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Evaluation of serum levels of Vitamin B12 , Vitamin 9 and zinc in Children Autism Spectrum Disorder

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ABSTRACT

Background: The complicated neurodevelopmental disease known as autism, or autism spectrum disorder, is often identified in a child's first three years of life. Cobalamin, another name for vitamin B12, is a water-soluble vitamin that is necessary for several bodily physiological functions. Adenosylcobalamin and methylcobalamin are two coenzyme forms of vitamin B12 that are involved in the metabolism of propionate, amino acids, and single-carbon units. The study aims to investigate whether serum Vitamin B12 , Vitamin 9 and zinc levels are associated with ASD and compare it with the control group in Kirkuk City.

Materials and Methods: From November 2024 to February 2025, a case-control research was conducted at the Pediatrics Hospital and two children's autism centers in Kirkuk City, Iraq: the Al-Irada Center for Autism and the Al-Tammauz Center for Autism. Kirkuk City, in northern Iraq, around 238 kilometers north of the capital, Baghdad, is where the study was carried out.

Results: The city is well-known for both its substantial economic significance and its diversified population. There are 56 patients with ASD in total. There were fifteen girls and forty-one males. They were between the ages of three and twelve. There were thirty-two healthy subjects in the control group of the current investigation. Nine of them were female, while twenty-three were male. They were between the ages of three and twelve. Provides the research participants' family history and demographic details. The median age (IQR) of the 88 participants was 6 years (4–8). The ASD group had a lower median age (5.00 years [IQR: 4.00, 8.00] vs. 7.00 years [IQR: 4.75, 9.50]; p = 0.058) than the control group. The median Zinc (Mg/dl) was significantly higher in the control group compared to the ASD group (106.35 (76.25, 133.60) Mg/dl vs. 76.60 (64.65, 96.65) Mg/dl; p = 0.004).

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INTRODUCTION

Autism, also known as an autism spectrum disorder, is a complex neurodevelopmental disorder usually diagnosed in the first three years of a child's life. A range of symptoms characterizes it and can be diagnosed at any age, including adolescence and adulthood. However, early diagnosis is crucial for effective management, prognosis, and care. Unfortunately, there are no established fetal, prenatal, or newborn screening programs for autism, making early detection difficult (1). It has largely been viewed as a lifelong condition (2). It is a spectrum where each individual presents differently (3).

Vitamin B12 (B12, cobalamin (Cbl)) is a water-soluble vitamin with a highly complex structure, which anticipates the complexity of the mechanisms by which the vitamin is assimilated and transported. Three proteins, intrinsic factor (IF), haptocorrin (HC), and transcobalamin (TC), and their respective membrane receptors are involved in these mechanisms. B12 deficiency due to malabsorption may produce a wide range of clinical symptoms, which include hematological manifestations ranging from macrocytosis to megaloblastic anemia, central and peripheral neurological disorders, psychiatric disorders, and thromboembolic outcomes (4). Vitamin B12 is essential for normal growth, development, and physiological functions. It plays an important role in neural myelination, synaptogenesis, and neurotransmitter synthesis.

Vitamin B12, also known as cobalamin, is a water-soluble vitamin essential for various physiological processes in the human body. It is primarily found in animal-derived foods such as meat, fish, dairy, and eggs and is crucial for DNA synthesis, red blood cell formation, and the proper functioning of the nervous system. Vitamin B12 exists in two coenzyme forms, adenosylcobalamin and methylcobalamin, which are involved in the metabolism of propionate, amino acids, and single-carbon units. Folate is important for neural development and is a coenzyme for the 'one-carbon' metabolic pathway, which is utilized in a number of processes, including DNA synthesis and regulation, cell proliferation, and immune function. It is also important in the realization and regulation of many neurological events, such as DNA damage repair, monoamine synthesis, and the synthesis of transmitters in the central nervous system (5). Zinc, a vital trace element, holds significant importance in numerous physiological processes within the body. It participates in over 300 enzymatic reactions, metabolic functions, regulation of gene expression, apoptosis, and immune modulation, thereby demonstrating its essential role in maintaining overall health and well-being. Zinc is essential for survival and zinc deficiency is linked to a variety of adverse conditions including growth retardation, impaired immune function, improper skin and bone formation and repair, as well as cognitive disfunction (6).

Trace elements play a critical role in the pathogenesis of autism spectrum disorders (ASD). Several studies have suggested a disturbance in the metabolism of zinc in ASDs (7). The study aims to determine whether there is a difference in serum levels of Vitamin B12, Folate, Zinc levels between children diagnosed with Autism Spectrum Disorder and healthy controls.

MATERIALS AND METHODS

A case-control study was performed at children's autism centers (Al-Irada Center for Autism and Al-Tammauz Center for Autism) and the Pediatrics Hospital in Kirkuk City-Iraq from November 2024 to February 2025. The study was conducted in Kirkuk City, which is located in northern Iraq, approximately 238 kilometers north of the capital, Baghdad. The city is known for its diverse population and significant economic importance. The total number of ASD patients is (56). Forty-one of them were males, while fifteen were females. Their ages ranged from 3 to 12 years. The present study contained a control group of thirty-two subjects who were healthy. Twenty-three of them were males and nine of them were females. Their ages ranged from 3 to 12 years. These individuals were attending different clinics of a pediatric hospital and were evaluated by a clinician.

Inclusion criteria: Previously diagnosed with ASD (for SG) patients within the specified age group.

Exclusion criteria: Has a chronic illness or a hematological disorder., and children outside the specified age group.

A volume of approximately 5 milliliters of venous blood was collected from both patient and control participants and transferred into a gel tube containing No EDTA to allow for clotting for 30 minutes at room temperature. Subsequently, the tubes were centrifuged at a speed of 3000 rpm for 15 minutes. Then, the acquired sera were aspirated by using an automatic micropipette, transported separately to two microtubes (Eppendorf), and stored at a temperature of -20℃ for late serological examination.

Statistical Analysis

All analyses were performed using R v4.4.2 (programming language). Shapiro-Wilk tests and Q-Q plot were used to test for normal distribution (Appendix-normality tests) . As normal distribution was mostly absent, medians with interquartile ranges (IQR) were shown for continuous variables and number (percentage) for categorial variables.

To test for differences between unpaired groups (ASD patients and Controls), Mann-Whitney U tests (Wilcoxon rank sum test) were performed for continuous variables and Pearson's Chi-squared test for categorical variables (8). Univariate and multivariable logistic regression were used to show associated factors with ASD patients with odd ratio and 95% CI for odd ratio. All p values less than 0.05 was considered statistically significant (8).

By using Medcalc software, Receiver-operator characteristic (ROC) curves were calculated to examine the cut-off for biomarkers with the best sensitivity and specificity. The optimal threshold was obtained by using Youden's *J* statistic; Youden's *J* statistic is (*J* = sensitivity + specificity -1(9).

RESULTS

Table 1 presents the demographic characteristics and family history of the study participants. Among the 88 participants, the median age (IQR) was 6 years (4–8). The median age was lower in the ASD group than in the control group (5.00 years [IQR: 4.00, 8.00] vs. 7.00 years [IQR: 4.75, 9.50]; p =0.058). Age was significantly associated with ASD in both the univariate and multivariable analyses. Each additional year of age was associated with a 16% decrease in the odds of ASD in the univariate model (OR =

0.84; 95% CI: 0.70–0.98; $p = 0.035$), and a 23% decrease in the multivariable model (aOR = 0.77; 95% CI: 0.58–0.98; $p = 0.043$).

Gender was not significantly associated with ASD in either model. Female participants had 20% lower odds of being in the ASD group in the univariate analysis (OR = 0.80; 95% CI: 0.31–2.13; $p = 0.7$), but this association remained non-significant after adjustment (aOR = 1.18; 95% CI: 0.28–5.42; $p = 0.8$). Vitamin B-12 (pg/mL) was significantly associated with ASD in both models. The univariate analysis showed a small but significant increase in odds per unit (OR = 1.00; 95% CI: 1.00–1.01; $p = 0.007$), which remained significant in the multivariable analysis (aOR = 1.01; 95% CI: 1.00–1.01; $p = 0.006$).

Vitamin B9 (ng/mL) was not significant in the univariate model (OR = 1.00; 95% CI: 0.92–1.08; $p > 0.9$). However, in the multivariable model, it showed a negative association with ASD (aOR = 0.89; 95% CI: 0.78–1.00; $p = 0.062$), approaching statistical significance. Zinc (mg/dL) showed consistent inverse associations with ASD. In the multivariable model, each unit increase was associated with a 3% decrease in the odds of ASD (aOR = 0.97; 95% CI: 0.95–0.99; $p = 0.004$).

Receiver operating characteristic (ROC) curve analysis was used to evaluate the diagnostic performance of each study variable as a potential biomarker for ASD. The performance metrics include the area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

For Vitamin B-12 (pg/mL), the AUC was 0.691 (95% CI: 0.584–0.786). At a cutoff value of >445.9 pg/mL, it demonstrated sensitivity of 51.79%, specificity of 84.37%, PPV of 85.3%, and NPV of 50.0% (Fig. 4.12). Vitamin B9 (ng/mL) had a relatively poor diagnostic performance with an AUC of 0.522 (95% CI: 0.413–0.630). At a threshold of ≤3.37 ng/mL, it showed sensitivity of 3.57%, specificity of 81.25%, PPV of 25.0%, and NPV of 32.5% (Fig. 4.13). Lastly, Zinc (mg/dL) showed an AUC of 0.687 (95% CI: 0.580–0.782), reflecting moderate diagnostic accuracy. At a cutoff of ≤85.1 mg/dL, Zinc yielded a sensitivity of 71.43%, specificity of 65.62%, PPV of 78.4%, and NPV of 56.8% (Fig. 4).

DISCUSSION

There were no significant differences in gender distribution between the groups (p = 0.7), with males comprising 73% of the ASD group and 69% of the control group. ASD is more common in males (10). Hodges et al., (2020) suggested that girls who meet the criteria for ASD are at higher risk of not receiving a clinical diagnosis. Among ASD group, positive family history of autism was reported in 11 (20%). The median age at symptom onset was 2 years (IQR: 1.5 – 4). Additionally, 8 (14%) had comorbid conditions other than autism (11). Regarding the symptoms in autistic children, speech difficulties were the most prevalent (91.1%). Attention deficits were reported in 82.1% of the children, and social interaction problems were reported in 67.9%. The core features of autism spectrum disorders (ASD) are communication impairments, social skills deficits, and the presence of repetitive or overly restricted behaviors (12).

In the present study, the median (IQR) concentration of Vitamin B9 was lower in the ASD group compared to the healthy control group; however, this difference did not reach statistical significance. This finding (7.42 [5.26, 13.19] ng/ml vs. 9.14 [4.95, 14.86] ng/ml; p = 0.7) suggests a trend toward lower folate levels in children with ASD.In the present study, the median (IQR) concentration of Vitamin B9 was lower in the ASD group compared to the healthy control group; however, this difference did not reach statistical significance. This finding (7.42 [5.26, 13.19] ng/ml vs. 9.14 [4.95, 14.86] ng/ml; p = 0.7) suggests a trend toward lower folate levels in children with ASD. On the other hand, Vitamin B12 levels showed a statistically significant difference, with higher concentrations observed in the ASD group compared to the healthy control group (448.75 [324.75, 789.75] pg/ml vs. 355.95 [267.00, 423.60] pg/ml; p = 0.003).

These results align with prior findings by Barnhill et al., (2018), who reported multiple B vitamin deficiencies, including folate, among children with ASD in a case-control study of 86 children with ASD and 57 neurotypical peers aged 2–8 years. The study highlighted that child with ASD often have restricted dietary patterns, which may contribute to insufficient intake of essential nutrients like B1, B2, B3, B6, and folate (13).

Further biochemical insight was provided by Belardo et al., (2019), who employed advanced UHPLC-mass spectrometry (Q-exactive analyzer) techniques to assess urine samples from children with ASD. Their findings demonstrated simultaneous deficiencies of vitamins B6, B9, and B12, proposing that intestinal dysbiosis may underlie poor absorption of these nutrients. Moreover, genetic mutations could further compromise B vitamin metabolism in this population (14).

Vitamin B12, in particular, plays a crucial role in neurological health. Its involvement in maintaining gut microbiota homeostasis has drawn attention, especially given the frequent presence of dysbiosis in individuals with ASD. Such imbalances can lead to increased intestinal permeability, chronic low-grade inflammation, and alterations in the gut–brain axis—all of which may exacerbate or contribute to ASD symptoms. In addition, B12 is vital for the metabolism of neurotransmitters like serotonin and dopamine, which are critical for mood regulation, cognitive function, and behavioral responses (15).

Supporting the importance of these nutrients, a systematic review by Hoxha et al., (2021) concluded that supplementation with folic acid or folinic acid in children with ASD led to notable improvements in both neurological and behavioral outcomes. These benefits were accompanied by favorable changes in one-carbon metabolism markers, highlighting the therapeutic potential of targeting B vitamin pathways in ASD management (16).

In the current study, the median Zinc (Mg/dl) was significantly higher in the control group compared to the ASD group (106.35 (76.25, 133.60) Mg/dl vs. 76.60 (64.65, 96.65) Mg/dl; p = 0.004) (Fig. 4.3c). These results are consistent with a previous case-control study of 23 children with autism who were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) and the Childhood Autism Rating Scales (CARS) and who did not have another medical condition. The control group included 23 seemingly

healthy children whose age and gender were matched to the patients. Revealed that Serum zinc was significantly decreased in the ASD group than the control group (P value <0.001) (Ahmad et al., 2023). (DK et al., 2019) reported that there was a mean difference of serum zinc levels between the ASD and non-ASD groups of 1·75 µmol/l (P<0·001, CI 1·2-2·1). suggesting zinc deficiency is likely to be common in ASD patients and is a potentially modifiable environmental factor associated with the condition. However, a case control study of 150 children with ASD who and 72 controls, results revealed that the mean (SD) zinc level was not different between the groups (ASD 11.7 [1.7] versus control 11.6 [2.1] µmol/L, p value = 0.86) (17).

Systematic review and meta-analysis concluded that although it cannot be stated that there is a significant difference in Zn concentrations between individuals with ASD and controls, the reviewed data point to a frequent occurrence of lower concentrations of Zn in individuals with ASD, and that this profile is also possibly related to the severity of the disorder (18). Another systematic review and meta-analysis reported that there was a significant statistical difference between plasma Zn concentration and autistic patients, besides healthy controls: -0.253 (95% CI: 0.498 to -0.007) (19). Additionally. A Meta-analysis showed that the Patients with ASD had lower levels of zinc compared to controls (p=0.021) (5).

In the current study, the median Zinc (Mg/dl) was significantly higher in the control group compared to the ASD group (106.35 (76.25, 133.60) Mg/dl vs. 76.60 (64.65, 96.65) Mg/dl; p = 0.004) (Fig. 4.3c). These results are consistent with a previous case-control study of 23 children with autism who were diagnosed using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) and the Childhood Autism Rating Scales (CARS) and who did not have another medical condition. The control group included 23 seemingly healthy children whose age and gender were matched to the patients. Revealed that Serum zinc was significantly decreased in the ASD group than the control group (P value <0.001) (20). Click or tap here to enter text. DK et al., (2019) reported that there was a mean difference of serum zinc levels between the ASD and non-ASD groups of 1·75 µmol/l (P<0·001, CI 1·2-2·1). suggesting zinc deficiency is likely to be common in ASD patients and is a potentially modifiable environmental factor associated with the condition (21). However, a case control study of 150 children with ASD who and 72 controls, results revealed that the mean (SD) zinc level was not different between the groups (ASD 11.7 [1.7] versus control 11.6 [2.1] µmol/L, p value = 0.86) (22).

CONCLUSION

This study demonstrates that children with ASD differ from controls in several biochemical and demographic factors. Younger age and lower zinc levels were independently associated with higher odds of ASD, while vitamin B12 levels were significantly higher in the ASD group and showed moderate diagnostic performance. Vitamin B9 tended to be lower in ASD but did not reach statistical significance. Overall, zinc and vitamin B12 showed the most promise as potential biomarkers, though their diagnostic accuracy was moderate. These findings support growing evidence that micronutrient imbalances are common in ASD and may play a contributory role, highlighting the need for

further large-scale studies to clarify their clinical relevance and potential role in early screening or targeted nutritional interventions.

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TABLES

Table 1: Descriptive Characteristics of Study Participants

Characteristics	Total (N = 88)	Study Groups		p-value
		ASD (N = 56)	HealthyControl (N = 32)	
Age (years)				0.058
Mean ± SD	6.39 ± 2.70	5.93 ± 2.43	7.20 ± 2.97	
Median (Q1, Q3)	6.00 (4.00, 8.00)	5.00 (4.00, 8.00)	7.00 (4.75, 9.50)	
Gender, n (%)				0.7
Male	63 (72%)	41 (73%)	22 (69%)	
Female	25 (28%)	15 (27%)	10 (31%)	
Family History, n (%)				
Yes	-	11 (20%)	-	
No	-	45 (80%)	-	
Age at Symptom Onset (years)				
Mean ± SD	-	2.72 ± 1.65	-	
Median (Q1, Q3)	-	2.00 (1.50, 4.00)	-	
Other Diseases, n (%)				
Yes	-	8 (14%)	-	
No	-	48 (86%)	-	

Table 2: Comparisons of Vitamin Levels Between ASD and Control Group

	Total (N = 88)	Study Groups		p-value
		ASD (N = 56)	Healthy Control (N = 32)	
VitB9 (ng/ml)				0.7
Median (Q1, Q3)	8.13 (5.26, 13.42)	7.42 (5.26, 13.19)	9.14 (4.95, 14.86)	
VitB12 (pg/ml)				0.003
Median (Q1, Q3)	389.10 (306.95, 525.75)	448.75 (324.75, 789.75)	355.95 (267.00, 423.60)	
Zinc (Mg/dl)				0.004
Median (Q1, Q3)	80.75 (67.35, 112.80)	76.60 (64.65, 96.65)	106.35 (76.25, 133.60)	

Table 3: Spearman correlation of characteristic

	Age	Vitamin B-12	Vitamin B9	Zinc
Age	1			
Vitamin B-12	-0.47***	1		
Vitamin B9	-0.29***	0.19	1	
Zinc	-0.23*	0.19	0.08	1

Table 4: Logistics regression for factors associated with ASD patients

Characteristic	Univariate			Multivariable		
	OR[1]	95% CI[1]	p-value	aOR[1]	95% CI[1]	p-value
Age	0.84	0.70, 0.98	0.035	0.77	0.58, 0.98	0.043
Gender						
Male	—	—		—	—	
Female	0.80	0.31, 2.13	0.7	1.18	0.28, 5.42	0.8
Vitamin B-12 (pg/ml)	1.00	1.00, 1.01	0.007	1.01	1.00, 1.01	0.006
Vitamin B9 (ng/ml)	1.00	0.92, 1.08	>0.9	0.89	0.78, 1.00	0.062
Zinc (Mg/dl)	0.98	0.97, 0.99	0.008	0.97	0.95, 0.99	0.004

Table 5: Area under the curve, sensitivity, specificity, and predictive values of biomarkers.

Biomarker	AUC	AUC 95 % CI	Threshold	Sensitivity	Specificity	PPV	NPV
Vitamin B-12 (pg/ml)	0.691	0.584 to 0.786	>445.9	51.79	84.37	85.3	50.0
Vitamin B9 (ng/ml)	0.522	0.413 to 0.630	≤3.37	3.57	81.25	25.0	32.5
Zinc (Mg/dl)	0.687	0.580 to 0.782	≤85.1	71.43	65.62	78.4	56.8

FIGURES

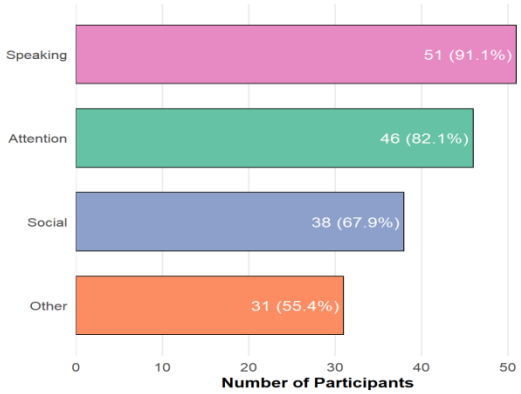

Figure 1: Percentages of symptoms among ASD patients

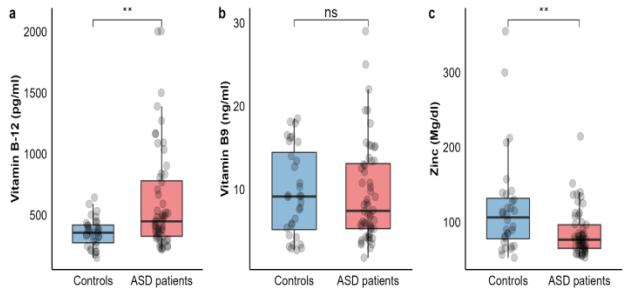

Figure 2: a-boxplot of VitB12 by study groups, b-boxplot of VitB9 by study groups, c- c-boxplot of Zinc by study groups.

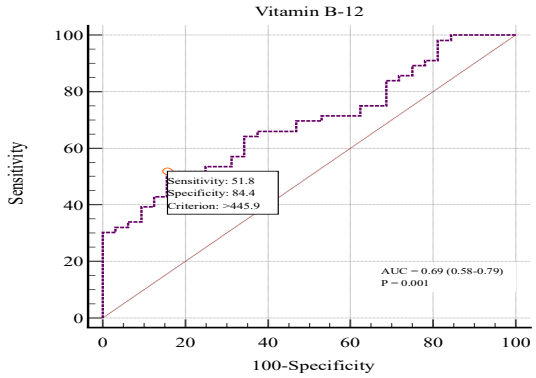

Figure 3: Receiver operating characteristic (ROC) curve of vitamin B9.

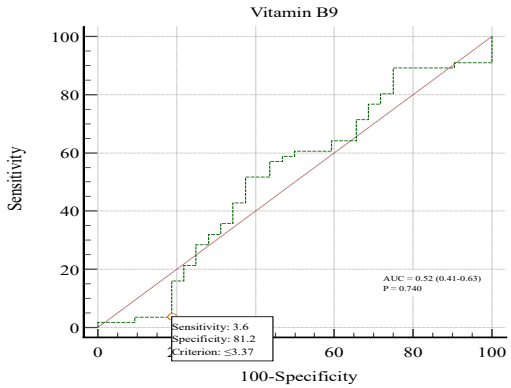

Figure 4: Receiver operating characteristic (ROC) curve of Zinc.

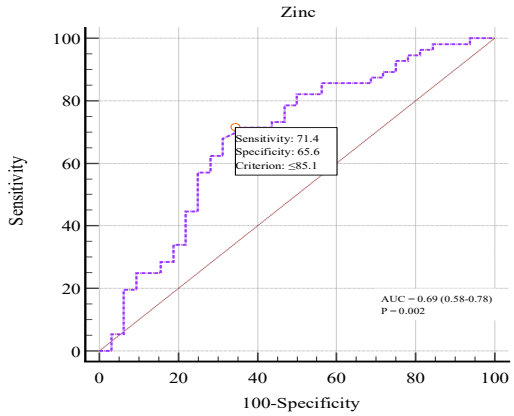

Figure 5: Receiver operating characteristic (ROC) curve of vitamin B12.