Original Paper

Micronutrient Research in Autism Spectrum Disorder. A Clinical Study

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ABSTRACT: Autistic spectrum disorders are part of the category of neurodevelopmental disorders, characterized by: difficulties in communication and social interaction, restrictive and repetitive patterns of behaviours and activities, which are present throughout the developmental period, and can be diagnosed in the first five years of life. Due to the increase in the incidence of this disorder in recent years, it has become a topic of great interest both to specialists in child and adolescent psychiatry and to researchers in the field. Given the polymorphism of Autism Spectrum Disorder and the need to discover factors that better explain the etiology of this disorder, studies related to biomarkers are extremely varied. One of the areas of study that have exercised particular interest is related to the involvement of metals in the pathology of autism spectrum disorder. Apart from the controversies related to heavy metals that according to studies affect the developmental process, there are studies that suggest that some micronutrients such as zinc, copper, selenium, iron, magnesium, may be involved in the etiology of autism spectrum disorder. Starting from these studies, we set out to investigate to what extent these essential metals for the body are involved in the etiology of autism spectrum disorder and how they influence the severity of symptoms.

KEYWORDS: *Autism spectrum disorder, etiology, micronutrients.*

Introduction

The increase of Autism Spectrum Disorders (ASD) incidence, in the recent years, has led to increased interest in researching this phenomenon [1].

Currently, existing studies address the issue of etiology, symptomatology and therapeutic approaches.

Although the etiology of ASD has been extensively studied, it remains unknown.

It cannot be said with certainty that a specific factor is involved in the expression of the symptomatology.

There are a few known biomarkers, but they do not apply to the whole variety of autism spectrum disorders. Studies describe a genetic influence, but there is wide heterogeneity in this regard.

There are of course genetic variants that have been described in relation to autism spectrum symptoms, but these represent only a fraction of this category [2].

The findings regarding potential biomarkers cover many areas, but they are not unified and do not lead to clear conclusions, and more research is needed to better understand the etiology of Autism Spectrum Disorder.

The role of metals in the incidence of ASD has been a subject of interest, starting from the hypothesis of the involvement of environmental factors in the appearance of this disorder.

A 2016 study by Kern and colleagues found that 74% of 100 peer-reviewed studies suggested that physiological levels of mercury were a risk factor for ASD [3].

Research led by Mold in 2018 highlighted the presence of high levels of aluminium in both the white and gray matter of ASD patients and it is assumed that it can cross blood-brain barriers and is taken up by microglial cells. [4].

Apart from heavy or toxic metals, which have been shown to disrupt the developmental process, it has been suggested that metal micronutrients may represent some of the causative factors of ASD.

Biometals such as Zinc (Zn), Copper (Cu), Selenium (Se), Iron (Fe), Magnesium (Mg), are involved in neurodevelopment and associated, in some studies, with the incidence of ASD [5,6].

Zinc is considered the second most abundant element in the human body [6].

It is involved in glutamatergic transmission during embryonic development and early childhood [7,8].

Zinc level is 10 times higher in the brain than Serum Zinc level and has a developmental role.

It is found in higher concentrations in denser neuronal areas playing an important role in: neuronal modulation, synaptic plasticity, learning and memory [9].

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It is involved in the gut-brain interaction, with prenatal zinc levels influencing even the morphology of the placenta [10,11].

Zinc also plays a role in both innate and adaptive immunity [12,13].

Selenium is involved in oxidative stress in the brain. Impaired selenium homeostasis has been associated in some studies with increased incidence of ASD [14,15,16].

Iron plays a role in neurotransmitters synthesis, myelin production and synaptogenesis. [15,17].

Iron deficiency is associated with: neurodevelopmental delays, but also with the severity of emotional or behavioural symptoms [18,19,20,21].

Magnesium is involved in the basic cellular process, nucleic acid formation, cellular energy metabolism, neurodevelopmental process [22,23,24].

It regulates glutamate activating channels in neuronal membranes with implications in the pathogenesis of ASD [6].

Copper contributes to cell growth, cellular immunity (15,25), placenta development [26].

Along with folic acid and vitamin A, copper participates in the formation of the neural plate and neural tube early in development. It also plays a role in neurotransmitter synthesis and neuromodulation [6,27,28,29].

Motivated by the need to identify potential biomarkers of Autism Spectrum Disorder, we set out to investigate the role that some micronutrients, namely: Iron, Magnesium, Selenium, Zinc, Copper, could have in the expression of ASD symptoms.

Material and Methods

Groups

The study included 75 children, 45 children with autistic spectrum disorder, who formed the study group, and 30 children without psychiatric pathology, who formed the control group.

Both boys and girls were included in the groups.

In the study group, 35 children were males and 10 children were females, and in the control group 13 children were males and 17 children were females.

The age of the children was between 2 and 12 years old.

The following inclusion criteria were considered: not having presented symptoms of acute illness at least one month before blood testing, not having undergone drug treatment, or other treatments with different food supplements.

Patients who were receiving treatments incompatible with the study were excluded.

Those who showed signs of infectious disease were rescheduled for another testing date.

The information was obtained from the child's parents or caregiver.

The medical diagnosis was formulated according to the criteria described in the Manual of Diagnostic and Statistical Classification of Mental Disorders DSM-5.

All parents or legal guardians were informed about the study and their informed consent was obtained for conducting the study and publishing the data.

Also, for the collection of the necessary samples, a contract was signed with the analysis laboratory.

The protocol of this study was approved by the University and Scientific Ethics and Deontology Commission of the University of Medicine and Pharmacy of Craiova, No. 154/24.09.2021, which certifies that the ethical principles underlying the Declaration of Helsinki and the Code of University Ethics regarding the proper conduct complied with the research process, together with the codes of practice established by the Code of Medical Ethics.

Screening

In order to carry out the screening of the autistic spectrum disorder and to support the diagnosis, we used the following instruments: SCQ (Social Communication Questionnaire), an instrument that helps to assess the abilities and social functioning of children [30] and ASRS (Autism Spectrum Rating Scale) test which is a set of rating scales built to measures behaviours associated with Autistic Spectrum Disorders [31].

Collection and testing of laboratory samples

Serum Iron was analysed by Photometry/ Spectophotometry; usual material: serum, transported at temperature of 2-8°C, Sample stability: 4 days at 18-25 ºC, 7 days at 2-8ºC, 60 days at-20ºC, Minimum quantity: 1ml

Serum Magnesium was analysed by Photometry/Spectophotometry; Usual material: serum, transported at temperature of 2-8°C, Sample stability: 7 days at 2-8°C, 1 year at-20°C, Minimum quantity: 1ml

Copper in serum was determined by ICP-MS (inductively coupled plasma mass spectrometry) Usual material: serum (tube with royal blue stopper), transported at temperature of 2-8°C,

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Sample stability: 14 days at 2-8°C, Minimum quantity: 1ml

Selenium in serum was determined by ICP-MS (inductively coupled plasma mass spectrometry), with common ancillaries being used: serum (tube with royal blue stopper), transported at temperature of 2-8°C, Sample stability: 14 days at 2-8°C, 1 year at-20°C, Minimum quantity: 1ml

Zinc in serum was determined by ICP-MS (Inductively Coupled Plasma Mass Spectrometry), with common ancillaries being used: Serum (tube with royal blue stopper, decant within two hours of collection), transported at temperature of 2-8 °C, Sample stability: 14 days at 2-8°C, Minimum quantity: 1ml

Statistical analysis

For data examination, Shapiro-Wilk and Kolmogorov-Smirnov tests, QQ Plots and Boxplots were used to check the type of data distribution.

To perform the comparative analysis, for groups with distribution approximated as Gaussian, the Student's t-test with a significance threshold of 0.05 was used for paired analysis.

For multiple comparisons, the ANOVA test was used with a significance threshold of 0.05 with the Bonferroni correction in the case of groups with equal coefficients of variation and Tamhane's for the situation when the variants were unequal.

For non-parametric groups (non-Gaussian), the comparative analysis of paired groups was done with the Mann-Whitney U test.

For multiple comparisons, the Kruskal-Wallis test was used.

To establish whether there are differences according to age, after cluster discrimination analysis, we divided each group into two different (ANOVA, $p<0.0001$) and homogeneous (Wilk's Lambda) subgroups: the group of patients with autism <5 years old, group of patients with autism >5 years old, control group <5 years, control group >5 years.

Results

After performing the data statistical analysis, we did not find statistically significant values between the study group and the control group, for any of micronutrients that we have studied: Iron ($p=0.324$), Magnesium ($p=0.643$), Selenium ($p=0.396$), Copper ($p=0.298$).

Regarding Zinc, we observed that 22.22% of patients in the study group had values below the reference values, but also 20% of children in the control group had low values.

Therefore, although we found low zinc values, we did not find statistical significance, $p=0.731$ (Figure 1, Table 1).

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of MAGNESIUM is the same across categories of LOT.	Independent-Samples Mann-Whitney U Test	,643	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is ,05.

Figure 1. Distribution of Magnesium, Mann-Whitney U Test.

Table 1. Mean and standard deviation for Iron, Magnesium, Zinc, Selenium, Copper.

Micronutrients	Study Group N=45	Control Group N=30	Score t	Z score	U score	p-score <0,05
	Mean±SD	Mean±SD				
Iron	85,377±35,497	77,633±29,097	-0,993			0,324
Magnesium	2,24±0,035	2,236±0,027		-0,463	633	0,643
Zinc	0,857±0,113	0,862±0,125	0.346			0,731
Selenium	86,346±17,285	86,806±17,035	0,854			0,396
Copper	113,397±21,06780	108,393±3,458	-1,048			0,298

Note: Statistical analysis performed with ANOVA (t-score, p-score), equal variances for Iron, Zinc, Selenium, Copper and Mann Whitney test for unequal variances (Z-score, U, p-score) for Magnesium, SD=Standard Deviation

To see if there were differences by age, we divided each group into two subgroups, with the reference age being 5 years.

In this case too, we did not find statistically significant values, the average of micronutrients for each subgroup being close (Table 2).

Table 2. Analysis of micronutrients by age.

Micronutrients	Study Group < 5Y N=20	Control Group < 5Y N=8	Study Group >5Y N=25	Control Group > 5Y N=22	p-score
	Mean±SD	Mean±SD	Mean ±SD	Mean±SD	
Iron	81,000±44,976	80,125±39,793	88,880±26,077	76, 727±25,260	0,651
Magnesium	2,205±0,187	2,287±0,145	2,268 ±0,267	2,218±0,150	0,636
Zinc	0,832±0,121	0,886±0,151	0, 868±±0,107	0,853±0,118	0,663
Selenium	85,285±19,679	88,628±10,598	81,796 ±15,348	86,059±10,598	0,726
Copper	114,900±21,398	118,250±10,333	112,196±21,398	104,809±20,235	0,278

Note: ANOVA test between groups, score p<0.05, SD=Standard Deviation, N=number of patients, <5Y=less than 5 years, >5Y=more than 5 years

We analysed micronutrients according to male and female gender for both the study group and the control group and found no statistically significant differences between groups (Table 3).

Table 3. Analysis of micronutrients according to male and female gender

Micronutrients	Study Group Gender F N=10	Control Group Gender F N=17	Study Group Gender M N=35	Control Group Gender M N=13	p-score
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
Iron	86,900±+23,900	74,411±29,381	84,942±38,453	81,846 ± 29,345	0,717
Magnesium	2,190±0,185	2,217±0,177	2,254±0,247	2,261 ±0,104	0,780
Zinc	0,829±0,096	0,849±0,137	0,859±0,118	0,879 ± 0,111	0,782
Selenium	84,890±19,416	84,723±16,559	82,905±16,911	89,530 ± 17,932	0,710
Copper	111,320±26,012	104,288 ±21,922	113,991±19,840	113,761 ±13,087	0,423

Note: ANOVA test between groups, score p<0.05, SD=Standard Deviation, N=number of patients, gender M=male, gender F=Female

Since we found that there is a high rate of comorbidity between Autism Spectrum Disorder (ASD) and Attention Deficit Hyperkinetic Disorder (ADHD), we performed a comparative analysis between the group of patients diagnosed with ASD, the group of patients diagnosed with ASD comorbid with ADHD and control group, for each micronutrient.

For Iron, the ANOVA test between groups showed no statistically significant differences between the 3 groups, Mean (SD) was: 84.85 (38.46) for ASD, 87.00 (25.67) for ASD/ADHD, 77.633 (29.09) for the control group, p-score=0.607.

In the comparative analysis of magnesium concentration for patients with ASD, ASD/ADHD and control group (ANOVA between groups) Mean (SD) was: 2.25(0.17) for ASD, 2.19(0.17) for ASD /ADHD, 2.23(0.14), p-Score=0.660, no statistical significance was found.

Comparative analysis of Zinc concentration in patients with ASD, ASD/ADHD and controls (ANOVA between groups), Mean (SD) was: 0.85 (0.10) for ASD, 0.84 (0.11) for ASD/ADHD, 0.86), p-Score=0.924, without a statistical significance.

For Copper, comparative analysis in patients with ASD, ASD/ADHD and controls (ANOVA between groups), Mean (SD) was: 113.44 (22.60) for ASD, 113.39 (16.33) for ASD /ADHD, 108.39 (18.94), p-Score=0.584, with no statistical significance.

Comparative analysis of Selenium concentration in patients with ASD, ASD/ADHD and control group (ANOVA between groups), Mean (SD) was: 83.00 (18.02) for ASD, 84.41 (15.51) for ASD /ADHD, 86.80 (17.03), p-Score=0.680, with no statistical significance.

Also, after analysing the three groups according to age (above and below 5 years) and according to gender (male and female), we did not find statistically significant correlations.

Discussions

To date there is evidence associating zinc deficiency with ASD. In a study that examined 1,967 children with ASD, nearly 30% had low zinc concentration in their hair samples [32].

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Another small study found lower levels of zinc in the saliva of children with autism compared to healthy controls.

Zinc levels may also be correlated with the severity of ASD presentation [33].

Li SO, in 2014, suggested that the zinc/copper ratio could be used as a diagnostic biomarker.

The authors state that Zinc/Copper cycles may play a role in the onset of ASD and the rhythmicity between these cycles and can be used as a diagnostic tool for ASD classification [34].

Although differences in Zinc and Copper have been reported in the literature.

In our study, although 22.22% of children with ASD had zinc concentration below normal reference values, we did not find significant differences between the group of children with ASD and the control group.

These results may be influenced by the design of all studies and the fact that they were not representative enough.

There is a large variability of results in previously performed studies, so a larger number of patients would be needed to make up statistically significant groups.

Regarding Selenium, there are studies in the literature, which have shown that there is a possibility of an association between some cases of ASD and micronutrients [35,41].

However, they are contradictory, the differences may come from the fact that the biological materials collected for highlighting the different metals studied were different (serum, hair, nails).

There are studies that have identified differences depending on gender and age, but there are also studies that have not observed these differences.

Behl et al.'s 2020 study identified significant differences between men and women in terms of Selenium concentration [6].

One of the explanations offered by the authors is that patients with ASD have more restrictive eating behaviours compared to the general population.

Regarding Iron, there is a consensus that it is involved in the neurodevelopment process and that a deficiency of it prevents the myelination process, leading to developmental delays.

Lower serum iron concentrations have been observed in patients with ASD [36,37,38,39].

In our study, we could not correlate iron concentrations with the expression of ASD symptoms as we found no differences between the study group and the control group.

The role of magnesium has been less studied compared to other micronutrients [40].

Some studies have found low levels of magnesium in ASD patients [42].

In the present study, we found no statistically significant differences between the two groups, but it was observed that in both groups there were slightly elevated magnesium values in more than half of the children, which could suggest an instability of magnesium ions, those results being most likely influenced by the emotional state during collection and the act of collecting the samples.

Limitations of the study

Insufficient number of subjects involved in the study.

Being a small number, the results cannot be generalized to all patients with ASD.

The groups of patients were not homegenous: there were large differences between the number of girls and boys involved in the study, especially regarding the control group; the same is applied to age group stratification

Several variables were not taken into account.

Conclusions

Although the pathophysiology of ASD is heterogeneous, differences in neural development are almost always evident in the first years of life.

Therefore, a more comprehensive examination of metal micronutrient levels in young children with ASD is needed to better understand their involvement in etiology.

Although we did not find statistically significant data, in order to confirm the hypothesis from which we started, namely that there could be the possibility of correlating some of the micronutrients with autism spectrum pathology, considering that regarding this subject the results are often contradictory or inconclusive, the questions remain open:

Do micronutrients play a role in the expression of ASD symptom severity?

If they do play a role, can micronutrients be considered when trying to explain the etiology of ASD?

Can micronutrients be some of the future biomarkers of ASD?

Informed consent

All parents or legal guardians were informed about the study and their informed consent was obtained for conducting the study and publishing the data.

Also, for the collection of the necessary samples, a contract was signed with the analysis laboratory.

The protocol of this study was approved by the University and Scientific Ethics and Deontology Commission of the University of Medicine and Pharmacy of Craiova, No. 154/24.09.2021, which certifies that the ethical principles underlying the Declaration of Helsinki and the Code of University Ethics regarding the proper conduct complied with the research process, together with the codes of practice established by the Code of Medical Ethics.

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Conflict of interests

We declare that there is no conflict of interests.

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