoms, despite there being no difference in AMH levels between the two groups, may be attributed to the receptor sensitivity in the AMH-related pathway rather than to serum AMH levels. In the light of the complex etiology of ASD, we do not discount the possibility that AMH may contribute to male preponderance in the condition.

As mentioned above, there are studies showing a relationship between AMH and socialization. In this study, it was thought that socialization, which is the main symptom of autism, may be related to AMH. Although there was no difference in AMH levels between ASD and control groups, a positive correlation was found between autism symptoms and AMH serum levels. Although there are studies showing that AMH is effective in socialization, as far as the researchers come across in the literature, there is no study examining the relationship between social phobia and anxiety disorders, which are another condition associated with socialization.

Baron-Cohen's 'Extreme Male Brain' proposes a link between male preponderance in ASD and fetal testosterone exposure. The 2D/4D finger length ratio is the most frequently used putative biomarker of fetal testosterone (29). In the present study, the 2D/4D finger length ratio was lower in the children diagnosed with ASD than in the healthy controls. In a recent review study, Fusar-Poli et al. reported a lower 2D/4D finger length ratio in individuals diagnosed with ASD compare to healthy controls (30). Similarly, a contemporary study comparing 2D/4D finger ratios between cases diagnosed with ASD and healthy controls reported an inverse association between 2D/4D and ASD (31). The 2D: 4D ratio remains stable for a lifetime, and represents an indirect, retrospective, and non-invasive

measure exhibiting negative correlation with intrauterine testosterone exposure. In current study, prematurity rates were significantly higher in the case group compared to the control group. This is an important confounding factor in terms of testosterone exposure during the fetal period. It would be useful to take this factor into consideration when evaluating the results regarding finger length ratio. However, it should be noted that fetal testosterone levels are particularly high in mid-pregnancy and testosterone levels decrease in late pregnancy (32).

In conclusion, the findings of the study revealed that InhB and prenatal testosterone exposure were associated with male preponderance in ASD. The research also shows that AMH may be related to the severity of ASD symptoms. These results suggest that InhB, AMH, and fetal testosterone might be factors associated with the male preponderance in ASD. Such results may also improve our understanding of the etiology of male preponderance in ASD. Further clinical longitudinal research should now be performed in order to more closely examine the links between male preponderance in ASD and Sertoli cell hormones.

There are a number of limitations to this study. These include the relatively low patient number, potential biases since randomization was not employed during sample selection, the cross-sectional study design and ignoring the intelligence quotient. In addition, another limitation is that the studies examining the relationship between AMH and socialization are animal studies, so the generalizability of these results is limited. Since anxiety disorders and prematurity could not be excluded as a confounding factor in our study, it would be more beneficial for the readers to evaluate the results of the study by considering this limitation. However, despite these limitations, reinforcing the diagnosis of ASD using KI-SADS, a semi-structured interview method recognized as the gold standard in the identification of psychiatric diseases in children, and with CARS, the reliability and validity of which have been structured to Turkish norms, represents a particular strength of the study. In addition, while several studies have evaluated the 2D/4D finger length ratio in children diagnosed with ASD, to the best of our knowledge no previous research has evaluated the 2D/4D finger ratio, a prenatal testosterone marker, together with AMH and InhB levels. More research with larger samples is now needed for a better understanding of the etiology of male preponderance in ASD.

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Ethics Committee Approval: This study was approved by the Istanbul University, Faculty of Medicine Clinical Research Ethics Committee (Case Number 2021/45, Date: 08/01/2021).

Informed Consent: The parents were informed about the study, and verbal consent was obtained from children and written and verbal consent was obtained from the parents.

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Conflict of Interest: The authors declared that there is no conflict of interest.

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