

Dysregulated Vitamin B12 levels in a large cohort of 3025 ASD children

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Abstract

Background

Vitamin B12 (B12) plays a crucial role in neurodevelopment, methylation pathways, mitochondrial function and its dysregulation has been implicated in Autism Spectrum Disorder (ASD). It is specifically required as a cofactor for the activity of two strictly B12 dependent enzymes: methionine synthase and methylmalonyl-CoA mutase thereby playing a crucial role in key metabolic pathways, including the conversion of homocysteine to methionine.

Methods

This observational study evaluated B12 levels in children from age 2 to 18 years diagnosed with ASD (according to DSM-5 criteria) and studied association of B12 levels with severity and behavioral features measured using Gilliam Autism Rating Scale (GARS) and social maturity using SQ scores.

Results

Our findings revealed that only 40% of children had optimum B12 levels. A substantial proportion 33% demonstrated elevated B12 levels, whereas 27% had B12 deficiency. In the profound ASD group 30.25% were B12 deficient, 31.9% had elevated B12 and 37.81% had optimum levels. Deficiency increased with age and elevated levels were common in younger age group. Deficiency was more prevalent among vegetarians than non-vegetarians, whereas elevated B12 levels were more frequently observed among non-vegetarians. Multivariable regression analysis also revealed that Age was significant predictors of both GARS and SQ scores, and Diet was a significant predictor of GARS whereas B12 was marginally associated with SQ after adjustment for covariates.

Conclusion

The heterogeneous distribution of B12 status across the ASD cohort suggests that B12 dysregulation may be more complex than a uniform deficiency state. The presence of elevated B12 levels may reflect impaired cellular utilization or altered cobalamin metabolism, highlighting the limitations of serum B12 as a sole indicator of functional cobalamin status. Since core symptoms and clinical severity were observed across all B12 categories, optimizing B12 levels alone may not produce predictable changes in ASD severity. Assessment of functional metabolic biomarkers and methylation related pathways is required in future studies. Optimizing B12 levels should be an adjunctive treatment strategy rather than a standalone.

INTRODUCTION

Vitamin B12 (B12) (cobalamin) is a water-soluble vitamin, consumed primarily through animal products such as meat, dairy, and eggs in diet.[1, 2] (Fig. 1) B12 is absorbed through complex procedure in terminal ileum.(Fig. 2) Methylcobalamin and 5-deoxyadenosylcobalamin, are the two metabolically active forms of B12 involved in metabolism.[3](Fig. 1) It plays an essential role in development and function of the nervous system by supporting myelin synthesis and is critical for the maturation of red blood cells in the bone marrow and DNA synthesis.[4, 5, 6] B12 plays a critical role for synaptogenesis, synthesis of neurotransmitters, and many cellular metabolic pathways for maintenance of genomic stability.[2, 7] B12 also acts as an essential cofactor for several enzymes involved in the biosynthesis of RNA and DNA.[2]

Figure 1 : Overview of Vitamin B12 (Cobalamin) metabolism.

Schematic representation of Vitamin B12 intake from dietary sources and its Active forms

Figure 2: Schematic representation of Absorption of Vitamin B12 (Cobalamin).

Dietary Vitamin B12 (Cobalamin) bound to Animal Protein (AP) enters the stomach along with salivary R protein. In the stomach gastric acid and pepsin release B12 from Animal proteins and B12 binds to R proteins. In the duodenum, pancreatic protease degrades B12-R complex, allowing B12 to bind intrinsic factor (IF) secreted by gastric parietal cells. B12-IF complex is absorbed in the terminal ileum via receptor mediated endocytosis. After endocytosis vitamin B12 dissociates from Intrinsic Factor and binds to transcobalamin (TC II) to form B12-TC II for transport through the bloodstream to the brain and liver.

B12 plays a vital role as a cofactor in two major metabolic pathways: the breakdown of methylmalonic acid and the methionine synthesis. [8] It is specifically required as a cofactor for the activity of two strictly B12 dependent enzymes: Methionine synthase (MS) and methylmalonyl-CoA mutase. [4, 5, 6, 8] In cytoplasm, Methionine synthase (MS) an enzyme encoded by the Methionine synthase gene (MTR gene), catalyzes the remethylation of homocysteine to methionine through a two-step cyclic process involving methylcobalamin.[4, 9] In the first step, cobalamin accepts a methyl group from 5-methyltetrahydrofolate (5-methyl THF), generating methylcobalamin and releasing tetrahydrofolate (THF). In the second step, methylcobalamin donates this methyl group to homocysteine, producing methionine. During this process, cobalamin is oxidized to an inactive cob(II)alamin form, which is reactivated by Methionine synthase reductase (MTRR), restoring it to the active cob(I)alamin state and maintaining continuous catalytic activity.[8, 10] (Fig. 3) Also MTRR, along with S-adenosylmethionine (S-AdoMet), vitamin B2 and vitamin B3 activates MTR gene thereby MS. Methionine is required to generate S-AdoMet, the primary methyl donor for methyltransferases (MTs) that methylate biomolecules DNA, RNA, proteins, and lipids, thereby linking cobalamin to folate dependent one carbon metabolism. [4, 8, 9] Disruption in Methylation cycle will reduce conversion of Inactive B12 to Methylcobalamin (Active) causing functional B12 deficiency.

Figure 3: Role of Methylcobalamin in Methionine synthesis

Schematic representation of conversion of homocysteine to methionine mediated by Methionine Synthase (MS) and Methylcobalamin as an active cofactor, with Cob(II)alamin intermediate during the transfer of a methyl group from 5-Methyl THF to homocysteine.

In mitochondria, inactive Cob(II)alamin is converted into active 5-deoxyadenosylcobalamin by the enzyme adenosyltransferase. This 5-deoxyadenosylcobalamin acts as a cofactor for L-methylmalonyl-CoA mutase(MMCoAMut), required for conversion of L-methylmalonyl-CoA to succinyl-CoA. This succinyl-CoA then enters the TCA cycle, which helps generate energy ATP and maintain metabolic balance in the cell. [4, 10] (Fig. 4) Disruption of this pathway leads to methylmalonic acid (MMA) accumulation, which has been associated with mitochondrial dysfunction and neurodevelopmental abnormalities.[11]

Figure 4: Role of Adenosylcobalamin in Methylmalonyl-CoA Metabolism

Schematic representation of vitamin B12 metabolism in the mitochondria. The inactive form cob(II)alamin is converted into its active form, adenosylcobalamin, by the enzyme adenosyltransferase. Adenosylcobalamin then acts as a cofactor for methylmalonyl-CoA mutase, which catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA which contributes to ATP production and maintaining metabolic balance.

Disruptions in one-carbon metabolism, dysregulation of methylation capacity, impaired mitochondrial energy metabolism and increased oxidative stress and redox imbalance have been majorly reported as biological features contributing to pathophysiology of Autism spectrum disorder (ASD).[4, 8, 9, 11, 12, 13]

ASD is an early onset neurodevelopmental condition characterized by deficits in verbal and nonverbal communication, repetitive behavior, and restricted social interaction.[14, 15] Both genetic predisposition and environmental factors have been proposed as major contributors to the etiology of ASD.[16] B12 is one of the environmental elements that influence neural development of the brain and its function.[6] Inadequate B12 levels during pregnancy or early childhood may increase ASD risk by impairing cognitive and behavioral development. Impairments in neurotransmitter synthesis and neuronal myelination may contribute to cognitive and behavioral features of ASD.[1]

The present study evaluates B12 levels in a largest cohort of children with ASD. Due to the limited and inconsistent evidence regarding its clinical correlation w.r.t. ASD severity, this study aims to investigate the clinical association between variability of B12 and clinical features, neurobehavioral characteristics of ASD.

MATERIALS AND METHODS

Study Design:

This study was conducted as a retrospective observational study including 3025 patients diagnosed with ASD. All participants were diagnosed according to established criteria, based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V). Diagnosis was confirmed through a comprehensive clinical assessment performed by qualified healthcare professionals with expertise in neurodevelopmental disorders, ensuring accuracy and consistency across the study population.

Patient Selection:

All procedures were conducted in accordance with ethical standards and approved by the Central Drugs Standard Control Organization (CDSCO) registered Institutional Ethics Committee (IEC). The study included male and female children between 2 to 18 years of age with a confirmed diagnosis of Autism Spectrum Disorder (ASD) based on DSM-V criteria. Participants were enrolled only after written informed consent was obtained from the patient's parents.

Vitamin B12 Assay:

For biochemical analysis of B12, 3–5 ml of blood samples was collected in EDTA tubes and transported under refrigerated conditions (2–8°C) to NDC Diagnostics. Serum was separated by centrifugation at 3,500 rpm for 10 minutes and subsequently analyzed. B12 levels were determined using ADVIA Centaur VB12 assay, a competitive immunoassay based on direct chemiluminescent technology on the ADVIA Centaur XP system. B12 analyses were performed using standard calibration curves and quality control samples in accordance with the manufacturer's instructions. The assay required 100 µL of sample per test and had a measurable analytical range of 45–2000 pg/mL. The system measures chemiluminescence, inversely proportional to the concentration of B12 in the sample. Results were reported in pg/mL. The reference interval for B12 according to the Siemens ADVIA Centaur XP assay was 211–911 pg/mL.[17] Within this reference interval, the study specific classification of B12 was determined using the mean $\pm$ 1 standard deviation (SD) of the study population. Accordingly, concentrations < 386 pg/mL are classified as deficient, optimum levels range from 386–736 pg/ mL, and levels beyond 736 pg/ mL are considered as elevated.

Clinical Scales:

To evaluate and assess the presence and severity of ASD related symptoms, standardized clinical scales, the Childhood Autism Rating Scale (CARS),[18] Gilliam Autism Rating Scales (3rd ed.) [19] were employed for quantifying behavioral characteristics of ASD. Vineland Social Maturity Scale (VSMS) Social Quotient (SQ) [20] was also used to assess social competence, adaptive functioning, and social maturity.

Analysis:

B12 levels were categorized into 3 different groups as deficient, optimum, or elevated, and their percentage analysis was carried out. Subgroup analysis was performed to examine the association between serum Vitamin D3 levels and ASD severity, with participants classified into GARS severity levels (Level I, Level II, and Level III). Additionally, the association between serum Vitamin D3 levels and social maturity was evaluated using Social Quotient (SQ) scores, with participants categorized into profound, severe, moderate, and mild impairment groups. An independent-samples t-test and a Chi-square test were performed to examine the association between dietary pattern (vegetarian vs. non-vegetarian) and B12 levels. Descriptive statistics such as mean, median, Range and Standard Deviation (SD) were carried out on D3 levels and GARS, SQ scores. Multivariable Regression analysis was used to assess the relationship between D3 levels and GARS, SQ scores. A percentage analysis was performed to determine the prevalence of clinical symptoms such as speech impairments, hyperactivity, aggression, social interaction, repetitive behaviours and sensory issues across different B12 levels.

RESULTS

Demographic characteristics of study population

This observational study included a total of 3025 individuals diagnosed with ASD. Of the total participants, the majority were male 2425 (80.14%), while females accounted for a smaller number 600 (19.83%). Chronological age of participants ranged from 2 to 18 years. where the majority of participants were between 5–10 years 1486 (49.12%) followed by 1182 (39.07%) participants in the 2–5 years of age and the remaining small percent 357 (11.80%) of patients were aged 10–18 years. With respect to dietary pattern, 1634 participants (54.02%) consumed a non-vegetarian diet, whereas 1391 (45.98%) followed a vegetarian diet (Table 1). Overall, the sample was predominantly male and largely composed of children under 10 years of age and had a relatively balanced distribution of dietary patterns.

Table 1
Demographic data

n = 3025		
Sex	No. of patients	%
Female	600	19.83
Male	2425	80.14
Age		
2–5	1182	39.07
5–10	1486	49.12
10–18	357	11.80
Diet		
Veg	1391	45.98
Non- Veg	1634	54.02

Classification of Vitamin B12 in our study population

Serum vitamin B12 concentrations were categorized as Deficient, Optimum, or Elevated based on the mean $\pm$ 1 SD of the study population with the Siemens ADVIA Centaur XP assay reference interval. Percentage analysis of B12 levels revealed that 796/3025 (26.3%) children had B12 deficiency. Optimum B12 levels were observed in 1230/3025 (40.66%) children making this the most prevalent category in the study population and 999/3025 (33.02%) of children have elevated levels of B12. Overall, the distribution showed higher representation in the optimum and elevated vitamin level groups compared to the deficient group. (Table 2)

Table 2
Distribution of Vitamin B12 levels for study participants

VIT B12	No. of patients	%
DEFICIENT (less than 386 pg/ mL)	796	26.31
OPTIMUM (386–736 pg/ mL)	1230	40.66
ELEVATED (greater than 736 pg/ mL)	999	33.02

Relationship between Vitamin B12 levels and Demographic characteristics

Analysis of B12 status across gender indicates that B12 levels are similarly distributed between males and females, suggesting that gender may not play a significant role in determining B12 status in this population. Age wise analysis showed a clear trend in B12 levels. In children aged 2–5 years, a higher proportion 38.58% exhibited elevated levels. In the 5–10 years group, more participants 30.95% had elevated levels. In contrast, the age group 10–18 years showed the highest prevalence of deficiency 33.90%. Overall, B12 deficiency increased with age, whereas elevated levels showed a decreasing trend. Dietary

analysis showed that B12 deficiency was more prevalent among vegetarians than non-vegetarians (28.80% vs. 24.18%). In contrast, elevated B12 levels were observed more frequently among non-vegetarians (34.27%) than vegetarians (31.60%). The proportion of participants with optimum B12 levels were similar between vegetarians (39.61%) and non-vegetarians (41.55%). (Table 3)

Table 3
Distribution of Vitamin B12 levels across Gender, Age and Diet

Vitamin B12 Levels				
Gender	n = 3025	DEFICIENT %	OPTIMUM %	ELEVATED %
Female	600	155 (25.84)	244 (40.66)	201 (33.5)
Male	2425	641 (26.43)	986 (40.66)	798 (32.91)
Age				
2–5	1182	273 (23.10)	453 (38.32)	456 (38.58)
5–10	1486	402 (27.05)	624 (42)	460 (30.95)
10–18	357	121 (33.90)	153 (42.85)	83 (23.25)
Diet				
Veg	1391	401 (28.8)	551 (39.61)	439 (31.6)
Non- Veg	1634	395 (24.18)	679 (41.55)	560 (34.27)

Percentage analysis of Vitamin B12 Levels and ASD Severity based on GARS

The clinical association between ASD severity and B12 levels was determined using GARS scores. Among participants with deficient B12 levels, the majority cases were categorized as GARS Level III (68.35%), followed by Level II (31.28%), while cases in Level I were minimal (0.37%). Among optimum B12 group, most participants were in GARS Level III (68.61%), with Level II accounting for (30.89%) and Level I comprising only (0.48%) population. In the elevated B12 group, GARS Level III had (67.87%) participants, followed by Level II (31.53%), while Level I had (0.60%). Overall, across all B12 categories, GARS Level III (severe ASD) was the most prevalent, followed by Level II (moderate ASD), with Level I (mild ASD) representing a negligible proportion. This suggests that severe ASD was predominant in our study. (Table 4)

Table 4
Analysis of Vitamin B12 levels with ASD severity according to GARS Scale

GARS	Vitamin B12 Levels		
	Deficient	Optimum	Elevated
Level 3	544 (68.35%)	844 (68.61%)	678 (67.87%)
Level 2	249 (31.28%)	380 (30.89%)	315 (31.53%)
Level 1	3 (0.37%)	6 (0.48%)	6 (0.60%)

Percentage analysis of Vitamin B12 Levels and Social Maturity of ASD children based on SQ scores

The association between B12 levels and impairment in social maturity was assessed using SQ scores. The distribution of B12 status was similar across all SQ categories, with optimum Vitamin B12 levels being the most frequently observed category followed by elevated and deficient levels. (Table 5)

Table 5
Analysis of Vitamin B12 levels and Social maturity of ASD children according to SQ scores

SQ	Vitamin B12 Levels		
	Deficient	Optimum	Elevated
Profound	36 (30.25%)	45 (37.81%)	38 (31.93%)
Severe	171 (28.17%)	246 (40.53%)	190 (31.30%)
Moderate	275 (25.70%)	432 (40.38%)	363 (33.64%)
Mild	314 (25.54%)	507 (41.25%)	408 (33.19%)

Relationship between Diet (Veg/ Non veg) and Vitamin B12

An independent-samples t-test was performed to compare mean Vitamin B12 levels between vegetarian and non-vegetarian participants. Levene's test indicated equality of variances ($F = 1.330$, $p = .249$). The analysis revealed a statistically significant difference in mean Vitamin B12 levels between the two dietary groups, $t(3025) = -2.791$, $p = .005$. The mean difference was -45.10 (95% CI: -76.77 to -13.42), indicating that Vitamin B12 levels differed significantly between vegetarians and non-vegetarians. (Table 6a)

Table 6
a: Comparison of Mean Vitamin B12 Levels Between Vegetarian and Non-Vegetarian Participants by Independent t-test

		Levene's test for equality of variances		t- test for equality of Means					
		F	Sig.	t	df	Sig.(2-tailed)	Mean Difference	Std. Error Difference	95% CI of the Difference
Lower									
Vit B12	Equal variances assumed	1.330	0.249	-2.791	3025	0.005	-45.09635	16.15565	-76.77362 -13.41918
	Equal variances not assumed			-2.800	2978.835	0.005	-45.09635	16.10448	-76.67338 -13.51932

A Chi-square test was performed to determine the association between dietary pattern (vegetarian and non-vegetarian) and Vitamin B12. The analysis demonstrated a statistically significant association between dietary pattern and Vitamin B12 status (Pearson's $\chi^2 = 8.556$, $p = .014$). The likelihood ratio test also indicated a significant association (Likelihood Ratio = 8.540, $p = .014$). Furthermore, the linear-by-linear association was also significant ($\chi^2 = 6.921$, $p = .009$), suggesting a significant linear trend across the ordered Vitamin B12 categories. (Table 6b)

Table 6
b: Association of Vitamin B12 Levels Between Vegetarian and Non-Vegetarian Participants by Chi-Square test

Pearson Chi- Square	Value	df	Asymptomatic significance (2- sided)
	8.556 ^a	2	0.14
Likelihood Ratio	8.540	2	0.14
Linear by Linear Association	6.921	1	0.009
N of valid cases	3025		

a. 0 cells(0%) have expected count less than 5. The minimum expected count is 366.03

Central Tendency and Variability for Vitamin B12 levels and SQ, GARS Scores

Descriptive analysis showed that the SQ and GARS scores exhibited a relatively balanced distribution, as indicated by a mean of 46.86 and a median of 45 and mean of 105.08 and a median of 106 respectively. In contrast, B12 levels showed substantial spread, with a mean of 685.93, a median of 555. Among the study variables, B12 (SD = 443.34; range = 1978) exhibited the greatest variability, followed by SQ scores (SD = 16.63; range = 121.2) and GARS scores (SD = 10.94; range = 183). The higher mean compared with the median for B12 suggests a slight positive skew in its distribution, whereas the close agreement between the mean and median for SQ and GARS scores indicates relatively symmetric distributions. (Table 7)

Table 7
Descriptive statistics for Vitamin B12 levels and SQ, GARS Scores

Mean	Vitamin B12	SQ Scores	GARS
	685.93	46.86	105.08
Median	555	45	106
Range	1978	121.2	183
SD	443.34	16.63	10.94

Multivariable linear Regression analysis of Association Between Vitamin B12 Levels, Age, Gender, Diet and GARS, SQ Scores

A multivariable linear regression analysis was conducted to evaluate the association of age, gender, Diet and B12 levels with GARS scores. The overall model was statistically significant ($p < 0.001$).

Age was significantly associated with GARS scores ($B = -0.172$, $\beta = -0.047$, $p = 0.011$), indicating that for every one unit increase in age, the GARS score decreased by 0.172 points.

Diet was also significantly associated with GARS scores ($B = 1.315$, $\beta = 0.032$, $p = < 0.001$) with non vegetarian participants having GARS scores that were 1.315 points higher than vegetarians.

In contrast, Gender did not significantly predict GARS scores ($B = 1.037$, $\beta = 0.038$, $p = 0.042$) also B12 levels were not significantly associated with GARS scores after adjustment for age, gender and diet ($B = 0.028$, $\beta = 0.033$, $p = 0.073$), indicating that Gender and B12 did not contribute meaningful independent predictive power to GARS scores.(Table 8)

Table 8
Multivariable Linear Regression Analysis of Factors Associated with GARS Scores

Coefficients ^a							
	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95% Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
(Constant)	105.094	0.737		142.639	.000	103.650	106.539
Vitamin B12	0.000	0.000	-0.015	-0.838	.402	-0.001	0.001
Diet	1.315	0.399	0.060	3.296	< 0.001	0.533	2.098
Gender	0.886	0.499	0.032	1.776	0.076	-0.092	1.865
Age	-0.172	0.067	-0.047	-2.555	0.011	-0.303	-0.040

a: Dependent variable: GARS

A multivariable linear regression analysis was conducted to evaluate the association of age, gender, Diet and B12 levels with the SQ Scores. The overall model was statistically significant ($p < 0.001$).

Among the variables, Age was the only significant predictor ($B = -2.328$, $\beta = -0.418$, $p < 0.001$), indicating that the SQ decreased by 2.32 points for every one unit increase in Age.

B12 was a marginally significant predictor of SQ scores ($B = -0.001$, $\beta = -0.032$, $p < 0.053$), suggesting a possible trend.

In contrast, neither Gender ($B = -0.363$, $\beta = -0.009$, $p = 0.600$) nor Diet ($B = -0.259$, $\beta = -0.008$, $p = 0.639$) showed a statistically significant association with the SQ after adjustment for the other variables.(Table 9)

Table 9
Multivariable Linear Regression Analysis of Factors Associated with SQ Scores

Coefficients ^a							
	Unstandardized Coefficients		Standardized Coefficients			95% Confidence Interval for B	
	B	Std. Error	Beta	t	Sig.	Lower Bound	Upper Bound
Constant	64.022	1.021		62.694	0.000	62.019	66.024
Vitamin B12	-0.001	0.001	-0.032	-1.935	0.053	-0.002	0.000
Gender	-0.363	0.692	-0.009	-0.525	0.600	-1.719	0.993
Age	-2.328	0.093	-0.418	-25.006	< 0.001	-2.511	-2.146
Diet	-0.259	0.553	-0.008	-0.469	0.639	-1.344	0.825

a- Dependent variable: SQ

Prevalence of Symptoms of ASD across Vitamin B12 Levels

Percentage analysis of the prevalence of symptoms in patients across B12 levels was carried out. (Table 10). In children with speech impairment 40.06% had optimum levels of B12, followed by 33.54% having elevated B12 and 26.40% had B12 deficiency. Among hyperactive children 26.09% had B12 deficiency, 32.85% had elevated levels of B12 and 41.06% had optimum B12 levels. In the aggression group 40.95% had optimum levels of B12, followed by 32.41% having elevated B12 and 26.64% had B12 deficiency. In children with difficulty in social interaction 40.35% had optimum levels of B12, 33.06% had elevated B12 levels and 26.60% had B12 deficiency. In repetitive behaviour group 40.42% had optimum levels of B12, 33.38% had elevated B12 levels and 26.20% had B12 deficiency, and in children who had sensory issues 40.65% patients had optimum levels of B12, 33.04% had elevated B12 levels and 26.31% had B12 deficiency. B12 status showed a similar distribution across participants with ASD related symptoms, suggesting variability of B12 across all symptoms. (Table 5)

Table 10
Percentage Symptomatic Analysis

Symptoms	Speech		Hyperactivity		Aggression		Social Interaction		Repetitive Behaviour		Sensory
	Non-functional speech	Functional speech	Observed	Not Observed	Observed	Not Observed	Observed	Not Observed	Observed	Not Observed	Observed
Vitamin B12 - Deficient	725	71	741	55	646	150	59	737	755	41	789
%	26.40	25.45	26.09	29.73	26.64	25.00	23.23	26.60	26.20	28.67	26.31
Vitamin B12- Optimum	1100	130	1166	64	993	237	112	1118	1165	65	1219
%	40.06	46.59	41.06	34.59	40.95	39.50	44.09	40.35	40.42	45.45	40.65
Vitamin B12- Elevated	921	78	933	66	786	213	83	916	962	37	991
%	33.54	27.96	32.85	35.68	32.41	35.5	32.68	33.06	33.38	25.87	33.04

Relationship Between profound impairment in social maturity and Vitamin B12 Deficiency

Among the 3025 participants, 119 (3.9%) demonstrated profound impairment in social maturity with SQ < 20. Within this subgroup, 36 participants (30.25%) were found to have B12 deficiency, 38 participants (31.9%) were found to have elevated B12 and 45 individuals (37.81%) had optimum levels of B12. Overall, the distribution of Vitamin B12 status remained similar across the profound group and requires further investigation into the potential relationship between B12 status and adaptive functioning.

A scatter plot was constructed to assess the relationship between B12 levels and ASD severity measured by the GARS. The distribution of points formed a diffuse, circular pattern without any linear trend, this indicates the absence of a correlation between the two variables.(Fig. 5)

Figure 5: Scatter plot illustrating the distribution of Vitamin B12 levels against GARS scores

The lack of statistical significance across tests such as Spearman correlation, Kruskal-Wallis test strongly suggest no correlation between B12 levels and GARS, SQ severity within our sample. Even after removing outliers and categorizing the data for a Linear by Linear association test and scatter plot the trend remained non-significant. This suggests that clinical interventions targeting only B12 may not yield predictable linear changes in GARS score or symptoms based on current data.

DISCUSSION

Autism spectrum disorder (ASD) is a neurodevelopmental condition of growing clinical concern due to its increase in prevalence, attributed to changes in diagnostic criteria, lifestyle and increased awareness.[21] ASD is recognized as a multifactorial disorder involving complex interactions among genetic, environmental, and biological factors that disrupt neurodevelopmental pathways. ASD imposes a significant socioeconomic burden and adversely affects the quality of life of affected individuals and their families.[22] Emerging evidence suggests that B12 plays an important nutritional and environmental role in ASD by influencing prenatal neurodevelopment and linking metabolic disturbances with immune dysregulation, thereby contributing to ASD pathophysiology. [1] Our study evaluated the variability of B12 levels and their association with ASD severity and core clinical symptoms and its role in pathophysiology of ASD.

Demographic characteristics

In our study of 3,025 children with ASD, a male predominance was observed, with males contributing to 80.1% of the cohort (2425 males vs. 600 females), having a 4:1 male-to-female ratio, this is consistent with previously reported findings.[23, 24] This sex disparity can be attributed to hormonal or genetic influence suggesting a protective effect in females against both inherited and de novo ASD risk variants, as well as the modulatory influence of sex chromosome related genes and sex hormones, particularly testosterone, on the expression and severity of autistic traits.[23] The majority of participants were in the range 5–10 years 49.12% and 2–5 years 39.07%, together accounting for 88.19% of the study population, while a smaller proportion in the 10–18 year age group 11.8%. This age distribution indicates that ASD is most commonly identified during early to middle childhood, likely due to heightened awareness, advances in diagnostic practices, and earlier recognition of symptoms. Early identification is crucial, as it enables timely intervention, which is associated with better management and developmental outcomes. In our study, a slightly higher proportion of participants followed a non-vegetarian diet (54.02%) compared with a vegetarian diet (45.98%). Despite the relatively similar distribution of dietary patterns, non-vegetarians exhibited a higher prevalence of elevated Vitamin B12 levels than vegetarians, emphasizing the influence of dietary intake on Vitamin B12 status.

Vitamin B12 Absorption

B12 absorption is a complex multistep process that begins in the stomach, where gastric acid releases cobalamin from dietary proteins, and cobalamin binds initially to protective R proteins. In the duodenum, pancreatic enzymes degrade these R proteins, enabling cobalamin to bind with Intrinsic Factor.

Intrinsic factor is a glycoprotein secreted by gastric parietal cells that is essential for absorption of cobalamin. The cobalamin-Intrinsic Factor complex then travels to the terminal ileum, where specific receptors recognize and absorb it into the intestinal cells via endocytosis. After receptor mediated endocytosis in the ileum, the cobalamin is released from Intrinsic Factor and binds to transcobalamin (TC II) for release into the blood stream for transport to the liver, Brain and other tissues. Transcobalamin enters the brain cells via endocytosis and subsequently is degraded via lysosomes to free cobllalamin.[4] Disruption of this pathway at any stage such as autoimmune destruction of parietal cells in pernicious anemia, inadequate dietary intake, or small intestinal bacterial/fungal overgrowth (SIBO/SIFO), intestinal malabsorption can reduce hepatic B12 stores over time, leading to neurological disorders. (Fig. 2) These problems are commonly seen in ASD, which could be one of the cause of B12 deficiency in our cohort.

Role of Vitamin B12 in One Carbon Cycle (Methylation)

B12 plays a crucial role in DNA synthesis, and epigenetic regulation through its involvement in one carbon metabolism and cellular energy pathways.[25] As a cofactor for methionine synthase, B12 facilitates the remethylation of homocysteine to methionine, leading to the formation of S-adenosylmethionine (SAME), the primary methyl donor required for DNA,[8] a process essential for regulating gene expression, preserving chromatin structure, and maintaining genomic stability.[25] (Fig. 3) In addition, B12 functions as a coenzyme for methylmalonyl-CoA mutase, enabling the conversion of methylmalonyl-CoA to succinyl-CoA, a key intermediate in the TCA cycle.[10] This process supports ATP generation and provides metabolic substrates essential for nucleotide and heme biosynthesis, the building blocks of DNA. These pathways highlight the central role of B12 in DNA synthesis & repair, cellular metabolism, and normal neurodevelopment. [25] (Fig. 4)

The improper functioning of methylcobalamin dependent MS may lead to diseases associated with the following pathophysiological mechanisms: the direct toxicity of metabolites, a deficiency of synthesis products, methylation disorders and increased oxidative stress.[9] Besides its function as an essential enzyme cofactor, B12 also acts as a scavenger of reactive oxygen species (ROS), with antioxidant properties comparable to those of superoxide dismutase-1 [9, 12]. Its reduced form (cob(II)amin) directly captures superoxide radicals. Additionally, cobalamin suppresses oxidative stress that is induced by activation of caspase-3 and thereby prevents cells from apoptosis and oxidative damage.[9, 13]

Role of Vitamin B12 in Neurotransmitter Synthesis and Neural connectivity

Adequate B12 levels help ease hippocampal damage, preserve blood brain barrier integrity, and enhance structural and functional neural connectivity.[26] Deficiency of B12 reduces SAME levels due to impaired methylation, leading to disruption of neurotransmitter balance, neuronal communication, and synaptic plasticity. Impaired methylation also results in homocysteine accumulation, which increases oxidative stress and neuronal toxicity, thereby contributing to cognitive dysfunction, mood disturbances, and neurodevelopmental abnormalities frequently observed in ASD.[27, 28] SAME dependent transmethylation is essential for the synthesis, metabolism and regulation of neurotransmitters such as dopamine, serotonin, and norepinephrine, noradrenaline, adrenaline, and histamine. [28, 29] Reduced SAME availability may produce a hyposerotonergic state associated with social communication deficits, aggression and theory of mind impairments observed in Autism Spectrum Disorder (ASD).[30–32] Altered SAME levels may disrupt dopamine turnover, contributing to aggressive, repetitive behaviors and behavioral inflexibility in ASD.[31, 32] Furthermore, SAME influences the expression of glutamate decarboxylase (GAD1), the enzyme responsible for converting excitatory glutamate into inhibitory gamma-aminobutyric acid (GABA). Reduced SAME levels may therefore contribute to excitation inhibition imbalance, a key neurobiological feature implicated in ASD, often manifesting as sensory hypersensitivity, seizures, anxiety, and impaired behavioral regulation.[32, 33] B12 is also essential for the formation, maintenance, and repair of the myelin sheath.[27, 34] B12 supports myelin synthesis by acting as a cofactor in the conversion of methylmalonyl-CoA to succinyl-CoA thereby regulating lipid and protein metabolism necessary for myelin integrity.[34] B12 also aids remyelination and nerve regeneration by supporting Schwann cell function.[35] Deficiency of B12 disrupts myelin maintenance, leading to demyelination, impaired nerve conduction.[36]

Functional Vitamin B12 Deficiency in ASD

Biologically active B12 exists in two coenzyme forms adenosylcobalamin and methylcobalamin which support the breakdown of methylmalonic acid and the synthesis of methionine, respectively. [8] Circulating B12 exists in multiple chemical forms, including inactive or functionally unavailable analogues that lack cofactor activity in human metabolism. Elevated or optimum B12 may not necessarily reflect functional sufficiency [37] and could represent inactive forms, impaired cellular utilization or underlying metabolic or inflammatory alterations.[38] In ASD elevated B12 levels reflect the accumulation of metabolically inactive cobalamin forms, resulting from impaired methylation pathways that disrupt normal cellular utilization. B12 concentrations within the optimum range do not necessarily indicate adequate levels of biologically active or functionally available cobalamin. These inactive forms may arise from dietary or microbial sources or from defects in intracellular cobalamin processing and inadequate conversion to active coenzyme forms. These are detected by standard B12 assays, thereby masking an underlying functional deficiency of B12 at the cellular level.[39, 40]

Disrupted Vitamin B12 Metabolism in ASD

Our findings revealed that 40.66% of children showed optimum levels of B12, followed by 33.02% participants who exhibited elevated levels of B12 and 26.31% were B12 deficient. This distribution indicates noticeable variability in B12 status among children with ASD, with a good percentage demonstrating either deficiency or elevation. These findings differ from previous studies that have reported a higher prevalence of B12 deficiency among individuals with ASD. [1, 41, 42, 43] A study by Nesa, A. *et al.* found remarkably lower B12 levels in children with ASD relative to healthy peers, highlighting the potential role of B12 insufficiency in ASD pathophysiology.[39] Observational studies by Yektaş Ç *et al.* and Erden S *et al.* have documented significantly lower B12 concentrations in ASD populations, suggesting that deficiency may be linked to metabolic disruptions and symptom severity in autism. [42, 43] A study by Sourander *et al.* stated that elevated maternal B12 levels (≥ 81 st percentile) in early pregnancy were associated with an increased risk of childhood autism, consistent with findings from a Finnish birth cohort showing a modest but significant association (adjusted OR 1.59, 95% CI 1.06–2.41) with ASD.[44] Additional studies from the Johns Hopkins Bloomberg School of Public Health, have reported that extremely high maternal B12 levels in new mothers substantially increases risk of ASD three times in children .[45] Lonsdale D further observed that elevated maternal folate and/or B12 at delivery may be

associated with increased ASD risk in offspring.[46] However, these observations do not support a direct causal or toxic effect of v B12; rather, markedly elevated circulating levels may reflect underlying metabolic dysregulation, altered transport or utilization, inflammation, or excessive supplementation.

Role of Supplementation

An important factor in interpretation of B12 levels is the influence of supplementation of B12. In our study, only 203 out of 3025 participants i.e. 6.7% reported receiving B12 supplementation, representing a very small percentage of the study population. Among the individuals that obtained supplements, 90 individuals had optimum B12 levels, 34 were deficient, and 79 had elevated B12 levels. This low prevalence of supplementation, along with the varied distribution of B12 status within this group, indicates that supplementation is unlikely to be a major contributor to the overall distribution of B12 levels in the study population. And presence of elevated B12 levels cannot be just attributed to intake of supplements. Instead, these findings support the possibility of altered B12 metabolism or impaired cellular utilization in children with ASD.

Distribution of Vitamin B12 levels across Gender and Age

Our study showed progressive increase in B12 deficiency with increasing age, while elevated levels declined. Younger children (2–5 years) showed a higher proportion of elevated vitamin B₁₂ levels, which may be attributed to dietary intake, supplementation, or metabolic factors during early growth. However, adolescents (10–18 years) exhibited the highest prevalence of deficiency, possibly due to increased nutritional requirements, dietary inadequacies, or lifestyle changes during this stage. These findings highlight the importance of monitoring B12 levels and implementing appropriate nutritional interventions, particularly in adolescent populations.

Correlation of Vitamin B12 levels and Diet

The present study demonstrated a significant association between dietary pattern and B12. Vegetarian participants had significantly lower mean B12 levels than non-vegetarians, as confirmed by the independent-samples t-test. Consistent with this finding, the Chi-square test also confirmed a significant association between dietary pattern and B12, indicating that the distribution of deficient, optimum, and elevated B12 levels differed between the two dietary groups. Notably, B12 deficiency was more prevalent among vegetarians (28.80%) than non-vegetarians (24.18%), whereas elevated B12 levels were more frequently observed among non-vegetarians (34.27% vs. 31.60%). These findings are probable biologically, and similar as B12 is predominantly obtained from animal-derived foods such as meat, fish, eggs, and dairy products.[1, 2] The similar proportion of participants with optimum B12 levels in both dietary groups suggests that factors such as dietary supplementation, food fortification, or variations in dietary habits may have played a role in maintaining adequate B12 status in a substantial proportion of vegetarian participants. Overall, these findings highlight dietary pattern as an important factor for determining B12 status and support the need for regular nutritional assessment and appropriate dietary counselling among individuals.

Association of Vitamin B12 levels and severity of ASD

Percentage analysis in our study cohort, the B12 levels across the GARS scale revealed a heterogeneous pattern of B12 status in individuals with ASD. Our study showed the highest number of participants clustered in GARS Level 3, representing more severe ASD. (Table 4) The existence of deficient, optimum, and elevated B12 levels across severity groups suggests dysregulated B12 metabolism rather than a uniform deficiency state. Our findings differ from previous studies that state a clear correlation between severity of ASD and B12 deficiency.[47, 48] Study by Avram OE *et al.* found that B12 insufficiency and elevated homocysteine correlate with ASD risk and severity. [47] A study by Yuan, W. U. *et al.* reported positive correlation between B12 levels and developmental outcomes based on CARS and SRS, particularly language and social functioning, support the biological relevance of B12 in neurodevelopment in ASD.[48] These findings contrast with our present study, in which severe ASD was observed across deficient, optimum, and elevated serum B12 levels, indicating no clear linear relationship between B12 levels and ASD severity. This suggests that serum vitamin B12 concentration alone does not directly reflect ASD severity. The heterogeneity in B12 status across severity levels in our cohort aligns with the study that suggests that nutritional profiles in ASD are variable and not simply explainable by deficiency alone. [31] Variability in B12 status in ASD individuals with more severe GARS scores including optimum or elevated serum levels may be influenced by factors such as dietary patterns, vitamin supplementation, altered absorption, or impaired intracellular utilization of cobalamin, highlighting the complexity of interpreting B12 levels in ASD.[37, 38]

Correlation of Vitamin B12 Levels and Severity, Social Maturity of ASD children based on GARS and SQ scores respectively

Our study examined the association between B12 levels and Autism severity as measured by GARS scores and social maturity, as measured by SQ scores in children with ASD. The multivariable regression analysis demonstrated that Age was the major independent predictor of both SQ and GARS. Increasing age was associated with lower SQ and GARS scores, suggesting that age contributes significantly to variations in these clinical measures. Although B12 showed a marginal association with SQ scores, indicating that its independent contribution to social maturity was minimal. But B12 was not independently associated with GARS scores, suggesting that B12 levels do not influence autism severity when covariates are considered. Dietary pattern was independently associated with GARS scores, with non-vegetarian participants exhibiting higher GARS scores than vegetarians, although the effect size was small. But Dietary pattern was not an independent predictor of SQ. Overall, these findings indicate that age is an important predictor of both social maturity and autism severity. And B12 levels alone are unlikely to be major predictors of social maturity or autism severity and these are influenced by other environmental, biological and developmental factors. But B12 is an essential nutrient as it acts as a cofactor in two critical metabolic pathways: the breakdown of methylmalonic acid and the methionine synthesis and plays a crucial role in epigenetic regulation. These pathways highlight the central role of B12 in DNA synthesis & repair, cellular metabolism, Neurotransmitter synthesis, energy formation and normal neurodevelopment. Therefore, maintaining adequate Vitamin B12 status remains important for optimal neurodevelopment and overall health.

Correlation with symptoms of ASD

Percentage analysis of clinical symptoms across different B12 levels in our study revealed that core ASD symptoms including non-functional speech, hyperactivity, aggression, social interaction difficulties, repetitive behaviors, and sensory issues were prevalent across deficient, optimum, and elevated B12 groups. This aligns with previous research that suggested that while B12 plays a critical role in neurodevelopment and methylation pathways, B12 deficiency do not directly correlate with specific behavioral manifestations of ASD, particularly when functional biomarkers are not assessed [48, 49]. The presence of speech and social interaction impairments even among individuals with optimum or elevated B12 levels in our cohort supports the concept of functional or dysregulated B12 metabolism rather than a simple deficiency state.

Vitamin B12 vs Functional B12

Functional B12 deficiency is defined by impaired activity of B12 dependent enzymes despite optimum B12 concentrations and is reflected by elevated levels of methylmalonic acid and homocysteine.[35, 49] Disruption of the adenosylcobalamin dependent pathway results in methylmalonic acid accumulation, which has been linked to mitochondrial dysfunction and neurotoxicity biological abnormalities increasingly reported in neurodevelopmental disorders, including ASD.[50] Consistent with these findings, studies have also demonstrated reduced methylcobalamin levels in individuals with ASD despite optimum total serum B12 concentrations, supporting the presence of a functional or inactive B12 deficiency rather than complete nutritional deficiency. [51] Therefore only patients with B12 deficiency do not require treatment, but individuals with optimum or elevated B12 levels may also require clinical evaluation and management, as these levels do not necessarily reflect functionally active cobalamin or normal metabolic status.

The findings of our study further support the concept of dysregulated B12 metabolism in ASD, as seen by the existence of optimum, deficient and elevated serum B12 levels within our cohort. Such variability suggests that serum B12 concentrations alone may not adequately reflect functional cobalamin availability or metabolic utilization in ASD. Collectively, these observations reinforce the limitation of relying solely on B12 levels and emphasize the need for comprehensive metabolic profiling, including functional biomarkers such as methylmalonic acid and homocysteine, to more accurately evaluate B12 status and its contribution to ASD pathophysiology and severity and to better understand the relationship between B12 status and ASD symptoms. [52, 53] Therefore, assessing specific active forms of B12 such as methylcobalamin and adenosylcobalamin may provide a more accurate understanding of its role in neurodevelopmental conditions such as ASD. Also through a therapeutic perspective, supplementation should be given in biologically active forms, particularly methylcobalamin, which has been implicated in supporting methylation pathways and neuronal function.

This dissociation between B12 levels and intracellular bioactivity highlights the limitations of total B12 measurements when assessing cobalamin status in ASD. Assessment of cobalamin levels in children with ASD should not rely solely on serum B12 levels, but also include key methylation markers such as methylcobalamin, homocysteine and folate. These biomarkers provide important insight into one-carbon metabolism and help identify functional B12 deficiency or impaired methylation, thereby offering a more comprehensive evaluation of metabolic status. Future studies incorporating functional biomarkers may provide greater insight into the heterogeneous metabolic profiles observed in ASD and support more individualized diagnostic and therapeutic approaches.

LIMITATIONS

Our study included a large cohort of patients, but it had few limitations. We were not able to collect data on genetic variations affecting B12 metabolism and functional biomarkers such as methylmalonic acid, folate and homocysteine were not evaluated.

CONCLUSION

The presence of ASD symptoms across deficient, optimum, and elevated B12 levels, suggests the variability and heterogeneous nature of B12 status in ASD. 33% of the study population exhibited elevated B12 levels who also showed higher ASD severity. B12 deficiency increased with age and elevated levels were common in younger age group. B12 deficiency was more prevalent among vegetarians than non-vegetarians, whereas elevated B12 levels were more frequently observed among non-vegetarians. Inadequate dietary intake, SIBO/SIFO, intestinal malabsorption may cause B12 deficiency in ASD. Age was a significant predictor of both GARS and SQ scores, Diet was only of GARS scores and B12 was a marginal predictor of SQ scores. Profound group also demonstrated a similar distribution of B12 levels. These findings indicate that B12 levels alone do not adequately explain its role in ASD pathophysiology. This suggests that clinical interventions targeting only B12 may not yield predictable linear changes in GARS score or symptoms based on current data.

Impaired methylation pathways in ASD that disrupt normal cellular utilization may cause accumulation of inactive cobalamin forms, resulting in elevated B12 in our cohort. Hence, elevated or optimal B12 levels may not necessarily indicate functional sufficiency. Assessment of specific active forms of B12 such as methylcobalamin and adenosylcobalamin may provide a more accurate understanding of its role in ASD. These findings emphasize the importance of functional biomarkers, such as methylmalonic acid and homocysteine, along with clinical assessments to better understand B12 bioactivity in ASD. Future research employing comprehensive metabolic profiling may guide individualized therapeutic interventions for ASD. Optimizing B12 levels should be an adjunctive treatment strategy rather than a standalone.

Abbreviations

Vitamin B12
B12
Autism Spectrum Disorder
ASD
Animal Protein
AP

Intrinsic factor
IF
Transcobalamin
TC II
Methionine synthase
MS
Methionine synthase gene
MTR gene
5
methyltetrahydrofolate – 5-methyl THF
Tetrahydrofolate
THF
Methionine synthase reductase
MTRR
S
adenosylmethionine-SAMe
Methyltransferases
MTs
L
methylmalonyl-CoA mutase-MMCoAMut
Methylmalonic acid
MMA
Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
DSM-V
Central Drugs Standard Control Organization
CDSCO
Institutional Ethics Committee
IEC
Childhood Autism Rating Scale
CARS
Vineland Social Maturity Scale
VSMS
Social Quotient
SQ
Gilliam Autism Rating Scale
GARS
Small intestinal bacterial/fungal overgrowth
SIBO/SIFO
Reactive oxygen species
ROS
Glutamate decarboxylase
GAD1
Gamma aminobutyric acid
GABA

Declarations

CATEGORY OF STUDY

Clinical Study

Ethics approval and consent to participate:

All procedures were conducted in accordance with ethical standards and approved by the Central Drugs Standard Control Organization (CDSCO) registered Institutional Ethics Committee (IEC). The patients were selected in accordance with the World Medical Association's Revised Declaration of Helsinki. Participants were enrolled only after written informed consent was obtained from the patient's parents.

Consent for publication:

NA

Clinical Trial No.

NA

Competing interests:

The authors declare that they have no competing interests

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Data Availability

The datasets generated during and analysed during the current study are not publicly available due to patient confidentiality and institutional restrictions but are available from the corresponding author on reasonable request and with permission from NeuroGen Brain and Spine Institute

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Figures

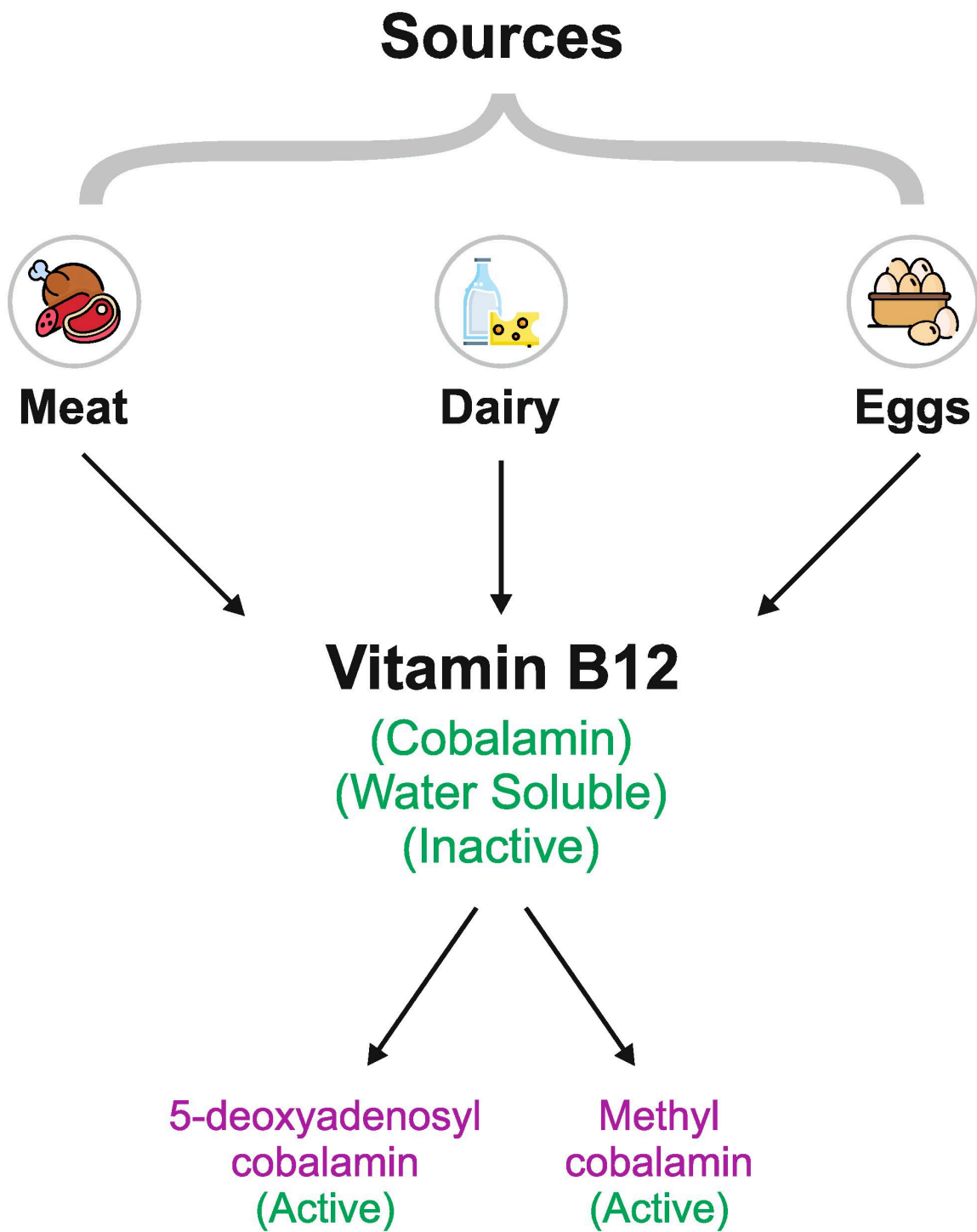


Figure 1

Overview of Vitamin B12 (Cobalamin) metabolism.

Schematic representation of Vitamin B12 intake from dietary sources and its Active forms

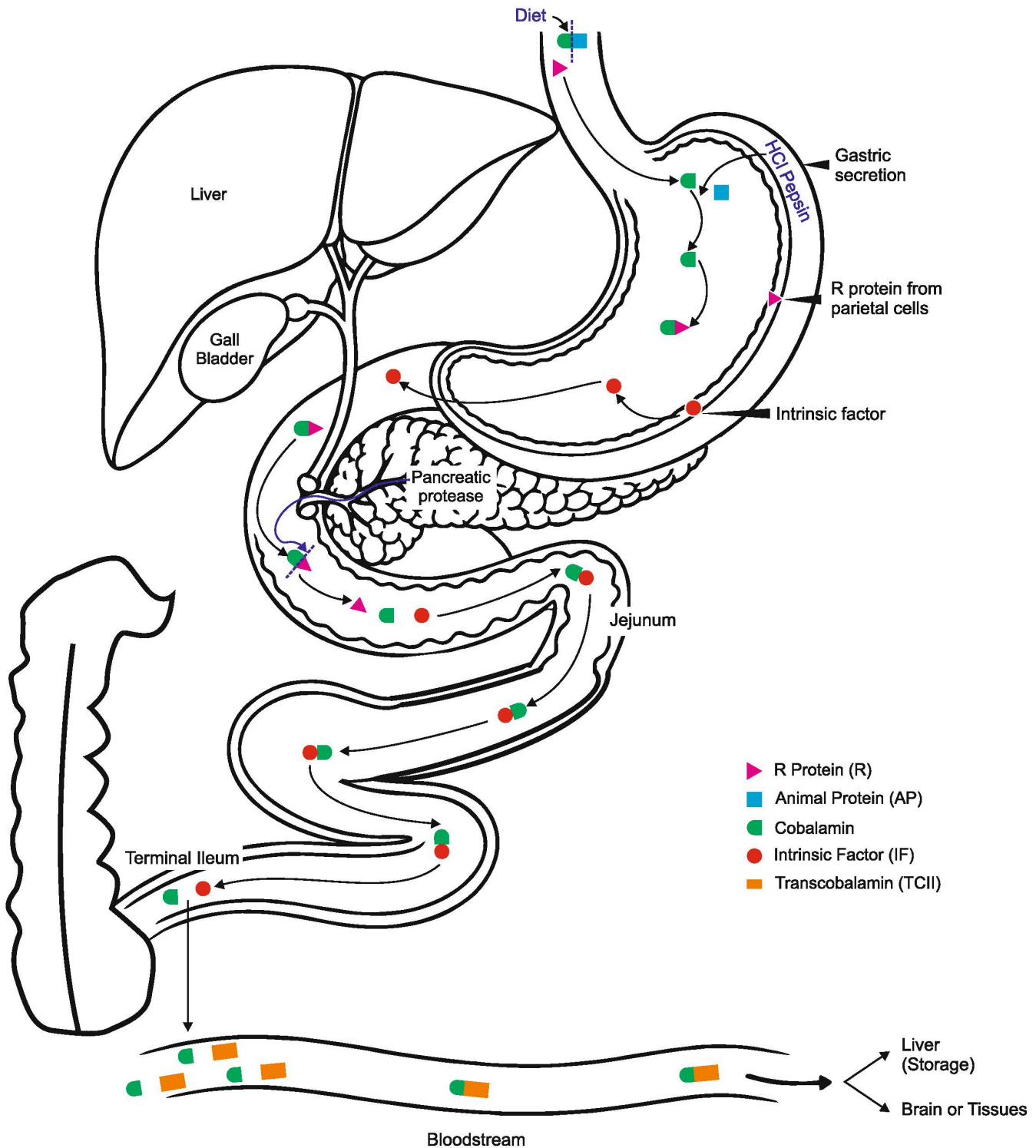


Figure 2

Schematic representation of Absorption of Vitamin B12 (Cobalamin).

Dietary Vitamin B12 (Cobalamin) bound to Animal Protein (AP) enters the stomach along with salivary R protein. In the stomach gastric acid and pepsin release B12 from Animal proteins and B12 binds to R proteins. In the duodenum, pancreatic protease degrades B12-R complex, allowing B12 to bind intrinsic factor (IF) secreted by gastric parietal cells. B12-IF complex is absorbed in the terminal ileum via receptor mediated endocytosis. After endocytosis vitamin B12 dissociates from Intrinsic Factor and binds to transcobalamin (TC II) to form B12-TC II for transport through the bloodstream to the brain and liver.

3) In cytoplasm

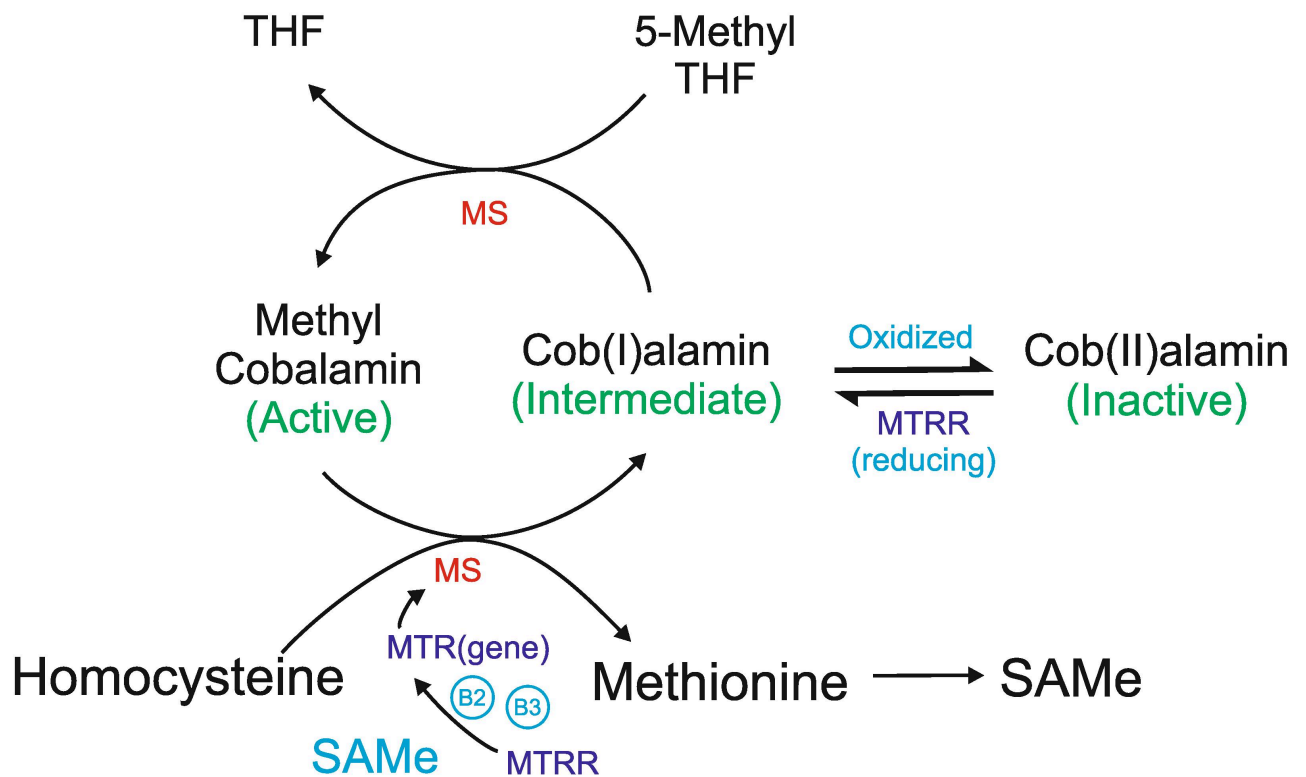


Figure 3

Role of Methylcobalamin in Methionine synthesis

Schematic representation of conversion of homocysteine to methionine mediated by Methionine Synthase (MS) and Methylcobalamin as an active cofactor, with Cob(I)alamin intermediate during the transfer of a methyl group from 5-Methyl THF to homocysteine.

4) In Mitochondria

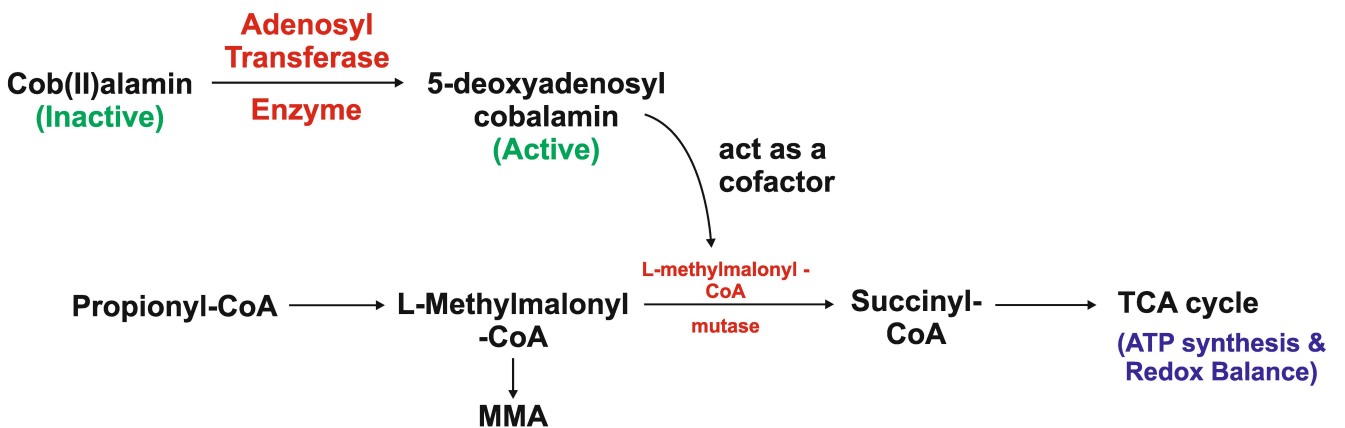


Figure 4

Role of Adenosylcobalamin in Methylmalonyl-CoA Metabolism

Schematic representation of vitamin B12 metabolism in the mitochondria. The inactive form cob(II)alamin is converted into its active form, adenosylcobalamin, by the enzyme adenosyltransferase. Adenosylcobalamin then acts as a cofactor for methylmalonyl-CoA mutase, which catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA which contributes to ATP production and maintaining metabolic balance.

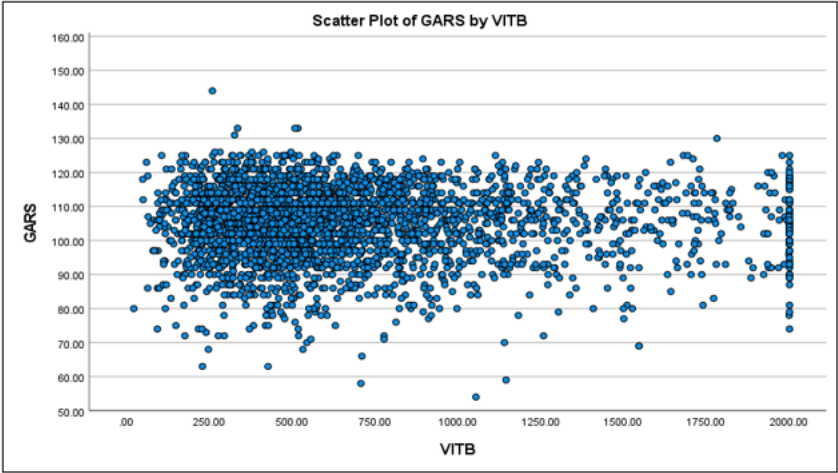


Figure 5

Scatter plot illustrating the distribution of Vitamin B12 levels against GARS scores