Academy of Romanian Scientists
Annals Series on Biological Sciences

Volume 15, No. 1, 2026, pp. 104 - 122
Online ISSN 2285 – 4177
ORIGINAL PAPERS

THE ROLE OF VITAMIN D AND VITAMIN B12 IN CHILDREN WITH AUTISM SPECTRUM DISORDER

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Abstract: Background. Autism spectrum disorders (ASD) include a heterogeneous group of neurodevelopmental disorders, characterized by a multifactorial etiology, in which the interaction between genetic, epigenetic, metabolic and environmental factors plays a determining role. In the last two decades, biomedical research has highlighted that ASD cannot be explained exclusively by neurocognitive or behavioral mechanisms, but involves systemic disorders affecting metabolism. **Objective.** We want to demonstrate that the presence of low vitamin D levels would be related to the worsening of symptoms in people diagnosed with autism spectrum disorders (ASD) and that the deficiency of those vitamins affects good physical and mental development. **Materials and methods.** The analysis of vitamin status aimed to evaluate serum vitamin D levels in neurotypical participants and those diagnosed with autism spectrum disorders (ASD), in order to identify possible differences associated with clinical status. Vitamin D was determined from serum obtained by centrifugation of venous blood collected in vacutainers without anticoagulant. Samples were collected in the morning, under standardized conditions and processed according to laboratory protocols. Serum was analyzed immediately or temporarily stored at refrigerated temperatures (2–8°C), respecting the analytical stability range specific to vitamin D determination. **Results.** The analysis of individual values revealed marked differences between the two groups. In the neurotypical group, a significant proportion of participants had vitamin D values within the biologically adequate range. More specifically, over 100% of participants had values ≥ 30 μg/L, and a small number had deficient values. The descriptive values (mean of 43.32 μg/L and median of 36.40 μg/L) support the existence of an adequate vitamin status in this group, despite a relatively large dispersion of values. In contrast, in the group of participants with ASD, most of the vitamin D values are below the sufficiency threshold. A significant proportion of participants had deficient values (< 20 μg/L) and another part fell into the insufficiency range (20–29 μg/L). Only a limited number of participants with ASD reached values considered biologically adequate. Regarding the analysis of the distribution of individual values indicates a clear contrast between the two groups. In the neurotypical group, most participants presented vitamin B1 concentrations within the normal biological range, with mean and median values comfortably above the lower limit of normal (mean = 407.63 pg/ml; median = 378.50 pg/ml). Only isolated cases were at or slightly below the lower limit of normal. In the ASD group, the distribution of vitamin B12 values was significantly shifted towards and below the lower limit of normal. A considerable number of participants presented values below 200 pg/ml indicating biological deficiency and an additional proportion fell into the risk zone (200–300 pg/ml).

Conclusion. The differences between the two groups are confirmed by the inferential analysis, with the Mann–Whitney U test indicating a highly statistically significant difference in the distributions of vitamin D ($p < 0.001$). These results suggest a significantly more frequent deficient status of vitamin D and vitamin B12 among participants with ASD compared to neurotypicals, consistent with the literature that frequently describes hypovitaminosis D in populations with ASD. Overall, the analysis of vitamin status by medication indicates that, within the ASD group, medication is not associated with statistically significant differences in serum levels of vitamin D or vitamin B12. However, the descriptive analysis reveals clinically important trends: participants on medication have, on average, lower vitamin D values and increased variability in vitamin B12 values.

Keywords: Autism Spectrum Disorders, Vitamin D, vitamin B12, biochemical analysis

DOI 10.56082/annalsarscibio.2026.1.104

1. Autism Spectrum Disorders (ASD) - Generalities

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders characterized by a multifactorial etiology, in which the interaction between genetic, epigenetic, metabolic and environmental factors plays a determining role. In the last two decades, biomedical research has highlighted that ASD cannot be explained exclusively by neurocognitive or behavioral mechanisms, but involves systemic disorders affecting energy metabolism, redox balance, endocrine function and intestinal homeostasis. The complex pathology of ASD is extremely challenging for the child, family and society, constituting a major public health objective. Although children with autism do not look different from others, they communicate, behave and learn differently than most other children. Currently, the diagnosis of ASD includes certain conditions that were previously diagnosed separately: autism, pervasive developmental disorder not otherwise specified and Asperger syndrome.

The causes of autism remain unknown, but intensive research is constantly being conducted in this field. The primary abnormality of this disease appears to be cognitive, affecting mainly symbolic thinking and language, with behavioral disorders secondary. "Organic brain disorders are indicated by increased complications of pregnancy and childbirth, as well as a link with epilepsy, and some individuals present with unlocalized neurological disorders" (Gelder, 1983).

The number of children diagnosed with autism spectrum disorders is increasing. Studies

that analyze the phenomenon have concluded that several factors are responsible for the increase. The prevalence of autism spectrum disorders (as determined by current diagnostic methods) appears to be about five to six per

1,000 young children. In the first years of life, there are usually no clear defining features, but parents need to be vigilant. In the second and third years of life, a rapid examination by specialists is necessary for the following areas of concern: communication, language development, especially comprehension, disturbance, unusual use of language, difficulty responding to name, non-verbal communication - insufficient (without pointing with the hand and difficulty following a point), no social smile to share good mood and respond to the smile of others (Mureşan, 2004).

Inappropriate, repetitive and ritualistic behavior, e.g. destroying objects, spinning objects (Jamieson, 2004), repeating movements, rocking the body, endless series of jumping on the mat or spinning movements around one's axis, are common characteristics of children with autism (Albano, 2003).

Children with autism are unable to decipher social intentions, a processing problem that will accompany them throughout their lives. As a result, they face difficulties in communicating with others and adapting to the environment. In addition, they find it very difficult to understand a certain situation from the point of view of others (Kanner, 1943).

Certain biochemical imbalances can be factors that contribute to the development of autism spectrum disorders. This is considered extremely important, and performing biochemical analyses during pregnancy and in the child after birth is essential. During pregnancy, there should be no heavy metal poisoning, electrolytes should be dosed appropriately, there should be no vitamin deficiencies, especially vitamin B12, and testosterone levels should be optimal.

By recognizing specific symptoms in a timely manner and by rapid and effective treatment, the consequences of autism can be reduced. Genetic testing can be useful for a couple who wants to have children and has or has had a family history of autism, as there are several determining factors that are believed to play a role in the development of the condition. Performing detailed biochemical analyses during pregnancy is extremely important because a metabolic imbalance can favor the development of autism in the child. It is desirable that family doctors are aware of the importance they have in the proper development of the pregnancy and must guide the mother at all times so that the result is the desired one.

Children with autism are unable to realize what is happening around them, and this will accompany them throughout their lives. As a result, they face difficulties in communicating with others and adapting to the environment.

Socialization plays a fundamental role in individual development, being the process by which people learn the norms, values, skills and behaviors necessary to function as effectively as possible. This learning process begins from the first moments of life and continues throughout life, continuously shaping individual identity, behavior and perceptions. This provides a framework in which individuals develop and refine their social skills. Through regular interaction with others, people learn to express their own thoughts and feelings in socially acceptable ways and to evaluate the complexity of human relationships. These skills are essential for success in many aspects of life (Pfeifer et al., 2021).The school integration of these children is quite difficult because they do not like to change their environment and prefer not to interact with anyone. Their integration is frequently carried out in special schools because in this education system the emphasis is placed on developing communication skills through speech therapy and behavioral therapies and activities are carried out to develop their care skills. However, integration into mainstream schools has its advantages, as these children can reach a high intellectual level, mainstream schools offering them the opportunity to learn alongside other children, but with an adapted curriculum. Care must be taken that the environment does not negatively influence the child with autism, so that he is not exposed to the phenomenon of bullying.

The biological basis of autism is complex and involved in the functioning of the brain. Neurotransmitters play an important role in transmitting signals between nerve cells and an imbalance in their levels can contribute to the symptoms of autism. On the other hand, the structure and function of the brain in autism differs from neurotypical individuals and changes in the cortex and other areas of the brain may affect the behavior and social skills of individuals with autism. In this context, the investigation of peripheral biological markers has become a major research direction, with the aim of identifying biological profiles associated with ASD, potential biomarkers and relevant pathogenic mechanisms. Recent studies suggest the existence of increased oxidative stress, metabolic and endocrine dysfunctions, as well as intestinal dysbiosis in individuals with ASD, but the results remain heterogeneous and often dependent on the characteristics of the samples analyzed.

2. The importance of vitamins D and B12 for the human body

Vitamin D

Vitamin D is a fat-soluble vitamin essential for maintaining the health of the human body. It is important in many physiological processes and plays a crucial role in maintaining the health of the skeletal system, the immune system and muscle functions. Vitamin D is classified as a fat-soluble vitamin, which is obtained from food or is produced by the skin from ultraviolet B (UVB, 290–320 nm) from sunlight (Nair and Maseeh, 2012). Due to vitamin D deficiency and the risk of disease in many countries, there is a legal obligation to fortify certain products (dairy products, especially milk and other dairy drinks, margarine) (Udristioiu, 2021). The classic role of vitamin D is to regulate calcium and phosphate metabolism, which is essential for bone remodeling. Based on research in recent decades, it has been shown that low sun exposure, limitation and vitamin D deficiency are associated with an increased risk potential for many other diseases, such as cancer (Bouillon et al., 2020). Currently, autism is a serious problem that unfortunately more and more people are facing and is spreading around the world like an epidemic. Although the disease cannot be attributed to a single factor, it is important to note that the prevalence of autism has begun to increase at the same time as the increase in the number of children with vitamin D deficiency. This is worrying because there seems to be a malabsorption of this vitamin in children with ASD. It has been found that vitamin D deficiency is strongly associated with the severity of ASD and in theory, affects the neurodevelopment of children with ASD. Due to its anti-inflammatory properties, it stimulates the production of neurotrophins, reduces the risk of seizures and regulates glutathione and serotonin levels, being very important for the proper development of the body.

There has been increasing evidence of an association between vitamin D and Autism Spectrum Disorders, with higher prevalence among children living in areas with low ultraviolet B radiation compared to those living in sunny areas (Cannell, 2017). Vitamin D is physiologically converted to its active form, 1,25(OH) D (Calcitriol), through two consecutive hydroxylation processes in the liver and kidneys (Gil et al., 2018). Calcitriol is a neuroactive hormone (Lee et al., 2019) that is responsible for various aspects of brain development and early cognitive development (Siracusano et al., 2020). Vitamin D helps in neuronal cell proliferation and neurotransmission functions (Sengenç et al., 2020), so theoretically its absence could lead to impaired neurodevelopmental processes.

In addition, vitamin D plays an important role in modulating inflammation by regulating the production of inflammatory cytokines and immune cells, which are essential for the pathogenesis of many immune-related diseases (Liu et al., 2018). Significantly higher concentrations of interleukin (IL)- 1β, IL-6, IL-8, interferon-gamma (IFN-γ), eotaxin, and monocyte chemotactic protein- 1 (MCP-1), along with significantly lower transforming growth factor-β1, have been reported in individuals with ASD compared to their healthy controls (Masi et al., 2015). Elevated IL-6 and TNF-α in children with ASD were positively correlated with ASD severity, as measured by the Childhood Autism Rating Scale (CARS) ($R = 0.638$ and $R = 0.699$, $p < 0.0001$, respectively), and are also used as biomarkers of ASD diagnosis (Yang et al., 2015). Animal model studies have revealed that elevated IL-6 levels in mice disrupt the balance of synaptic transmissions and mediate autism-like behaviors, including decreased social interaction, impaired cognitive skills, and learning deficits (Xu et al., 2015).

Vitamin D status has been reported to exert a negative association with inflammation, as summarized in a systematic review of typically developing (TD) children and adolescents (Filgueiras et al., 2020); while another systematic review of immune cell studies reported consistent observations of suppression in MCP-1, IL-6 and IL-8 with vitamin D supplementation (Calton et al., 2015).

Vitamin D is also involved in increasing the level of glutathione peroxidase 1 (GP1) (Ansari et al., 2020), thus reducing oxidative stress. Glutathione redox imbalance contributes to ASD because it increases the expression of proinflammatory cytokines and can have a significant impact on neuroinflammation (Bjørklund et al., 2020). Vitamin D plays a role in calcium and phosphorus metabolism and the process of bone formation. The body can synthesize vitamin D by transforming provitamin D in the skin under the influence of solar or artificial ultraviolet rays. It is found in small amounts in butter, milk, egg yolk. Vitamin D needs are 400-500 i.u./day under conditions of proper nutrition and sun exposure (Suditu, 2023) To improve vitamin D levels, sun exposure is recommended first of all, since it is synthesized mainly in the skin under the influence of ultraviolet rays. Vitamin D3 supplements are recommended for people who do not get enough sunlight. In case of imbalance or deficiency of vitamin D in the body, various health problems can occur. Symptoms associated with vitamin D deficiency include brittle bones, rickets in children, osteomalacia in adults, muscle pain, weakness, fatigue, increased risk of infections and inflammation, depression or sleep disorders. Vitamin D deficiency has also been associated with an increased risk of some chronic diseases, such as cardiovascular disease, diabetes, cancer and various autoimmune diseases. It is important to maintain adequate levels of vitamin D in the body through sun exposure, consumption of foods rich in vitamin D or vitamin D supplementation, especially during cold seasons and in people at increased risk of deficiency. It is also recommended to talk to your doctor before taking vitamin D supplements to determine the correct dosage and avoid possible drug interactions that could affect your health.

Because children with autism do not spend much time outdoors and become more agitated due to excessive heat, we find this high vitamin D deficiency in these children. They also have problems with nutrition, sometimes eating the same foods every day for a long time, which is a serious problem. A balanced diet could regulate the problems that have arisen at the biochemical level and could lead to an improvement in symptoms, which would improve their health and increase the chances of good social integration.

Vitamin B12

The B vitamins include various chemical substances that participate in the activity of enzymes, without which some syntheses and cellular activity could not take place. Some B vitamins play an important role in the formation of red blood cells, especially folic acid and vitamin B12. Reduced intake of vitamin B12 or insufficient absorption in the intestine becomes evident after a longer period of time (Suditu, 2023). Vitamin B12 plays a decisive role in lipid metabolism and in the regeneration and formation of myelin sheaths, which protect nerves from the loss of electrical impulses, ensuring a correct and efficient nerve flow in the transmission of transmitted information. This "isolation of the nerves responsible for tactile sensation and motor function is not only important, but simply necessary for the optimal functioning of the central nervous system, the brain and spinal cord. Vitamin B12 helps regulate the levels of neurotransmitters that control brain activity and indirectly regulate perception and mood. When vitamin B12 imbalances occur in the body, some health problems can occur, such as megaloblastic anemia, which is characterized by anemia (decreased red blood cell count) and extreme fatigue. Vitamin B12 deficiency can also lead to neurological problems, such as peripheral nerve damage, memory impairment and difficulty concentrating. In addition, a lack of vitamin B12 can affect mood, causing depression and anxiety.

One of the groups at high risk of vitamin B12 deficiency is vegetarians and vegans, as it is found in animal products, and they are advised to compensate for any deficiency with supplements. The elderly can also have vitamin B12 deficiency, as its absorption decreases with age. People with certain gastrointestinal diseases, such as celiac disease or Crohn's disease, are at higher risk of deficiency, as these diseases can affect the absorption of vitamin B12. People who have had surgery on their stomach or small intestine may also have difficulty absorbing vitamin B12. Vitamin B12 is found in foods of animal origin, being stored in the liver of the animal's body. Meat, fish, eggs and dairy products are also rich in vitamin B12. If a vitamin B12 deficiency is detected, treatment according to the doctor's prescription should include appropriate medications.

3. Demographic characteristics of the groups

The analysis of the gender distribution of participants, depending on status (neurotypical versus diagnosis of autism spectrum disorder – ASD), highlights an identical structure of the analyzed samples. Thus, within both groups, the share of male participants is 75.0% (n = 27), while female participants represent 25.0% (n = 9), each group having a total of 30 subjects.
This distribution reflects a predominantly male representation in both status categories, ensuring the structural comparability of the groups from the perspective of the gender variable. Maintaining the same proportion between sexes in the two subsamples contributes to reducing potential confounding effects associated with gender in subsequent comparative analyses, allowing a clearer interpretation of the differences that can be attributed to developmental

status.Also, the observed structure is consonant with data reported in the literature, which indicate a higher prevalence of the male gender in samples that include people with ASD, however, in the context of the present study, this distribution is also replicated in the neurotypical group, as a result of the selection and balancing strategy of the samples.

4. Vitamin status

Vitamin D and vitamin B12

Vitamin D and vitamin B12 play essential roles in supporting neurological and metabolic functions in children with autism spectrum disorder (ASD). Their deficiency is common, and supplementation can contribute to regulating inflammation, improving immune function, brain development, and optimizing serotonin levels, often improving behaviors.

Vitamin status

Differences in vitamin status between children and youth with ASD and neurotypicals specifically aimed to integrate possible deficiencies/under optimizations (vitamin D, B12) within the hypothesis of a multifactorial biological vulnerability in ASD.

The data analyzed in the statistical report indicate that some components of the mineral profile may show biologically relevant differential trends (e.g., as total with borderline significance in medication subgroup analyses, $p \approx 0.056$), suggesting the need for cautious interpretation in relation to sample size, variability, and potential diet/supplementation effects.

Vitamin status should be interpreted as a determinant/modulator of neuro-immune homeostasis and mitochondrial functioning, with a possible role in "amplifying" vulnerability to oxidative stress and supporting a dysbiosis profile; from this perspective, even small differences may be clinically relevant in subgroups, and future investigations should include systematic control of supplements, diet and seasonality (especially for vitamin D).

The present study was conducted on a pediatric sample and young people with autism spectrum disorders compared to a group of neurotypical children and consists of biochemical analysis of vitamin D, biochemical markers essential for good physical and mental development of children.

Vitamin D determination was performed by a standardized immunological method, used in clinical biochemistry to evaluate the serum concentration of 25-hydroxy-vitamin D, the main circulating metabolite of vitamin D.

5. The principle of the analytical method

The concentration of vitamin D was determined by a chemiluminescence immunoassay, based on the specific antigen–antibody reaction. The method is of the competitive type, in which 25-hydroxyvitamin D in the biological sample competes with a labeled analogue for the binding sites of specific antibodies.

The light signal generated following the chemiluminescence reaction is inversely proportional to the concentration of vitamin D in the sample and is measured by the analytical system according to the parameters of the method used.

Biological material and preanalytical conditions

Vitamin D

Vitamin D determination was performed from serum obtained by centrifugation of venous blood collected in vacutainers without anticoagulant. Samples were collected in the morning, under standardized conditions, and were processed according to laboratory protocols.

Serum was analyzed immediately or temporarily stored at refrigerated temperatures (2–8°C), respecting the analytical stability range specific to vitamin D determination.

Apparatus and reagents

The analyses were performed using an automated immunoassay analyzer with chemiluminescence technology, compatible with vitamin D determination. The reagents used were certified commercial kits, containing:

– antibodies specific for 25-hydroxyvitamin D;
– labeled vitamin D analog;
– chemiluminescence reaction triggering reagents;
– calibrators and internal controls.

All determinations were performed according to the manufacturer's instructions, respecting the incubation conditions and reaction parameters.

Expression of results

The concentration of vitamin D was expressed in micrograms per liter (µg/L), the unit of measurement used in the laboratory for reporting this parameter. The values were automatically generated by the analyzer based on the calibration curve.

Analytical quality control

Rigorous quality control procedures were applied for the determination of vitamin D, including:

– use of internal controls of known concentration;
– periodic verification of the analyzer calibration;
– validation of the results according to the laboratory's internal criteria;
– exclusion of inappropriate or degraded samples.

Vitamin B12

The determination of vitamin B12 was performed by a standardized immunological method, used in clinical biochemistry to evaluate the serum concentration of this water-soluble vitamin.

Principle of the analytical method

The concentration of vitamin B12 was determined by an electrochemiluminescence immunological method. The method is competitive and is based on the specific reaction between vitamin B12 in the biological sample and specific antibodies, in the presence of a labeled analog.

The signal emitted following the electrochemiluminescence reaction is inversely proportional to the concentration of vitamin B12 in the sample and is measured by the analytical system according to the parameters of the method used.

Biological material and preanalytical conditions

The determination of vitamin B12 was performed from serum obtained by centrifugation of venous blood collected in vacutainers without anticoagulant. Sample collection was performed under standardized conditions, and the serum was carefully separated to avoid contamination with figured elements.

The samples were analyzed immediately or temporarily stored at refrigeration temperatures, respecting the stability intervals specified by the kit manufacturer.

Apparatus and reagents

The analyses were performed using an automated immunoassay analyzer compatible with electrochemiluminescence technology. The reagents used were certified commercial kits, containing:

– specific antibodies for vitamin B12;
– labeled vitamin B12 analog;
– electrochemiluminescence reaction triggering reagents;

– calibrators and internal controls.

The determinations were performed according to the manufacturer's instructions, respecting the incubation times and reaction conditions.

Expression of results

The concentration of vitamin B12 was expressed in picograms per milliliter (pg/mL), the standard unit used in reporting this parameter in clinical biochemistry. The values were automatically calculated by the analytical system based on the calibration curve.

Analytical quality control

Quality control procedures were applied for the determination of vitamin B12, including:

– use of internal controls with known concentrations;
– periodic calibration of the analyzer;
– validation of results according to the laboratory's internal procedures;
– exclusion of samples with signs of hemolysis or degradation.

6. Statistical analysis of vitamin status

Vitamin status analysis aimed to assess serum levels of vitamin D and vitamin B12 in neurotypical participants and those diagnosed with autism spectrum disorder (ASD), in order to identify possible differences associated with clinical status. Shapiro–Wilk normality tests indicated significant deviations from normal distribution for both parameters, both in the neurotypical group and in the ASD group ($p < 0.05$), which is why comparisons between groups were performed using the nonparametric Mann–Whitney U test.

Vitamin D

For the interpretation of the results, internationally recognized biological reference ranges were used, according to which serum values of 25-hydroxyvitamin D are considered adequate at concentrations ≥ 30 µg/L, insufficient between 20 and 29 µg/L and deficient below 20 µg/L (Holick et al., 2011; Endocrine Society).

The analysis of individual values revealed marked differences between the two groups. In the neurotypical group, a significant proportion of participants had vitamin D values within the biological sufficiency range. More precisely, over half of the participants had values ≥ 30 µg/L, and a small number had deficient values. The descriptive values (mean of 43.32 µg/L and median of 36.40 µg/L) support the existence of an adequate vitamin D status in this group, despite a relatively large dispersion of the values.

In contrast, in the group of participants with ASD, most of the vitamin D values are below the sufficiency threshold. A significant proportion of participants have deficient values (< 20 µg/L), and another part falls within the deficient range (20–29 µg/L). Only a limited number of participants with ASD reach values considered biologically adequate. This profile is also reflected by the lower descriptive values of the mean (27.06 µg/L) and median (24.80 µg/L).

The differences between the two groups are confirmed by the inferential analysis, with the Mann–Whitney U test indicating a highly statistically significant difference in the vitamin D distributions ($p < 0.001$). These results suggest a significantly more frequent vitamin D deficiency status among participants with ASD compared to neurotypicals, found in accordance with the literature that frequently describes hypovitaminosis D in populations with ASD.

Vitamin B12

For vitamin B12, the biological reference range used in the interpretation was between 200 and 900 pg/ml, with values below 200 pg/ml being considered suggestive of deficiency, and values between 200 and 300 pg/ml being interpreted as the lower limit area (O'Leary & Samman, 2010).

Analysis of the distribution of individual values indicates a clear contrast between the two groups. In the neurotypical group, most participants have vitamin B12 concentrations located within the normal biological range, with mean and median values comfortably exceeding the lower limit of normal (mean = 407.63 pg/ml; median = 378.50 pg/ml). Only isolated cases are located at the lower limit of normal or slightly below it.

In the ASD group, the distribution of vitamin B12 values is significantly shifted towards the lower limit of normal and below it. A considerable number of participants have values below 200 pg/ml, indicating biological deficiency and an additional proportion fall into the risk zone (200–300 pg/ml). The significantly lower mean (275.97 pg/ml) and median (231.50 pg/ml) reflect this deficient profile.

The difference between the two groups is statistically supported by the Mann–Whitney U test ($p = 0.001$), indicating a statistically significant difference in vitamin B12 levels between participants with ASD and neurotypicals. Overall, the analysis of vitamin status highlights a profile characteristic of participants with ASD, marked by an increased prevalence of vitamin D deficiency and low vitamin B12 values. These findings may be correlated with nutritional particularities, food selectivity, changes in intestinal absorption or metabolic factors specific to ASD.

Table I.1. Shapiro–Wilk normality test for serum vitamin D and vitamin B12 levels, according to clinical status

Test for normality of data distribution

		Shapiro-Wilk		
	Status	Statistics	df	p
Vitamin D (µg/L)	Neurotipic	,912	30	,017
	with TSA	,875	30	,002
Vitamin B12 (pg/ml)	Neurotipic	,917	30	,022
	with TSA	,841	30	,000

Source: own data, statistically processed

Table I.2. Results of the Mann–Whitney U test for comparing vitamin D and vitamin B12 levels between groups

Test statistics[a]

	Vitamin D (µg/L)	Vitamin B12 (pg/ml)
Mann-Whitney U	193,000	221,500
Wilcoxon W	658,000	686,500
Z	-3,802	-3,380
p – Significance asymptotic (bilateral)	,000	,001

a.Grouping variabile: Status

Source: own data, statistically processed

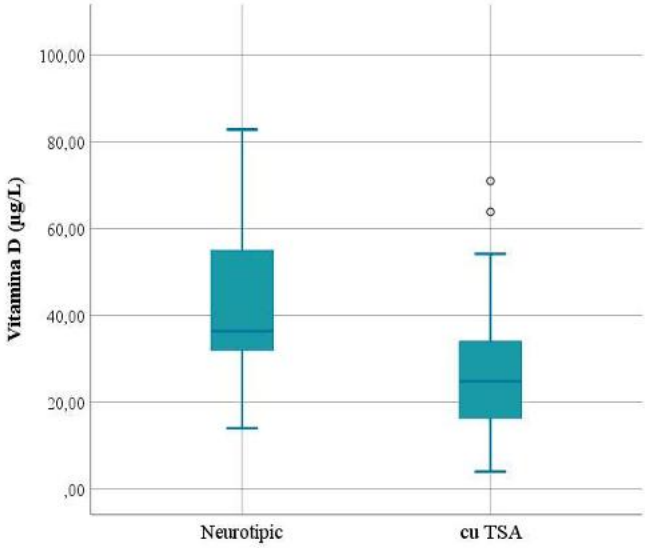

Figure I.1 Distribution of vitamin D values (µg/L) in neurotypical and ASD children
Source: own data, statistically processed

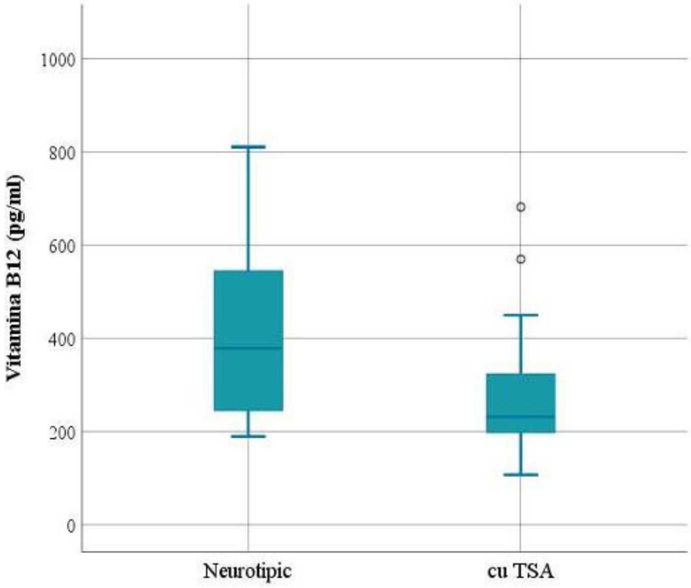

Figure I.2. Distribution of vitamin B12 values (pg/mL) in neurotypical and ASD children
Source: own data, statistically processed

To assess the potential influence of medication on vitamin status in the group of participants with autism spectrum disorder (ASD), serum levels of vitamin D and vitamin B12 were analyzed, comparing the non-medication and medicated subgroups. Given the non-normal distributions of the data and the small size of the subsamples, differences between the two subgroups were tested using the non-parametric Mann–Whitney U test.

Vitamina D

The interpretation of vitamin D results was performed using internationally recognized biological thresholds, according to which serum 25-hydroxyvitamin D concentrations are considered adequate at values ≥ 30 μg/L, insufficient between 20 and 29 μg/L and deficient below 20 μg/L (Holick et al., 2011).

Analysis of the distribution of individual values indicates that, in the subgroup without medication, a relevant part of the participants falls into the biological range of sufficiency or mild insufficiency. Approximately one third of the participants have values ≥ 30 μg/L, and the rest are distributed predominantly in the insufficiency range (20–29 μg/L) and, to a lesser extent, in the deficiency range. The descriptive values (mean = 31.70 μg/L; median = 28.00 μg/L) suggest a vitamin status at the lower limit of sufficiency, but characterized by a large dispersion of values.

In the subgroup with medication, the distribution of vitamin D values is shifted towards lower levels. Most participants have values below the threshold of 30 μg/L, with a significant proportion of deficient values (< 20 μg/L). The mean (22.43 μg/L) and median (19.00 μg/L) indicate, at group level, a status of insufficiency towards vitamin deficiency, and the minimum value of 4.00 μg/L highlights the presence of severe deficiencies.

However, the inferential analysis does not indicate a statistically significant difference between the two subgroups in terms of vitamin D levels ($p = 0.164$). This finding suggests that, within the present sample, medication is not associated with systematic differences in vitamin D levels, although the distribution of values indicates a descriptive trend towards a more deficient status in the medication subgroup.

Vitamin B12

For vitamin B12, the interpretation was made using the biological reference range of 200–900 pg/ml, with values below 200 pg/ml being considered suggestive of deficiency, and those between 200 and 300 pg/ml being interpreted as a risk area or lower limit of normal (O'Leary & Samman, 2010).In the subgroup without medication, the distribution of vitamin B12 values indicates a concentration of results in the lower area of the normal biological range. Some of the participants have values below 200 pg/ml, indicating biological deficiency, while the majority fall between 200 and 300 pg/ml. The mean (246.60 pg/ml) and median (230.00 pg/ml) reflect a marginal vitamin status, located close to the lower limit of normal, but with relatively low variability.In the subgroup with medication, vitamin B12 values are more heterogeneous. Although there are cases with deficient values, some of the participants have much higher values, significantly exceeding the lower limit of normal and, in some cases, reaching high values. This distribution is reflected in a higher mean (305.33 pg/ml), but also in a considerably higher standard deviation, indicating a pronounced variability in vitamin B12 status among participants under medication. The inferential analysis does not reveal statistically significant differences between the two subgroups ($p = 0.708$), suggesting that medication, in itself, is not associated with systematic changes in vitamin B12 levels, but rather with a greater dispersion of values, possibly influenced by factors such as intermittent supplementation or dietary particularities. Overall, the analysis of vitamin status according to medication indicates that, within the ASD group, medication is not associated with statistically significant differences in serum levels of vitamin D or vitamin B12. However, descriptive analysis reveals clinically important trends: participants on medication have, on average, lower vitamin D levels and increased variability in vitamin B12 levels.

Table I.3. Results of the Mann–Whitney U test for comparing serum levels of vitamin D (µg/L) and vitamin B12 (pg/ml) according to medication status, within the experimental group

Test statistics[a]

	Vitamin D (µg/L)	Vitamin B12 (pg/ml)
Mann-Whitney U	79,000	103,500
Wilcoxon W	199,000	223,500
Z	-1,392	-,374
p – asymptotic significance (bilateral)	,164	,708
The exact significance [2 × (unilateral significance)]	,174[b]	,713[b]

a. Grouping variabile: Medication
b.No correction was made for equal values

Source: own data, statistically processed

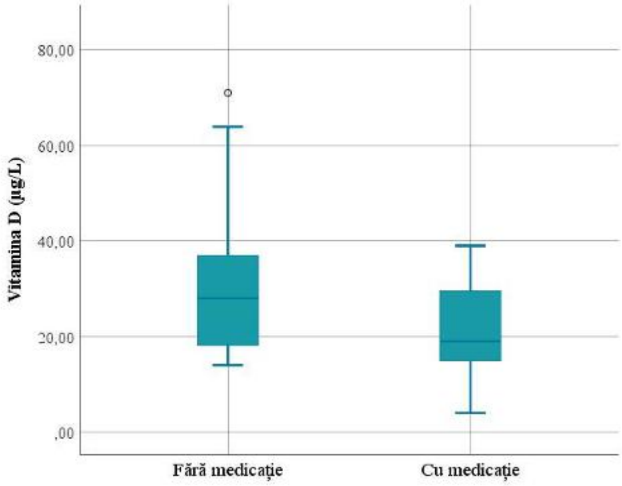

Figure I.3. Distribution of vitamin D values according to drug treatment in children with ASD

Source: own data, statistically processed

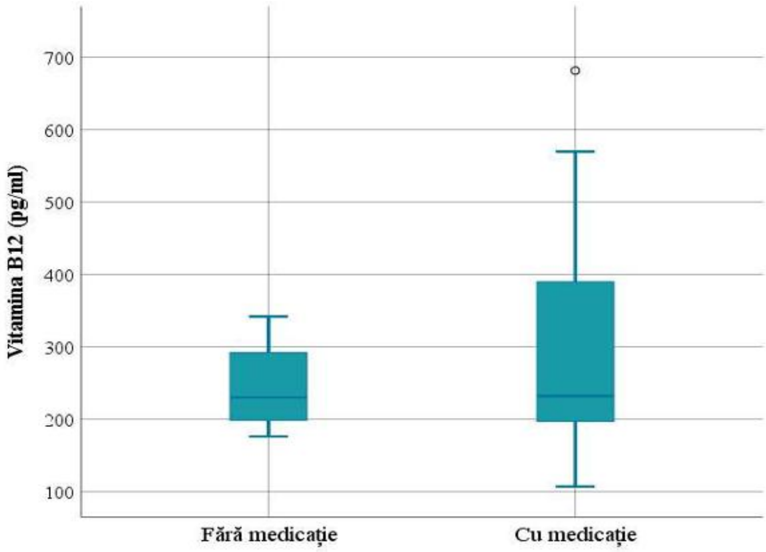

Figure I.4. Distribution of vitamin B12 values (pg/mL) in children with ASD, depending on drug treatment administration

Source: own data, statistically processed

Conclusions

Some biochemical changes in autism spectrum disorders are seen at the brain level, but the availability of diagnostic tools is more difficult due to the sensitivity of the brain to possible external confounding factors and due to technological ones. A complexity of testing tools for use at this level is observed and less complex and expensive testing methods can be used at the blood level. Thus, the identification of blood markers with a determining role in autism spectrum disorders could be a significant progress in identifying new methods of therapeutic intervention.

Vitamin status should be interpreted as a determinant/modulator of neuro-immune homeostasis and mitochondrial functioning, with a possible role in "amplifying" vulnerability to oxidative stress and supporting a dysbiosis profile; from this perspective, even small differences may be clinically relevant in subgroups, and future investigations should include systematic control of supplements, diet and seasonality (especially for 25-OH vitamin D).

Future research will certainly uncover the causes of autism and find new methods to improve the lives of children diagnosed with this serious developmental disorder.

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