FAMILY ANALYSIS OF IMMUNOGLOBULIN CLASSES AND SUBCLASSES IN CHILDREN WITH AUTISTIC DISORDER

Mirko Spiroski[1]*, Vladimir Trajkovski[2],
Dejan Trajkov[1], Aleksandar Petlichkovski[1],
Olivija Efinska-Mladenovska[1], Slavica Hristomanova[1],
Eli Djulejic[1], Meri Paneva[1], Jadranka Bozhikov[3]

[1] Institute of Immunobiology and Human Genetics,
Faculty of Medicine, 1109 Skopje, PO Box 60, Macedonia

[2] Institute of Special Education and Rehabilitation, Faculty of Philosophy,
Bull. Krste Misirkov bb, 1000 Skopje, Macedonia

[3] Andrija Stampar School of Public Health, Rockefeller St. 4, HR-10000 Zagreb, Croatia

* Corresponding author

Abstract

Autistic disorder is a severe neurodevelopment disorder characterized by a triad of impairments in reciprocal social interaction, verbal and nonverbal communication, and a pattern of repetitive stereotyped activities, behaviours and interests. There are strong lines of evidence to suggest that the immune system plays an important role in the pathogenesis of autistic disorder. The aim of this study was to analyze quantitative plasma concentration of immunoglobulin classes, and subclasses in autistic patients and their families. The investigation was performed retrospectively in 50 persons with autistic disorder in the Republic of Macedonia. Infantile autistic disorder was diagnosed by DSM-IV and ICD-10 criteria. Plasma immunoglobulin classes (IgM, IgA, and IgG) and subclasses (IgG1, IgG2, IgG3, and IgG4) were determined using Nephelometer Analyzer BN-100. Multiple comparisons for the IgA variable have shown statistically significant differences between three pairs: male autistic from the fathers (p = 0,001), female autistic from the mothers (p = 0,008), as well as healthy sisters from the fathers (p = 0,011). Statistically significant differences found between three groups regarding autistic disorder (person with autistic disorder, father/mother of a person with autistic disorder, and brother/sister) independent of sex belongs to IgA, IgG2, and IgG3 variables. Multiple comparisons for the IgA variable have shown statistically significant differences between children with autistic disorder from the fathers and mothers (p < 0,001), and healthy brothers and sisters from the fathers and mothers (p < 0,001). Comparison between healthy children and children with autistic disorder from the same family should be tested for immunoglobulin classes and subclasses in order to avoid differences between generations.

KEY WORDS: autistic disorder, immunoglobulin classes, immunoglobulin subclasses, family analysis

Introduction

Autistic disorder is a severe neurodevelopment disorder characterized by a triad of impairments in reciprocal social interaction, verbal and nonverbal communication, and a pattern of repetitive stereotyped activities, behaviours and interests (1). A number of factors have been implicated in the pathogenesis of autistic disorder including genetic (2), environmental (3), and immunological factors (4). There are strong lines of evidence to suggest that the immune system plays an important role in the pathogenesis of autistic disorder. These include changes in lymphocyte subsets (5), alteration in serum concentration of immunoglobulin classes and subclasses (6, 7) and cytokine production (8), presence of auto antibodies to neural antigens (9), increased frequency of the C4b null allele (10), and linkage with some immune response genes (11). Abnormal immunoglobulin (low IgA, increased IgE), decreased natural killer (NK) cells and other T-cell abnormalities may reflect "disregulation" of the immune system in persons with autistic disorder. These findings may also occur in parallel to changes in the developing CNS, and both may have the same aetiologies that underlie autistic disorder (9, 12-14). The immune abnormalities would therefore give the appearance of being causative (15). Individuals with autistic disorder had significantly lower serum concentrations of cortisol ($p < 10–6$), and significantly higher concentrations of ACTH ($p = 0,002$) than control age- and sex-matched subjects. The observed hormonal changes may indicate dysfunction of the hypothalamo-pituitary-adrenal axis in individuals with autistic disorder (16). We investigated plasma concentration of IgA, IgM, IgG classes, and IgG1, IgG2, IgG3, and IgG4 subclasses in children with autism and found that children with autism have increased plasma concentration of immunoglobulines (17). In addition, we investigated specific IgA, IgG, and IgE antibodies to food antigens in 35 participants with autistic disorder and 21 of their siblings in the Republic of Macedonia and found increased levels for some of the food antigens (18). The aim of our study was to analyze quantitative plasma concentration of immunoglobulin classes, and subclasses in autistic patients and their families to confirm the differences found in previous studies between the affected and the healthy control groups. The main point of our study was to examine whether there was a correlation in plasma concentration of immunoglobulin classes, and subclasses between autistic patients and their parents,

brothers, and sisters, because no such analysis of autistic patients and their family members has been made before.

Material and Methods

Subjects

The investigation was performed retrospectively in 50 persons with autistic disorder registered by the social institutions of the Republic of Macedonia. Infantile autistic disorder was diagnosed based on DSM-IV (1) and ICD-10 (19) criteria. The sample included 30 autistic patients (22 male and 8 female patients 10,14 ± 5,81 years of age). We also analyzed all family members who consented to participate during the continual check-ups of their children. There were 30 mothers and 26 fathers aged between 25 and 70 years; and 15 sisters and 7 brothers aged between 3 and 30 years. In most families the autistic child was the only child, although there were some families with two autistic children. The diagnosis of autistic disorder in children-patients and their siblings was reached in all cases by a specialist assessment of a senior pediatrician and with contributions from specialist in mental health care where appropriate.

Immunoglobulin determination

Blood for immunogenetic investigations was taken from the subjects after obtaining their informed consent. Ten millilitres of venous blood was drawn from each donor by a standard venipuncture using vacutainer. At the time of blood drawing, none of autistic children were receiving any prescription medication or antipsychotic drug. The test requests were properly documented and kept in a confidential file. All persons gave their informed consent and allowed inclusion of their data in the manuscript without disclosure of their identity in the publication. Blood samples were separated by centrifugation, and stored at - 20°C until the determination. Serum immunoglobulin classes (IgA, IgG, and IgM) and subclasses (IgG1, IgG2, IgG3, and IgG4) were determined immunonephelometricallly by an automated Nephelometer Analyzer BN-100 (Dade-Behring, Vienna, Austria) as anonymous samples using reagents and methods supplied by the analyzer's manufacturer.

Statistics

The analysis included descriptive statistics (means ± standard deviations), multivariate analysis of variance (MANOVA) with Tukey HSD *post hoc* test for multiple comparisons and discriminant analysis for eight groups of examinees (20, 21). Interclass correlation coefficients

were calculated for paired familial data (22). P-values lower than 0,05 were considered statistically significant.

Results

We analyzed all immunoglobulin classes and subclasses and presented the results as mean ± SD separately for male (Table 1), and female participants (Table 2).

Variables (g/L)	Male persons with autistic disorder (n=22)	Fathers (n=26)	Healthy brothers (n=7)
IgA	1,43 ± 0,78	2,47 ± 1,02	1,61 ± 0,29
IgM	1,05 ± 0,39	1,12 ± 0,63	0,90 ± 1,53
IgG	11,98 ± 2,34	12,25 ± 2,28	12,51 ± 1,53
IgG1	7,66 ± 1,65	7,08 ± 1,53	8,73 ± 1,34
IgG2	2,17 ± 0,85	3,24 ± 1,03	1,98 ± 0,43
IgG3	0,42 ± 0,19	0,51 ± 0,17	0,41 ± 0,17
IgG4	0,42 ± 0,19	0,53 ± 0,34	0,51 ± 0,33

TABLE 1. Plasma concentration of immunoglobulin classes and subclasses (mean ± SD) in male persons with autistic disorder, their fathers, and brothers

Variables (g/L)	Female persons with autistic disorder (n=8)	Mothers (n=30)	Healthy sisters (n=15)
IgA	1,50 ± 1,09	2,35 ± 0,96	1,43 ± 0,77
IgM	1,18 ± 0,55	1,49 ± 0,60	1,32 ± 0,28
IgG	12,18 ± 2,18	14,08 ± 2,42	12,24 ± 2,34
IgG1	8,03 ± 1,77	8,07 ± 1,93	7,72 ± 1,14
IgG2	2,24 ± 0,76	2,18 ± 0,83	2,54 ± 1,29
IgG3	0,48 ± 0,22	0,55 ± 0,17	0,46 ± 0,22
IgG4	0,71 ± 0,30	0,52 ± 0,37	0,41 ± 0,30

TABLE 2. Plasma concentration of immunoglobulin classes and subclasses (mean ± SD) in female persons with autistic disorder, their mothers, and sisters

Seven variables (IgA, IgM, IgG, IgG1, IgG2, IgG3, and IgG4) entered into factorial multivariate analysis of variance (MANOVA) with two factors, sex (male/female), and three groups regarding autistic disorder (person with autistic disorder, father/mother of a person with autistic disorder, and brother/sister), giving altogether six groups. *Post hoc* multiple comparisons with Tukey HSD test were made to reveal statistically

significant pairs of groups for the IgA variable. We can see statistically significant differences between three pairs: male autistic from the fathers (p = 0,001), male autistic from the mothers (p = 0,008), as well as healthy sisters from the fathers (p = 0,011) (Table 3). The results of multivariate discriminate analysis for six examined groups (1= male autistic; 2=female autistic; 3=fathers; 4=mothers; 5=healthy brothers; 6=healthy sisters) and seven variables revealed that the model is significant only for the variables IgA (p=0,034) and IgG4 (p=0,004) (Table 4).

N=98	Discriminant function analysis summary for all persons with autistic disorder (No of vars in model: 7; Grouping: 6 groups; Wilks' lambda: 0,399; approximate F (35,364) = 2,537; P<0,001)					
	Wilks' Lambda	Partial Lambda	F-remove (5,86)	*P*-level	Tolerance	1-Tolerance (R-Sqr.)
IgA	0,458	0,871	2,540	0,034*	0,843	0,157
IgM	0,430	0,929	1,309	0,268	0,931	0,069
IgG	0,425	0,939	1,115	0,359	0,057	0,943
IgG1	0,418	0,956	0,788	0,561	0,084	0,916
IgG2	0,431	0,927	1,351	0,251	0,220	0,780
IgG3	0,430	0,929	1,311	0,267	0,446	0,554
IgG4	0,486	0,822	3,724	0,004*	0,586	0,414

*, Statistically significant.

TABLE 4. Discriminant function analysis summary for all persons with autistic disorder

Structure matrix gives the correlation between original variables and discriminant functions (variables were sorted by ascending absolute correlation between each variable and any discriminant function). In the last two rows are given Eigenvalues, and cumulative proportion of variance. Standardized canonical discriminant function coefficients are given in Table 5. Graphical presentation of group centroids on discriminant functions 1 and 2 is shown in Figure 1. Discriminant function 1 (horizontal) clearly separated mothers and fathers (left) from children (right), whereas function 2 (vertical) separated fathers and autistic male (negative coordinates of group centroids) from the rest of the participants (positive coordinates).

Sex*Family member	Male autistic 1,374	Father 2,531	Healthy brother 1,562	Female autistic 1,505	Mother 2,330	Healthy sister 1,472
Male autistic						
Father	0,001*					
Healthy brother	0,998	0,196				
Female autistic	0,999	0,076	0,999			
Mother	0,008*	0,971	0,427	0,224		
Healthy sister	0,999	0,011*	0,999	1,000	0,056	

Tukey HSD test, variable IgA for all autistic disorder (Probabilities for post hoc tests; error: between MS = 0,827, df = 92,000)

*, Statistically significant.

TABLE 3. Multivariate analysis of variance (MANOVA) for 6 variables, factorial model for sex (male/female), and group membership regarding autistic disorder (person with autistic disorder, father/mother of a person with autistic disorder, and brother/sister)

FIGURE 1. Graphical presentation of group centroids on discriminant functions 1 and 2. Discriminant function 1 (horizontal) clearly separated mothers and fathers (left) from children (right), whereas function 2 (vertical) separated fathers and autistic male (negative coordinates of group centroids) from the rest of the participants (positive coordinates)
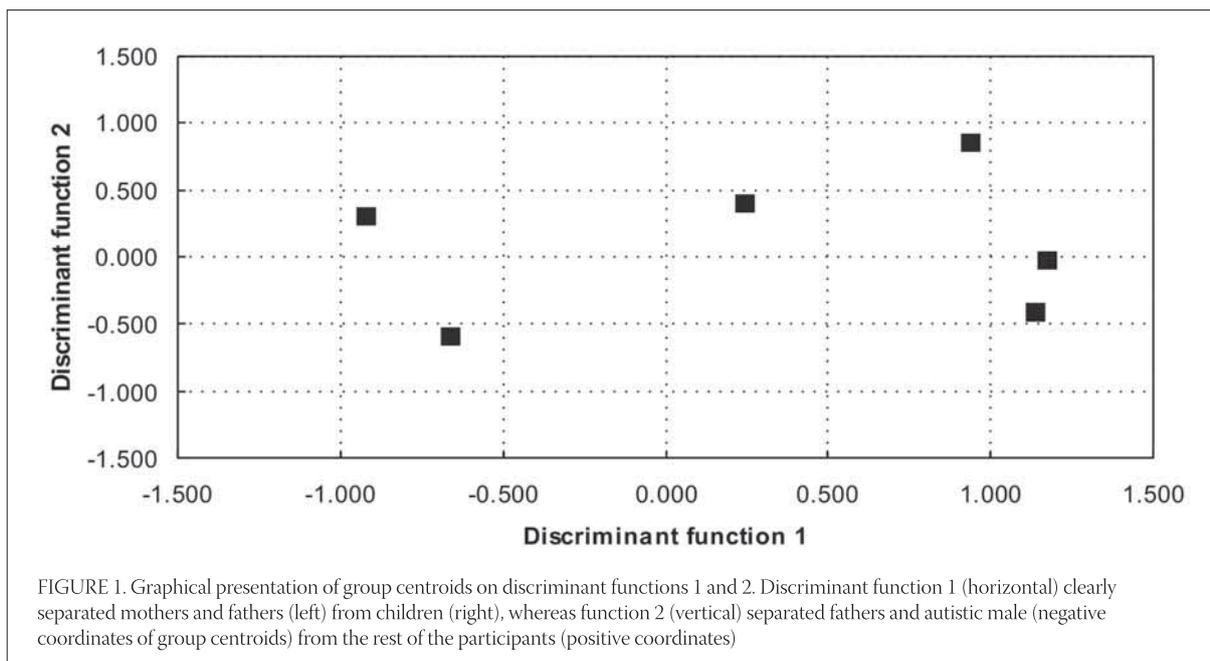
Variable	Discriminat function (standardized coefficients for canonical variables of all persons with autistic disorder)				
	1	2	3	4	5
IgA	-0,445	-0,402	0,696	-0,313	0,380
IgM	-0,140	0,495	-0,468	-0,453	0,119
IgG	-0,702	1,920	-0,125	-1,521	1,948
IgG1	0,787	-0,721	0,791	1,197	-2,084
IgG2	-0,717	-0,417	-0,099	0,979	-1,566
IgG3	0,168	-0,773	-0,657	-0,097	0,747
IgG4	0,685	-0,568	-0,248	-0,843	-0,262
Eigenvalues	0,815	0,237	0,060	0,039	0,013
Cummulative % of variance	70,1	90,4	95,5	98,9	100,0
Chi-square test when preceding roots were removed	83,06	29,09	9,85	4,61	1,13
P (chi-square test)	<0,001	0,217	0,829	0,799	0,769

TABLE 5. Standardized canonical discriminant function coefficients, enabling transformation of standardized values of original variables into scores on discriminant functions

FIGURE 2. Graphical presentation of group centroids on discriminant functions 3 and 4. Discriminant function 3 (horizontal) clearly separated sisters, mothers, and autistic male (left) from brothers, fathers, and autistic female (right), whereas function 4 (vertical) separated autistic male, mothers, and autistic female (negative coordinates of group centroids) from sisters, brothers, and fathers (positive coordinates)
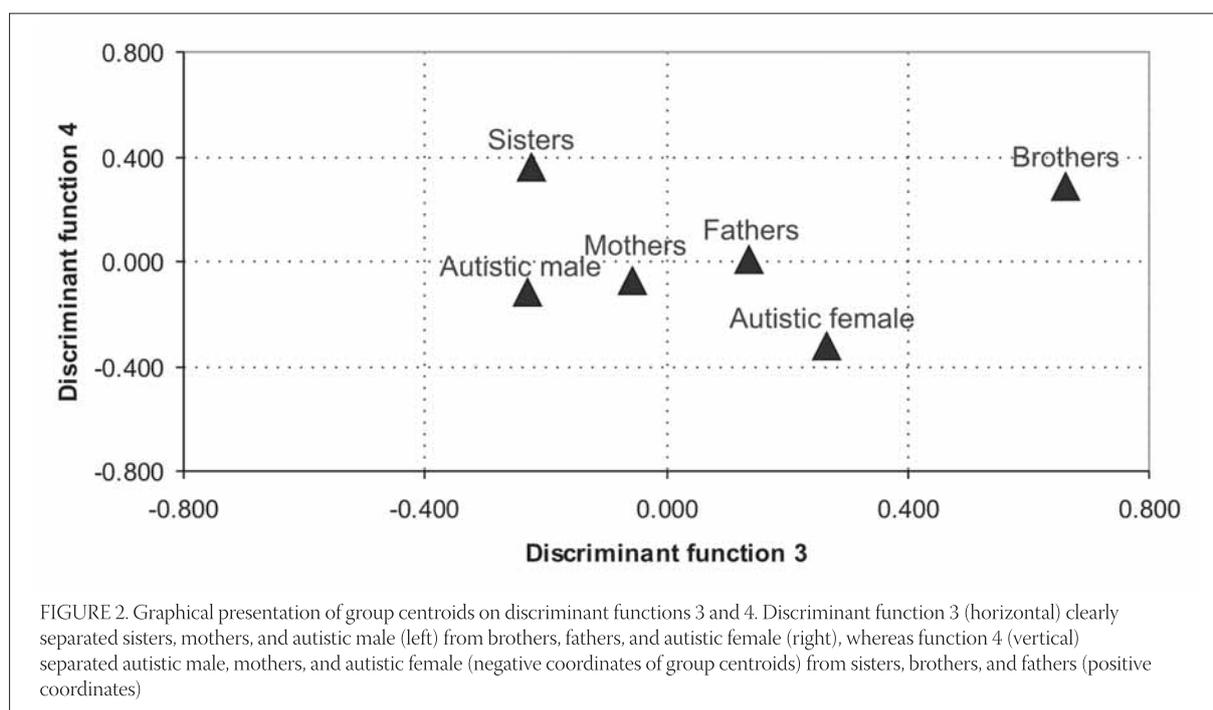
Graphical presentation of group centroids on discriminant functions 3 and 4 is shown in Figure 2. Discriminant function 3 (horizontal) clearly separated sisters, mothers, and autistic male (left) from brothers, fathers, and autistic female (right), whereas function 4 (vertical) separated autistic male, mothers, and autistic female (negative coordinates of group centroids) from sisters, brothers, and fathers (positive coordinates).

DISCUSSION

Our study showed that statistically significant differences found with factorial multivariate analysis of variance with two factors, sex (male/female), and three groups regarding autistic disorder (person with autistic disorder, father/mother of a person with autistic disorder, and brother/sister) belong to IgA, IgM, and IgG2 variables. *Post hoc* multiple comparisons with Tukey HSD reveal statistically significant pairs of groups for the IgA variable. We found statistically significant differences between three pairs: male autistic from the fathers, female autistic from the mothers, as well as healthy sisters from the fathers. The results should be interpreted very carefully. We can interpret that IgA, IgM, and IgG2 are connected somehow with the autistic disorder in the male and female persons, but we cannot interpret connection of healthy sisters with the fathers. There is also possibility that found differences are rather between generations (older and younger generations), and/or due to the small sizes of the groups (there are only 8 female with autistic disorder, and only 7 healthy brothers). Discriminant functions at group centroids showed that we did not find any significant difference between healthy children and children with autistic disorder, as well as with their parents (fathers, and mothers). It could be simply because the immunoglobulin classes and subclasses are significantly dependent of age (and sex), that in this case differences between generations are dominant (differences between parents and children), and differences in the sex are on the second place (the second discriminant function is not significant).

The analysis of three groups regarding autistic disorder, independent of sex, have shown that statistically significant differences with factorial multivariate analysis of variance belongs to IgA, IgG2, and IgG3 variables. *Post hoc* multiple comparisons with Tukey HSD test reveal statistically significant differences between children with autistic disorder from the fathers and mothers, and healthy brothers and sisters from the fathers and mothers. Again, we found statistically significant differences between generation, independent of sex (parents:children), but we did not find statistically significant differences between the healthy and children with autistic disorder (20 healthy brothers and sisters, and 28 children with autistic disorder). This investigation is the first family analysis of immunoglobulin classes and subclasses in children with autistic disorder. There are few papers in the literature dealing with immunoglobulin classes and subclasses in patients with autistic disorder. Decreased serum IgA (23), higher plasma concentration of IgM (17), higher plasma concentration of IgG (7, 17), increased plasma concentration of IgG1 in autistic males and autistic females (17), increased IgG2 (7), increased IgG4 (7, 17), and increased total IgE (18) were reported (Table 6). There is potential that such aberrant immune activity during vulnerable and critical periods of neurodevelopment could participate in the generation of neurological dysfunction characteristic of autistic disorder (24).

Immune system component	Findings	Reference
IgA	Decreased serum IgA	23
IgM	Significantly higher (p=0,031) plasma concentrations of IgM in autistic children in comparison with their healthy brothers or sisters.	17
IgG	Significantly higher (p=0,023) plasma concentrations of IgG in autistic children in comparison with their healthy brothers or sisters. Increased (p=0,012) plasma concentrations of IgG in autistic females in comparison with their healthy sisters.	17
	Serum IgG was significantly elevated.	7
IgG1	Increased plasma concentration of IgG1 (p=0,027) in autistic males, as compared with their healthy brothers. Increased (p=0,012) plasma concentrations of IgG1 in autistic females in comparison with their healthy sisters.	17
IgG2	Serum IgG2 significantly elevated.	7
IgG4	Serum IgG4 significantly elevated.	7
	Significantly higher plasma concentrations of IgG4 (p<0,001) compared to their siblings (healthy brothers or sisters).	17
IgE	Plasma concentration of total IgE was statistically significantly higher in the participants with autistic disorder.	18

TABLE 6. Studies of immunoglobulin classes and subclasses in children with autistic disorder

Circulating autoantibodies against various neuronal antigens have been found at a higher frequency in ASD groups compared to controls. Some of the targets include serotonin receptors (25), myelin basic protein (MBP) (26, 27), glial fibrillary acidic protein (GFAP), neuron-axon filament protein (NAFP) and neurofilament proteins (28), neuron-specific antigens (29), antibrain endothelial cell proteins and neurotrophic factors (9), cerebellar neurofilaments (30), and nerve growth factor (31). Serum autoantibodies to human brain, identified by ELISA and Western immunoblotting, were evaluated in children with autistic disorder, non-autistic siblings and controls. More autistic subjects than controls had bands at 100 kDa in caudate, putamen and prefrontal cortex as well as larger peak heights of bands at 73 kDa in the cerebellum and cingulate gyrus. Both autistic disorder subjects and their matched non-autistic siblings had denser bands (peak height and/or area under the curve) at 73 kDa in the cerebellum and cingulate

gyrus than did controls. Results suggest that children with autistic disorder and their siblings exhibit differences compared to controls in autoimmune reactivity to specific epitopes located in distinct brain regions (32). Specific IgA, IgG, and IgE antibodies to food antigens in 35 participants with autistic disorder and 21 of their siblings in the Republic of Macedonia were examined. Statistically significant higher plasma concentration of IgA antibodies against alpha-lactalbumin, beta-lactoglobulin, casein, and gliadin were found in the children with autistic disorder. Plasma concentrations of IgG antibodies against alpha-lactalbumin, beta-lactoglobulin, and casein in participants with autistic disorder were significantly higher. IgE-specific antibodies (alpha-lactalbumin, beta-lactoglobulin, casein, and gluten), as well as plasma concentration of total IgE, also were statistically significantly higher in the participants with autistic disorder. Gender differences were found for select IgA, IgG, and IgE (but not for total IgE) food-specific antibodies (kU/L) in the participants with autistic disorder and their siblings (18).

Conclusion

We investigated retrospectively 50 persons with autistic disorder from the Republic of Macedonia. Infantile autistic disorder was diagnosed by DSM-IV and ICD-10 criteria. Plasma immunoglobulin classes (IgM, IgA, and IgG) and subclasses (IgG1, IgG2, IgG3, and IgG4) were determined using Nephelometer Analyzer BN-100. The most important findings were found for multiple comparisons for the IgA variable between three pairs: male autistic from the fathers, female autistic from the mothers, as well as healthy sisters from the fathers. We also found statistically significant differences between three groups regarding autistic disorder (person with autistic disorder, father/mother of a person with autistic disorder, and brother/sister) independent of sex belongs to IgA, IgG2, and IgG3 variables. Multiple comparisons for the IgA variable also have shown statistically significant differences between children with autistic disorder from the fathers and mothers, and healthy brothers and sisters from the fathers and mothers. In the conclusion, we could say that comparison between healthy children and children with autistic disorder from the same family should be tested for immunoglobulin classes and subclasses, because these children are from the similar generations.

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