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ORIGINAL ARTICLE

The Relationship Between Acetylcholinesterase, Neurotransmitter Levels, Calcium, Vitamin D3, and Trace Minerals in Autism Spectrum Disorder

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ABSTRACT

Abstract. This study investigates potential associations between acetylcholinesterase (AChE) activity, neurotransmitters, trace minerals, and autism spectrum disorder (ASD). A comparative analysis was performed on 96 ASD patients and 96 healthy controls. Results indicated significantly reduced AChE activity and specific activity in ASD patients compared to controls, supporting prior findings that link AChE dysregulation to neurological disorders. Elevated levels of GABA and GRIA1 were observed in ASD patients, alongside decreased oxytocin and HDAC-2 levels. In the ASD group, calcium and vitamin D3 levels were markedly reduced, while magnesium and zinc concentrations were higher. Copper levels showed a significant decline in ASD patients. Correlation analysis revealed negative associations between AChE activity and oxytocin, and positive associations between AChE activity and vitamin D3, GABA, and zinc. These findings highlight dysregulation in the cholinergic, GABAergic, glutamatergic, and oxytocinergic systems, along with trace mineral imbalances that may contribute to ASD pathophysiology. Further research is needed to clarify these mechanisms and assess potential therapeutic interventions targeting these biochemical markers.

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INTRODUCTION

Acetylcholine, a neurotransmitter with diverse functions, plays a critical role in cognitive processes, learning, and memory. Acetylcholinesterase (AChE), which degrades acetylcholine, is vital for maintaining the balance of cholinergic neurotransmission. Several studies have documented changes in AChE activity in individuals diagnosed with Autism Spectrum Disorder (ASD). For instance, elevated levels of AChE in the cerebrospinal fluid of individuals with autism suggest that disruptions in the cholinergic system may contribute to the core symptoms of ASD[1].

Neurotransmitter imbalances are increasingly recognized as contributing factors in the pathophysiology of ASD. Gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the brain, plays a vital role in regulating neuronal activity. ASD has been associated with diminished GABAergic activity, as indicated by lower GABA levels in the brains of affected individuals. In contrast, glutamatergic dysfunction, characterized by excessive glutamate signaling, has also been implicated in ASD. Glutamate receptors, especially NMDA receptors, are essential for synaptic plasticity and learning, and aberrant receptor function may underlie cognitive and behavioral impairments observed in ASD[2,3].

Reduced oxytocin levels and altered oxytocin receptor expression have been reported in individuals with ASD. Oxytocin, often referred to as the "love hormone," is integral to social bonding, trust, and empathy. Dysregulation of oxytocin may play a role in the social deficits characteristic of ASD[4].

Trace minerals are crucial for the proper functioning of enzymes and proteins that support brain development and activity. Magnesium, for example, is required by several enzymes involved in the synthesis and release of neurotransmitters[3,4]. Low magnesium levels have been linked to ASD, and deficiencies in magnesium are associated with impaired cognitive function and behavioral challenges. These findings highlight the potential role of magnesium supplementation in managing ASD symptoms[5].

Zinc is an essential trace mineral that plays a critical role in brain development and neurotransmission. Zinc deficiency has been linked to various neurological disorders, including autism spectrum disorder (ASD). Studies have observed lower zinc levels in the blood and hair of autistic children, indicating a potential association between zinc deficiency and ASD[6,7].

Copper, while vital for brain function, can become toxic at elevated levels. Copper-dependent enzymes, through their role in oxidative stress, have been implicated in the pathogenesis of ASD. Research has reported increased copper levels in the blood and hair of individuals with autism, suggesting a role for copper dysregulation in the disorder[8].

Epigenetic mechanisms, such as histone modifications, are integral to gene expression and brain development. Histone deacetylases (HDACs), enzymes responsible for histone deacetylation, regulate gene transcription. Emerging evidence indicates that HDAC inhibitors may offer therapeutic potential for ASD by reducing repetitive behaviors and improving social interactions, as demonstrated in animal models of ASD[9].

MATERIALS AND METHODS

Study case and Subjects

The study included a cohort of 96 patients diagnosed with autism spectrum disorder (ASD), aged between 3 and 12 years. Blood samples were collected at Kirkuk Pediatric Hospital, located in Kirkuk, Iraq. Diagnoses were conducted by a skilled pediatrician fol-

lowing established protocols. Patients underwent a detailed assessment, which included inquiries about medical history, parental consanguinity, age at ASD diagnosis, prescribed medications, and measurements of weight and height.

BIOCHEMICAL ASSAYS

The method mentioned in the text was used to measure the activity of acetylcholinesterase. There is a path length of 1 cm in the quartz cuvette, which holds 3 mL of reactor mix. It has 0.075 M of ATI, 0.01 M of DTNB, and 0.1 M of phosphate buffer with a pH of 7.4. Over the course of three minutes, measurements were taken every thirty seconds to check the optical density at a wavelength of 412 nm. The extinction coefficient $\Delta A \times$ D.F. $\times 10^6$ /13600 $\times 3$ $\times 10^3$ μmol/min/ml was used to measure the enzyme activity. It was expressed as units (U) or moles of acetylthiocholine hydrolysed mL^{-1} min^{-1} [10]. The enzymatic activity was measured and expressed as units per milligramme (U mg^{-1}). The protein content was determined using the Lowry method and Ciocalteu's Phenol reagent. In addition, protein quantification was conducted using the Folin phenol reagent. The research used bovine serum albumin as the benchmark. The absorbance of the blue-colored complex was measured at a wavelength of 620 nm[11].

BLOOD COLLECTION

A venipuncture technique was employed to obtain a volume of 5 ml of blood using a disposable syringe. The blood was thereafter transferred into gel tubes for separation. The tubes underwent centrifugation for a duration of 10 minutes. The serum was isolated from the cells and thereafter kept at a temperature of -20°C.

ELISA METHOD

Elisa method was used to determination the level of [MMP9, GABA, GRIA1, OT and HDAC-2] according to their procedures[12–16].

Determination of Trace Minerals

Magnesium

When magnesium is exposed to calmagite in an alkaline solution, it produces a complex that has a purple tint. When aminopolycarboxylic acid (EGTA) is present, the response becomes specific. The magnitude of the purple hue is directly proportional to the quantity of magnesium[17,18].

Zinc

The chromogen in the reagent has a chemical reaction with zinc, resulting in the formation of a colorful compound. The strength of this color is directly related to the quantity of zinc[19]

Copper

The cupric ions react with the chromogen Di-Br- PAESA forming a blue compound, which intensity is proportional to the cooper concentration present in the sample

Determination of Calcium and Vitamin D3

The Moorehead and Briggs CPC (O-Cresol Phthalein Complex One) Method was employed to determine total calcium concentration in serum. In this method, an alkaline CPC solution reacts with calcium to produce a dark red complex. The absorbance of this complex is measured at a wavelength of 570 nm, with the intensity directly proportional to the calcium concentration in the specimen[18].

The LIAISON® 25 OH Vitamin D_3 test utilized a chemiluminescence immunoassay (CLIA) to accurately measure the total concentration of 25 OH vitamin D_3 in serum. During the initial incubation, the binding protein was detached from 25 OH Vitamin D_3, allowing the vitamin to bind to a specific antibody immobilized on a solid surface. After 10 minutes, a tracer—comprising vitamin D_3 conjugated to an isoluminol derivative—was added, followed by a second 10-minute incubation period. Unbound material was removed via a washing procedure. Subsequently, reagents were introduced to trigger a rapid chemiluminescent reaction. The photomultiplier quantified the light signal in relative light units (RLU), which exhibited an inverse correlation with the concentration of 25 OH vitamin D_3 in calibrators, controls, or samples[20]

RESULTS AND DISCUSSION

isplays the enzyme activity and specific activity (enzyme activity divided by total protein) of serum AChE, presented as the mean value plus or minus the standard deviation in U/ml and U/mg, respectively. The measures were acquired from individuals who have been diagnosed with ASD and were then compared to the measurements of persons who are in good health.

Table 1. The (activity and specific activity) of the serum AChE for all studied groups

Parameters	Controls (n=96)	Patients (n=96)	P value
Serum AChE U/ ml	5.023 ± 0.883	4.224 ± 1.160*	0.0369
S.A in Serum U/mg	0.722±0.151	0.57 ± 0.145*	0.001

***Significant ($p \leq 0.05$)**

The findings in Table 1 indicated that the patients group exhibited notably reduced levels of acetylcholine esterase activity and specific activity of ASD patients' serum in comparison to the healthy individuals. Furthermore, the findings align with prior research that has demonstrated a decrease in the activity of acetylcholinesterase (AChE). The research revealed a significant decrease in acetylcholinesterase (AChE) activity among individuals diagnosed with neurological disorders. The decline in AChE activity has been correlated with the presence of despair, anxiety, boredom, and sadness, hence further substantiating the connection between this condition and mental health and psychiatric issues[21,22]. Estrogen's effect on brain function, particularly in improving choline uptake and acetylcholine synthesis, could explain the rise in acetylcholinesterase activity[23,24]. The observed decrease in specific activity might be due to certain impurities, achieved by dividing the activity of AChE by the concentration of TP in the serum. The specific function of an enzyme can serve as a dependable indicator of its purity[25,26]. The findings indicated above align with the research undertaken by which investigated the parameters in children diagnosed with autism[26–29].

Table 2. Levels of MMP9, GABA, GRIA1, OT and HDAC-2 in the serum of patients with autism spectrum disorder compared to control

Mean± SD		Parameters
Control(n=96)	**Patient(n=96)**	
0.457± 0.137	0.499± 0.118*	**MMP9 ng/mL**
1.223± 0.638	1.052± 0.559*	**GABA μmol/L**
35.115± 8.563	39.253± 6.603**	**GRIA1 μmol/L**
68.115± 16.160	56.521± 13.982**	**OT pg/mL**
1.177± 0.312	1.512± 0.426**	**HDAC-2 ng/mL**

MMP-9 is a zinc-dependent protease involved in tissue remodeling and extracellular matrix degradation. Matrix metalloproteinase-9 contributes to synaptic plasticity, dendritic spine dynamics, and neuronal migration. Dysregulation of MMP-9 may disrupt neural circuitry and contribute to ASD pathogenesis. Synapses are the connections between neurons in the brain. They have a vital function in the processing of information, acquisition of knowledge, and retention of memories. Synaptic plasticity is the capacity of synapses to alter their strength over a period of time. It encompasses mechanisms like as long-term potentiation (LTP) and long-term depression (LTD). Matrix metalloproteinase-9 has a role in altering the extracellular matrix (ECM) around synapses, leading to synaptic remodellingThere is no text provided. Research has shown that there are changes in the expression of MMP-9 in the brains of people with autism spectrum disorder (ASD)[30,31]. Elevated MMP-9 activity may result in synaptic Over-Pruning, which involves the excessive elimination of synapses at crucial stages of brain maturation. Additionally, it may result in disturbed connectivity, characterised by aberrant neuronal circuitry caused by excessive remodelling of the extracellular matrix (ECM) and an imbalance between excitation and inhibition. This dysregulation affects both excitatory and inhibitory synapses, ultimately

impacting the overall functioning of the neural network. Elevated MMP9 expression is associated with an increased chance of developing ASD[31].

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the brain. GABAergic Dysfunction in ASD has shown altered GABAergic signaling has been observed in individuals with ASD and reduced GABAergic inhibition may contribute to hyperexcitability and sensory processing abnormalities[32]. Disrupted GABAergic function may be the cause of sensory processing difficulties, repetitive behaviours, and deficiencies in social communication. GABAergic interneurons have multiple important functions. They regulate the activity of nearby excitatory neurones in specific areas of the brain. They also contribute to neural synchrony and rhythmic oscillations, such as gamma oscillations. During brain development, GABAergic interneurons shape neural circuits and synaptic plasticity. Additionally, they provide feedback inhibition to ensure stable network dynamics[32,33].

AMPA is an ionotropic receptor for glutamate. Subunit 1 is responsible for encoding a component of ionotropic glutamate AMPA receptors. These receptors are ion channels that are activated by certain molecules (ligands) and allow the passage of positively charged ions. They play a crucial role in transmitting signals between nerve cells in the central nervous system. They have a vital function in the processes of synaptic plasticity that are associated with learning and memory. Uncommon mutations in the GRIA1 gene have been linked to a neurodevelopmental disorder characterised by cognitive impairment, linguistic difficulties, and aberrant behaviour[34].

Oxytocin is a remarkable neuropeptide produced in the hypothalamus and released into the bloodstream by the pituitary gland. When explore its functions and effects, particularly in the context of ASD and AChE. Some studies propose that oxytocin has neuroprotective properties, it may protect neurons against injury during childbirth and other stressors, these effects could indirectly impact AChE function. Skin-to-skin contact between parents and infants raises oxytocin levels and Affectionate touch and emotional support play a role in oxytocin release, while affectionate conversation with parents can boost oxytocin in children[35,36].

Histone deacetylase 2 is an enzyme that removes acetyl groups from lysine residues on histones and non-histone proteins. It plays a critical role in gene expression regulation by controlling chromatin structure. Studies have shown altered HDAC-2 expression in the brains of individuals with ASD. Histone deacetylase 2 is involved in epigenetic processes that influence neural development and function. Dysregulation of HDAC-2 may impact the expression of genes associated with ASD. Altered HDAC-2 levels may lead to changes in chromatin structure and gene accessibility[37,38].

Calcium (Ca) and Acetylcholinesterase (AChE) have an indirect but important relationship in the nervous system. Here's a breakdown of their individual roles and how they might interact. Calcium is a crucial signaling molecule in the nervous system. It helps trigger the release of neurotransmitters, including acetylcholine, from nerve cells. During the propagation of an action potential, which is an electrical signal, calcium channels are activated,

Table 3. Levels of Calcium, Vit D3, Mg, Cu, Zn in the serum of patients with autism spectrum disorder compared to control

Mean± SD		Parameters
Control(n=96)	**Patient(n=96)**	
9.87±0.840	9.53±0.854**	**Ca mg/dL**
33.3±8.822	27.8±6.595**	**Vit. D ng/ml**
1.585±0.152	1.536±0.099**	**Mg mg/dL**
58.323± 12.298	64.104± 11.510**	**Cu mg/dL**
96.948± 11.459	92.490± 9.311**	**Zn mg/dL**

leading to the influx of calcium ions into the neurone. The entry of calcium ions stimulates the secretion of neurotransmitters into the synaptic cleft, which is the gap between neurones. Reduced calcium levels may result in a diminished release of acetylcholine, which can weaken nerve impulses and cause muscular weakness, weariness, and poor cognitive function[39]. While Ca directly affects the release of acetylcholine, AChE doesn't directly influence calcium levels. However, both play a role in regulating the overall level and duration of acetylcholine signaling. One theory suggests that Alzheimer's may involve both decreased AChE activity and abnormal calcium signaling[40]. This may result in modified levels of acetylcholine, which may contribute to a reduction in cognitive function. Calcium is the predominant element in the human body and plays a crucial function in the process of skeletal mineralisation. Serum calcium exists in both free and bound forms, mostly with proteins such as albumin. Excess of calcium guide to kidney stones, impair renal functions, and prostate cancer[41]. The decreased in calcium concentration in ASD patients is in agreement with those of done by Wang Q (2021)[42] and Guo M (2020)[43], in which they indicated that Hypocalcemia seems to be related to combination of hypoparathyroidism and osteocalcin resulting from deficient calcium intake, a well-known clinical condition associated with ASD patients. Vitamin D is a fat-soluble vitamin with diverse functions. It is essential for bone health, immune function, and neurological well-being. In the brain, vitamin D receptors are present in various regions, including those involved in cognition and behavior. Vitamin D has anti-inflammatory properties and supports the production of neurotrophins (growth factors for neurons). Vitamin D_3 deficiency is strongly correlated with ASD severity. Supplementation with vitamin D_3 has been explored as a potential intervention. Vitamin D_3 has anti-inflammatory effects, which may be relevant in ASD and Chronic inflammation is associated with neurodevelopmental disorders. By modulating immune responses, vitamin D_3 may influence brain health in ASD. Neurotrophins are growth factors essential for neuronal survival and function, Vitamin D_3 stimulates neurotrophin production, potentially supporting neural networks. Vitamin D3 is involved in serotonin synthesis which are linked to ASD symptoms and adequate vitamin D3 may help maintain serotonin balance[44].

Individuals with autism spectrum disorder have a range of issues including stunted development, endocrine disorders, and metabolic abnormalities. Several factors can con-

tribute to the development of Vitamin D_3 deficiency in children and teenagers with autism spectrum disorder (ASD). These factors include deficiencies in insulin-like growth factor 1 (IGF-I), hypoparathyroidism caused by iron buildup in the parathyroid gland, delayed puberty and hypogonadism, reduced bone mass, and decreased synthesis of 25-OH-D due to liver siderosis[45].

Vitamin D deficiency or insufficiency was more common in patients with autism spectrum disorder than in control group. This finding is similar to the result of the study done by Sadia Sultan et al[46] and Siracusano M[47]. Their findings were ascribed to hepatic dysfunction, which caused impaired hydroxylation of vitamin D_3 and thus reduced blood levels. Research reported a deficiency of vitamin D_3 (25-OH Vitamin D_3) in people with autism spectrum disorder (ASD). This deficit has been ascribed to the impaired absorption of vitamin, as well as insufficient dietary intake. Research found that individuals with autism spectrum disorder (ASD) had lower levels of vitamin D_3 (specifically 25-OH Vitamin D_3) during winter compared to summer. This difference is attributed to factors such as geographical latitude, air quality, cloud cover, clothing, time of day, and usage of sunscreen[44,48,49]. Individuals with Autism Spectrum Disorder (ASD) are more likely to have a higher risk of vitamin D_3 insufficiency and, as a result, require more vitamin D_3 treatment. The research was carried out by Brain K. Lee[50]. Emerging evidence suggests a potential association between vitamin D_3 levels throughout early life and an increased susceptibility to neurodevelopmental disorders, such as ASD. The research authors, Mazahery H. and Camargo A. C., used the blood 25(OH)D_3 level during pregnancy or infancy as a more accurate measure of vitamin D_3 status[51] are found growing evidence for a relationship between vitamin D_3 and ASD. Their conclusions are substantiated by compelling data derived from experimental research that examine the mechanistic processes. Nevertheless, the absence of sufficient primary and secondary preventative intervention studies makes it impossible to establish this correlation, unless there are randomized placebo-controlled trials of vitamin D_3 specifically designed to assess its effectiveness as a preventive or disease-modifying treatment for individuals with ASD[50].

Magnesium concentration in the ASD patients significantly decreased ($P \leq 0.01$) than that of the control group. It is an essential mineral involved in various physiological processes. It plays a pivotal role in immune regulation, enzyme function, and neurotransmission. Magnesium modulates the immune response through its effects on cytokines and immune cells and it decreases cytokine production in monocytes after toll-like receptor (TLR) stimulation, immune balance is crucial for neurodevelopment. Magnesium is a cofactor for enzymes involved in neurotransmitter synthesis and release, adequate Mg levels support normal neuronal communication and altered Mg availability may impact synaptic plasticity. Magnesium has anti-inflammatory effects, potentially mitigating neuroinflammation and chronic inflammation is associated with ASD pathogenesis[52].

But Skalny AV[53] indicated that the levels of magnesium in ASD, patients were higher than that of control groups, which is inconsistent with the findings of the present study. There is no clear explanation for the hypomagnesemia of ASD, which could be severe

enough to stimulate clinically related neuromuscular symptoms

Based on the results of the present study, there was a high significant decrease in the Zn concentration in the serum of ASD patient s and this was in agreement with previous studies[54,55]. Zinc, a metal belonging to the IIB group, is a crucial micronutrient for human health that does not undergo any net change in its oxidation state during chemical reactions. It can be compared to the behind-the-scenes team of a large theater show, which is sometimes overlooked but absolutely necessary. Zinc serves as a cofactor for more than 300 enzymes. Envision it as the overseer of a complex ensemble, guaranteeing the harmonic execution of biochemical compositions. These enzymes are involved in a wide range of functions, including DNA replication and immunological responses. Zinc is crucial for the formation of more than 2000 transcription factors. These molecular maestros regulate gene expression. Zinc is a critical cofactor for AChE, meaning its presence is essential for the enzyme to function properly. Zn binds to the active site of AChE, facilitating the breakdown of ACh. Studies have shown that Zn deficiency can lead to decreased AChE activity, resulting in an accumulation of ACh in the synaptic cleft. Conversely, excess Zn might lead to excessive AChE activity and subsequent depletion of ACh. A delicate balance of ACh levels is crucial for proper neuronal signaling. Disruptions in this balance, either due to altered AChE activity or ACh synthesis, can have significant consequences for brain function[56].

Some studies report lower Zn levels in children with ASD compared to typically developing controls. This could potentially lead to decreased AChE activity and subsequent ACh accumulation. Genes associated with Zn transport and metabolism have been implicated in ASD susceptibility. Variations in these genes might affect cellular Zn homeostasis, impacting AChE activity and cholinergic signaling[54,57].

Serum Cu level was found to be significantly lower in ASD patients when compared with control, this was in agreement with previous studies[54,58]. Copper is an essential trace element involved in various physiological processes. It plays a critical role in enzyme function, neurotransmitter synthesis, and antioxidant defense. Copper serves as a cofactor for several enzymes, including AChE. AChE requires Cu for its proper activity in breaking down acetylcholine (ACh). But dysregulation of Cu may contribute to oxidative stress and neuroinflammation. Copper level linked to Cu is necessary in human nutrition for normal iron metabolism. Also, could be explained by the antagonistic effect of the Zn, as Zn depletion in autism spectrum disorder could greatly increase the Cu absorption via the gastrointestinal tract.

Results shown a negative correlation between AChE activity with copper (r= - 0.3012), significant weak positive correlation between serum AChE activity with zinc (r=0.2357), significant positive correlation between serum AChE activity and serum Vit. D (r = 0.3416), significant positive correlation between serum AChE activity and serum GABA (r = 0.2505), a weak positive correlation between serum AChE activity and serum GRIA1 (r = 0.1462), very weak positive correlation between gABA and Vit D_3 in ASD patients (r= 0.1198), significant negative correlation between serum AChE activity and serum Oxytocin (r = - 0.377) and weak positive correlation between serum AChE activity and serum Mg

(r = 0.1164) as showed below:

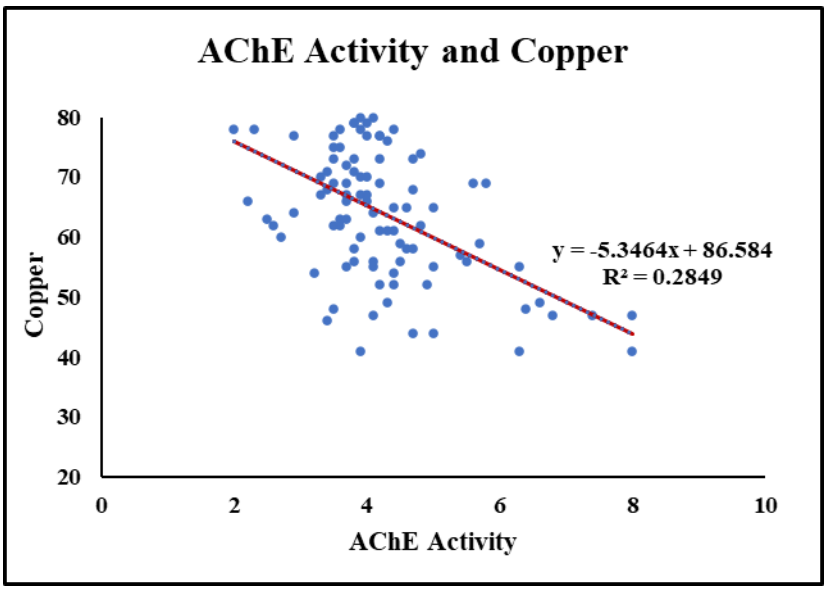

Figure 1

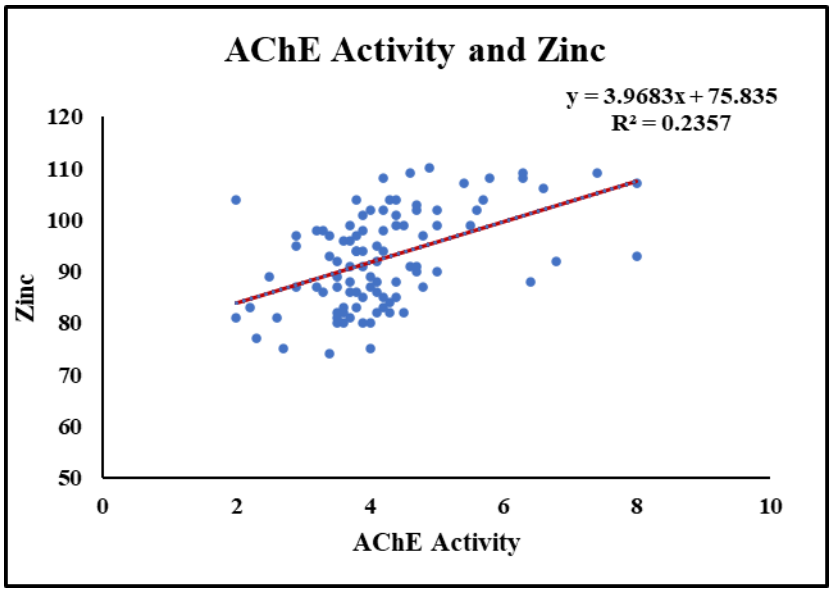

Figure 2

CONCLUSION

This study examined the biochemical profile of autistic children by analyzing acetylcholinesterase (AChE) activity, neurotransmitter levels, trace minerals, and matrix

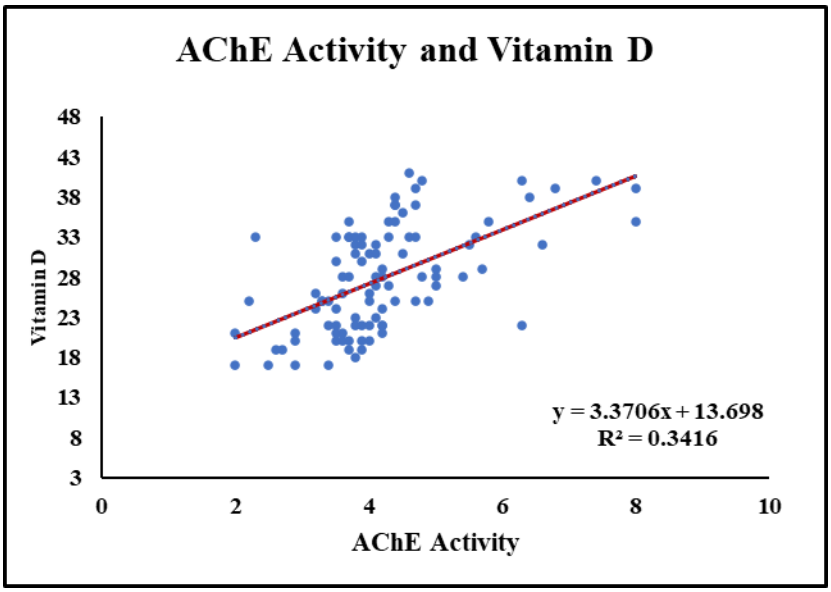

Figure 3

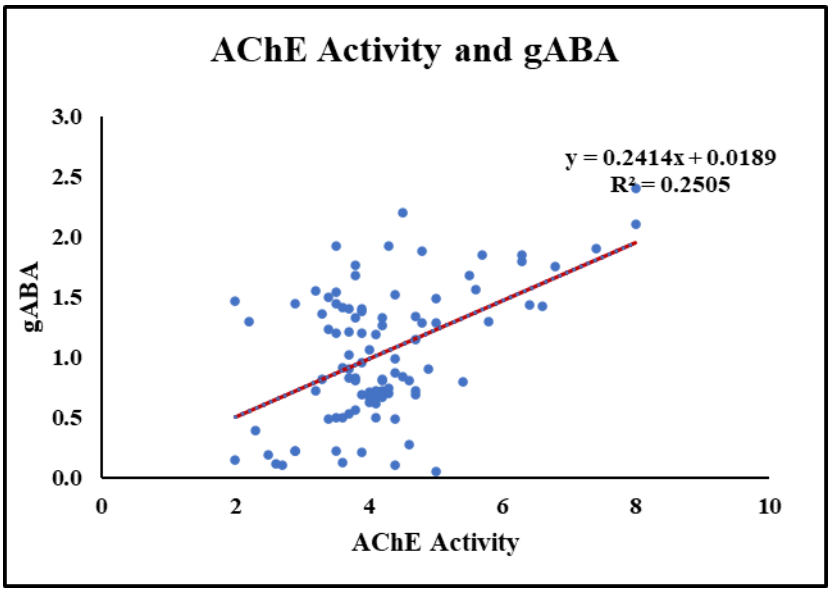

Figure 4

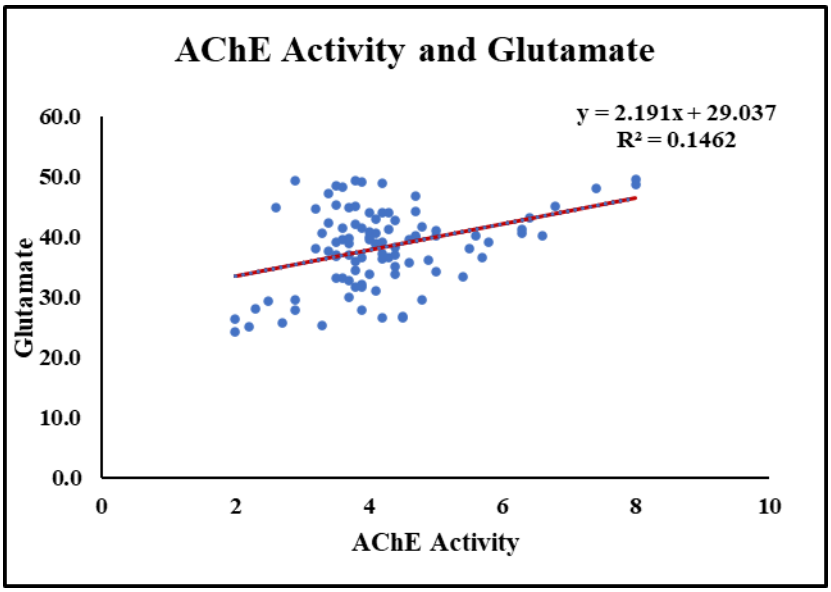

Figure 5

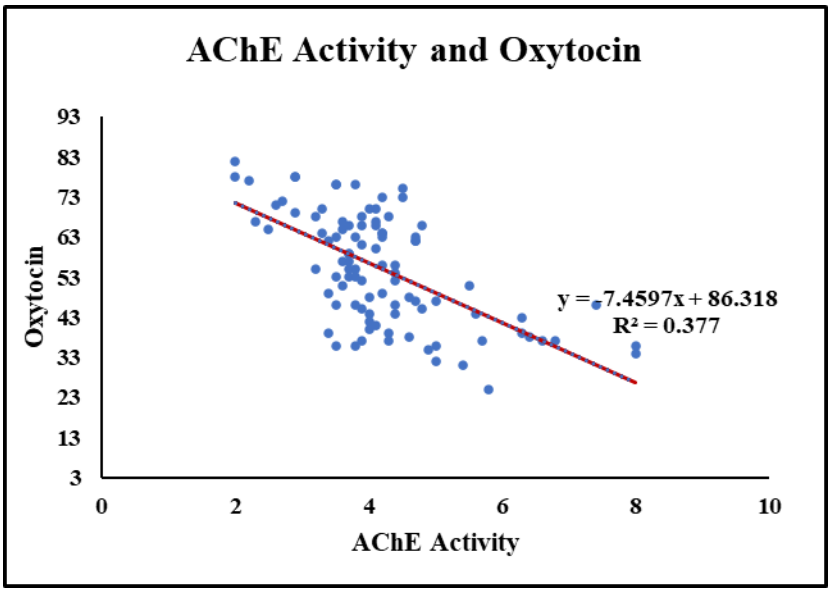

Figure 6

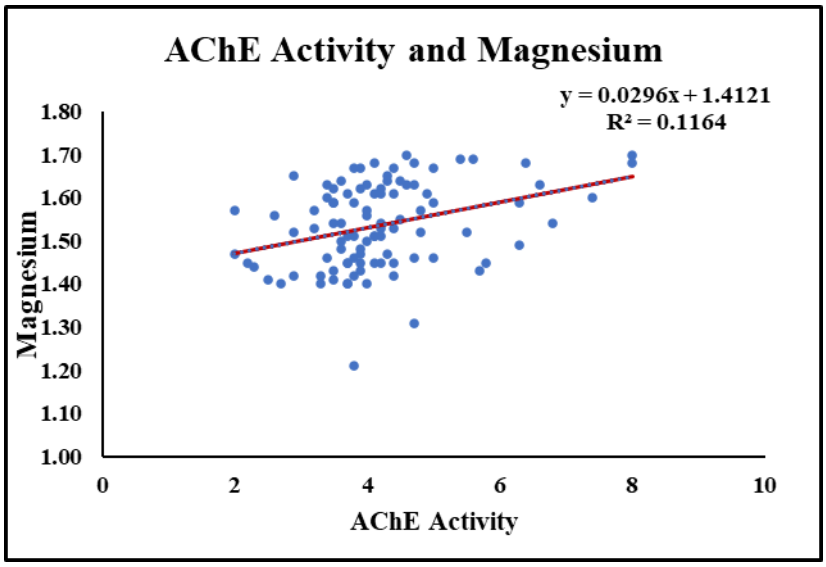

Figure 7

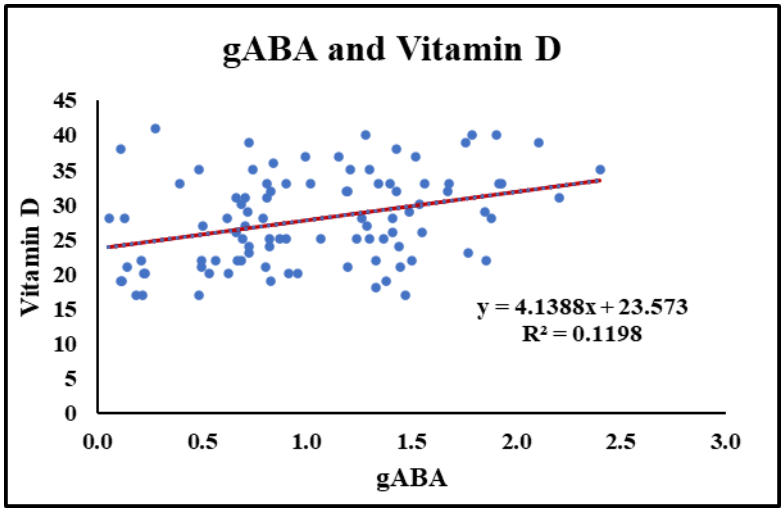

Figure 8 **The Correlation Between some of the biomarkers in Autism Spectrum Disorder Group**

metalloproteinase-9 (MMP-9). Results demonstrated significant reductions in AChE activity and specific activity in autistic patients compared to controls, consistent with prior research findings. Elevated levels of GABA and GRIA1, alongside decreased levels of oxytocin and HDAC-2, were observed in autistic individuals. Additionally, trace minerals such as calcium, vitamin D_3, and zinc were significantly lower in the autism group.

These findings suggest a complex interplay of neurochemical and metabolic factors in the pathophysiology of autism spectrum disorder (ASD). The observed biochemical alterations underscore the potential for developing targeted therapeutic strategies. Further research is essential to uncover the underlying mechanisms and evaluate the clinical implications of

these findings for the effective management of ASD.

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Authors' Declaration

No conflicts of interest present. We confirm ownership of all the Figures and Tables in the manuscript. Additionally, all figures and images included in the manuscript have the required permission for re-publication attached.

The project received approval from the local ethical committee at the University of Kirkuk and Kirkuk Health Directorate.

REFERENCES

1. Huang Q, Liao C, Ge F, Ao J, Liu T. Acetylcholine bidirectionally regulates learning and memory. Journal of Neurorestoratology. 2022;10(2):100002–100002. 10.1016/j.jnrt.2022.100002.
2. Zhao H, Mao X, Zhu C, Zou X, Peng F, Yang W, et al. GABAergic System Dysfunction in Autism Spectrum Disorders. Front Cell Dev Biol . 2022;9:781327–781327. 10.3389/fcell.2021.781327.
3. Nisar S, Bhat AA, Masoodi T, Hashem S, Akhtar S, Ali TA, et al. Genetics of glutamate and its receptors in autism spectrum disorder. molecular psychiatry . 2022;27:2380–2392. 10.1038/s41380-022-01506-w.
4. Baker E, Stavropoulos KK. The effects of oxytocin administration on individuals with ASD: Neuroimaging and behavioral evidence. Prog Mol Biol Transl Sci . 2020;173:209–238. 10.1016/bs.pmbts.2020.04.009.
5. Botturi A, Ciappolino V, Delvecchio G, Boscutti A, Viscardi B, Brambilla P. The Role and the Effect of Magnesium in Mental Disorders: A Systematic Review. Nutrients. 2020;12(6):1661–1661. 10.3390/nu12061661.
6. Shayganfard M. Are Essential Trace Elements Effective in Modulation of Mental Disorders? Update and Perspectives. Biological Trace Element Research. 2022;200(3):1032–1059. 10.1007/s12011-021-02733-y.
7. Sauer AK, Hagmeyer S, Grabrucker AM. Prenatal Zinc Deficient Mice as a Model for Autism Spectrum Disorders. International Journal of Molecular Sciences. 2022;23(11):6082–6082. 10.3390/ijms23116082.
8. Usui N, Doi M, Berto S, Matsuoka K, Ishida R, Irie K, et al. Copper-deficiency is associated with impairments in social behavior and oligodendro-

cyte development via mTOR signaling pathway. medRxiv. 2023;p. 2023–2035. 10.1101/2023.12.16.23300061.

9. Park J, Lee K, Kim K, Yi SJ. The role of histone modifications: from neurodevelopment to neurodiseases. Signal Transduction and Targeted Therapy . 2022;7(217). 10.1038/s41392-022-01078-9.
10. Singh S, Tiwari RK, Pandey RS. Acute toxicity evaluation of triazophos, deltamethrin and their combination on earthworm, Eudrilus eugeniae and its impact on AChE activity. Chemistry and ecology. 2019;35(6):563–575. 10.1080/02757540.2019.1600679.
11. Junior A, De Jesus EJ, Borges PC. Whey Protein Analysis Using the Lowry Assay and 96-Well-Plate Digital Images Acquired Using Smartphones. Journal of Chemical Education. 2023;100(6):2329–2338. 10.1021/acs.jchemed.2c00830.
12. Alrehaili AA, Gharib AF, Karam RA, Alhakami RA, Sawy WHE, Elrahman TMA. Clinical significance of plasma MMP-2 and MMP-9 levels as biomarkers for tumor expression in breast cancer patients in Egypt. Molecular Biology Reports. 2020;47(2):1153–1160. 10.1007/s11033-019-05216-5.
13. Nishiyama T, Sulistyaningdyah WT, Ueda K, Kusakabe H. GABA enzymatic assay kit. Bioscience, Biotechnology, and Biochemistry. 2020;84(1):118–125. 10.1080/09168451.2019.1661768.
14. Gnanadesikan GE, Hammock EA, Tecot SR, Carter CS, Maclean EL. Specificity of plasma oxytocin immunoassays: A comparison of commercial assays and sample preparation techniques using oxytocin knockout and wildtype mice. Psychoneuroendocrinology. 2021;132:105368–105368. 10.1016/j.psyneuen.2021.105368.
15. Alim MA, Grujic M, Ackerman PW, Kristiansson P, Eliasson P, Peterson M, et al. Glutamate triggers the expression of functional ionotropic and metabotropic glutamate receptors in mast cells. Cellular & molecular immunology. 2021;18:2383–2392. 10.1038/s41423-020-0421-z.
16. Tang Y, Lin YH, Ni HY, Dong J, Yuan HJ, Zhang Y, et al. Inhibiting Histone Deacetylase 2 (HDAC 2) Promotes Functional Recovery From Stroke. Journal of the American Heart Association. 2017;6(10):7236–7236. 10.1161/JAHA.117.007236.
17. Al-Dujaili AH, k Al-Hakeim H, Twayej AJ, Maes M. Total and ionized calcium and magnesium are significantly lowered in drug-naïve depressed patients: effects of antidepressants and associations with immune activation. Metab Brain Dis. 2019;34(5):1493–1503. 10.1007/s11011-019-00458-5.
18. Hua BL. Spectrophotometric determination of calcium, magnesium and inorganic phosphate in cerebrospinal fluid. University of Windsor Chemistry and Biochemistry; 1983. Available from: https://scholar.uwindsor.ca/etd/3555.
19. Al-Hasan AS. Correlation between Zinc and Iron with Psoriatic Patientsin Al-Anbar Governorate. Annals of RSCB;25(4):3589–3597.
20. Padro CA, Farmer CA, Thurm A, Shebl FM, Ilieva J, Kalra S, et al. Serum and cerebrospinal fluid immune mediators in children with autistic disorder: a longitudinal study. Mol Autism. 2017;8(1). 10.1186/s13229-016-0115-7.

21. Sattar SS, Rifaat SE. Effects of ethanolic Moringa oleifera extract on melatonin, liver and kidney function tests in alloxan-induced diabetic rats. Indian Journal of Forensic Medicine & Toxicology. 2019;13(4):1015–1019. 10.1186/s13229-016-0115-7.
22. Liu S, Li Z, Yu B, Wang S, Shen Y, Cong H. Recent advances on protein separation and purification methods. Advances in Colloid and Interface Science. 2020;284(102254):102254–102254. 10.1016/j.cis.2020.102254.
23. Bravo RKD, Angelia MRN, Uy LYC, Garcia RN, Torio MAO. Isolation, purification and characterization of the antibacterial, antihypertensive and antioxidative properties of the bioactive peptides in the purified and proteolyzed major storage protein of pigeon pea (Cajanus cajan) seeds. Food Chemistry: Molecular Sciences. 2022;4(30):100062–100062. 10.1016/j.fochms.2021.100062.
24. Alwahab RMA, Zainal IG. Measurement of Protein Oxidation Levels in Serum of Women with Pregnancy Complications. Egyptian Journal of Chemistry. 2021;64(11):6449–6455. 10.21608/ejchem.2021.72125.3685.
25. Yıldız S, Bilen Ç, Karakuş E. Purification and Characterization of Polyphenol Oxidase Enzyme from Damson Plum (Prunus insititia) by Spectrophotometrically. research square. 2021;p. 1–30. 10.21203/rs.3.rs-803684/v1.
26. Pant DR, Aryal B, Pun D, Sharma S, Joshi GP. Inhibition of α-amylase and α-glucosidase activities in vitro by extracts of selected medicinal plants. Biodiversitas: Journal of Biological Diversity. 2021;22(3). 10.13057/biodiv/d220314.
27. Kaur D, Behl T, Sehgal A, Singh S, Sharma N, Chigurupati S, et al. Decrypting the potential role of α-lipoic acid in Alzheimer's disease. Life Sciences. 2021;284(1):119899–119899. 10.1016/j.lfs.2021.119899.
28. Oki S, Toiyama Y, Okugawa Y, Shimura T, Okigami M, Yasuda H, et al. Clinical burden of preoperative albumin- globulin ratio in esophageal cancer patients. The American Journal of Surgery. 2017;214(5):891–898. 10.1016/j.amjsurg.2017.04.007.
29. Suzuki K, Sugihara G, Ouchi Y, Nakamura K, Tsujii M, Futatsubashi M, et al. Reduced Acetylcholinesterase Activity in the Fusiform Gyrus in Adults With Autism Spectrum Disorders. Archives of general psychiatry. 2011;68(3):306–313. 10.1001/archgenpsychiatry.2011.4.
30. Al-Sadi R, Engers J, Haque M, King S, Al-Omari D, Ma TY. Matrix Metalloproteinase-9 (MMP-9) induced disruption of intestinal epithelial tight junction barrier is mediated by NF-κB activation. PloS One. 2021;16(4). 10.1371/journal.pone.0249544.
31. Lord JR, Mashayekhi F, Salehi Z. How matrix metalloproteinase (MMP)- 9 (rs3918242) polymorphism affects MMP-9 serum concentration and associates with autism spectrum disorders: A case-control study in Iranian population. Dev Psychopathol. 2021;34(3):882–888. 10.1017/S0954579420002102.
32. Purkayastha P, Malapati A, Yogeeswari P, Sriram D. A Review on GABA/Glutamate Pathway for Therapeutic Intervention of ASD and ADHD. Curr Med Chem . 2015;22(15):1850–1859. 10.2174/0929867322666150209152712.

33. Zhao H, Mao X, Zhu C, Zou X, Peng F, Yang W, et al. GABAergic System Dysfunction in Autism Spectrum Disorders. Front Cell Dev Biol. 2021;9. 10.3389/fcell.2021.781327.
34. Soto D, Altafaj X, Sindreu C, Bayes A. Glutamate receptor mutations in psychiatric and neurodevelopmental disorders. Communicative & Integrative Biology. 2014;7(1). 10.4161/cib.27887.
35. Pichugina YA, Maksimova IV, Berezovskaya MA, Afanaseva NA, Pichugin AB, Dmitrenko DV, et al. Salivary oxytocin in autistic patients and in patients with intellectual disability. Front Psychiatry. 2022;13. 10.3389/fpsyt.2022.969674.
36. Huang Y, Huang X, Ebstein RP, Yu R. Intranasal oxytocin in the treatment of autism spectrum disorders: A multilevel meta-analysis. Neurosci Biobehav Rev. 2021;122:18–27. 10.1016/j.neubiorev.2020.12.028.
37. Guan FJ, Peng QQ, l Wang L, Yan XB, Dong C, Jiang XH. Histone deacetylase-2 expression and activity in children with nephrotic syndrome with different glucocorticoid response. Pediatric Nephrology . 2017;33:269–276. 10.1007/s00467-017-3791-4.
38. Tseng C, Mcdougle CJ, Hooker JM, Zürcher NR. Epigenetics of Autism Spectrum Disorder: Histone Deacetylases. Biol Psychiatry. 2022;91(11):922–933. 10.1016/j.biopsych.2021.11.021.
39. Goncharov NV, Belinskaia DA, Avdonin PV. Organophospate-Induced Pathology: Mechanisms of Development, Principles of Therapy and Features of Experimental Studies. J Evol Biochem Physiol. 2023;109(10):1349–1397. 10.31857/S0869813923100059.
40. Luo TY, Cai S, Qin ZX, Yang SC, Shu Y, Liu CX, et al. Basal Forebrain Cholinergic Activity Modulates Isoflurane and Propofol Anesthesia. Front Neurosci. 2020;14. 10.3389/fnins.2020.559077.
41. Reddi AS. Calcium, Phosphorus, and Magnesium Disorders and Kidney Stones. In: and others, editor. Absolute Nephrology Review. vol. 2022; 2024. p. 173–209. 10.1007/978-3-030-85958-9$_3$.
42. Wang Q, Kong Y, Wu DY, Liu JH, Jie W, You QL, et al. Impaired calcium signaling in astrocytes modulates autism spectrum disorder-like behaviors in mice. nature communications . 2021;12(3321). 10.1038/s41467-021-23843-0.
43. Guo M, Li L, Zhang Q, Dai Y, Chen L, Liu L, et al. Vitamin and mineral status of children with autism spectrum disorder in Hainan Province of China: associations with symptoms. Nutr Neurosci . 2018;23(10):803–810. 10.1080/1028415X.2018.1558762.
44. Rihal V, Khan H, Kaur A, Singh TG, Abdel-Daim MM. Therapeutic and mechanistic intervention of vitamin D in neuropsychiatric disorders. Psychiatry Res . 2022;317:114782–114782. 10.1016/j.psychres.2022.114782.
45. El-Hawy MA, Saleh NY. Effect of cyclic pamidronate administration on osteoporosis in children with β-thalassemia major: a single-center study. Clin Exp Pediatr. 2022;65(8):405–409. 10.3345/cep.2019.00535.

46. Ambelil M, Sultana S, Roy S, Gonzalez MM. Anaplastic Transformation in Mandibular Metastases of Follicular Variant of Papillary Thyroid Carcinoma: A Case Report and Review of the Literature. Ann Clin Lab Sci. 2016;46(5):552–556.
47. Siracusano M, Riccioni A, Abate R, Benvenuto A, Curatolo P, Mazzone L. Vitamin D Deficiency and Autism Spectrum Disorder. Curr Pharm Des . 2022;26(21):2460–2474. 10.2174/1381612826666200415174311.
48. Ersfeld DL, Rao DS, Body JJ, Sackrison JL, Miller AB, Parikh N, et al. Analytical and clinical validation of the 25 OH vitamin D assay for the LIAISON automated analyzer. Clin Biochem. 2004;37(10):867–874. 10.1016/j.clinbiochem.2004.06.006.
49. Guo M, Zhu J, Yang T, Lai X, Lei Y, Chen J, et al. Vitamin A and vitamin D deficiencies exacerbate symptoms in children with autism spectrum disorders. Nutr Neurosci . 2018;22(9):637–647. 10.1080/1028415X.2017.1423268.
50. Lee BK, Eyles DW, Magnusson C, Newschaffer CJ, McGrath JJ, Kvaskoff D, et al. Developmental vitamin D and autism spectrum disorders: findings from the Stockholm Youth Cohort. Mol Psychiatry. 2021;26(5):1578–1588. 10.1038/s41380-019-0578-y.
51. Mazahery H, Camargo CA, Conlon C, Beck KL, Kruger MC, v Hurst PR. Vitamin D and Autism Spectrum Disorder: A Literature Review. Nutrients. 2016;8(4):236–236. 10.3390/nu8040236.
52. Kardani A, Soltani A, Sewell R, Shahrani M, Rafieian-Kopaei M. Neurotransmitter, Antioxidant and Anti-neuroinflammatory Mechanistic Potentials of Herbal Medicines in Ameliorating Autism Spectrum Disorder. Curr Pharm Des. 2019;25(41):4421–4429. 10.2174/1381612825666191112143940.
53. Skalny AV, Mazaletskaya AL, Ajsuvakova OP, Bjørklund G, Skalnaya MG, Chernova LN, et al. Magnesium Status in Children with Attention-Deficit/Hyperactivity Disorder and/or Autism Spectrum Disorder. Soa Chongsonyon Chongsin Uihak . 2020;31(1):41–45. 10.5765/jkacap.190036.
54. El-Meshad GM, El-Khair MSA, Moharam NM, El-Nabi SAA. The plasma zinc/serum copper ratio as a biomarker in children with autism spectrum disorders. Menoufia Medical Journal. 2017;30(3):727–733. 10.4103/1110-2098.218255.
55. Hawari I, Eskandar MB, Alzeer S. The Role of Lead, Manganese, and Zinc in Autism Spectrum Disorders (ASDs) and Attention-Deficient Hyperactivity Disorder (ADHD): a Case-Control Study on Syrian Children Affected by the Syrian Crisis. Biol Trace Elem Res. 2020;197(1):107–114. 10.1007/s12011-020-02146-3.
56. Sarasamma S, Audira G, Juniardi S, Sampurna BP, Liang ST, Hao E, et al. Zinc Chloride Exposure Inhibits Brain Acetylcholine Levels, Produces Neurotoxic Signatures, and Diminishes Memory and Motor Activities in Adult Zebrafish. Int J Mol Sci. 2018;19(10):3195–3195. 10.3390/ijms19103195.
57. Zieminska E, Ruszczynska A, Augustyniak J, Toczylowska B, Lazarewicz J. Zinc and Copper Brain Levels and Expression of Neurotransmitter Receptors in Two Rat ASD Models. Front Mol Neurosci. 2021;14. 10.3389/fnmol.2021.656740.

58. Li S, l Wang J, Bjørklund G, Zhao W, Yin C. Serum copper and zinc levels in individuals with autism spectrum disorders. Neuroreport . 2014;25(15):1216–1220. 10.1097/WNR.0000000000000251.