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Diagnostic Performance of Serum Homocysteine Compared With Vitamin B12 in Infantile Tremor Syndrome: A Case-Control Study

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Abstract

Background

Infantile tremor syndrome (ITS) presents with nutritional, neurological, developmental, and hematological abnormalities in infancy. The diagnostic performance of serum homocysteine and its relationship with different clinical presentations across the ITS spectrum remain insufficiently characterized. This study aimed to evaluate the diagnostic performance of serum homocysteine and compare it with serum vitamin B12 in children with ITS/pre-ITS.

Methods

This observational unmatched case-control study was conducted in the Department of Pediatrics of Gandhi Medical College over 19 months. Forty children with ITS/pre-ITS and 40 apparently healthy controls were enrolled. Serum homocysteine, vitamin B12, folate, electrolytes, and hematological parameters were assessed. Between-group comparisons and receiver operating characteristic (ROC) curve analyses were performed to evaluate the diagnostic performance of serum homocysteine and vitamin B12.

Results

Serum homocysteine was significantly higher in cases than controls (median {interquartile range, IQR}: 35.70 {30.53-42.92} versus 5.52 {4.70-6.20} µmol/L; $p < 0.001$). ROC analysis demonstrated excellent apparent discrimination for serum homocysteine (area under the curve {AUC}, 0.995; 95% confidence interval {CI}, 0.987-1.000), with 100% sensitivity and 92.5% specificity at an exploratory ROC-derived threshold of ⩾19.2 µmol/L. Serum vitamin B12 showed substantially lower discrimination (AUC, 0.604; 95% CI, 0.464-0.744). Homocysteine concentrations were significantly higher in children with ITS than in those with pre-ITS (41.61 ± 9.64 versus 32.82 ± 8.05 µmol/L; $p = 0.003$).

Conclusion

Serum homocysteine demonstrated greater apparent case-control discrimination than vitamin B12 among children with ITS/pre-ITS. However, substantial nutritional differences between cases and healthy controls may have inflated the observed diagnostic performance. The exploratory ROC-derived threshold of ⩾19.2 µmol/L requires independent validation in clinically representative populations before routine clinical application.

Categories: Neurology, Nutrition, Pediatrics
Keywords: homocysteine, hyperhomocysteinemia, infantile tremor syndrome, neurodevelopmental delay, pre-its, vitamin b12

Introduction

Infantile tremor syndrome (ITS) is a well-known neuro-nutritional disorder that occurs mostly in infants and young children in the Indian subcontinent [1]. It is associated with developmental delay or regression, tremors, hyperpigmentation of the skin, and hypopigmented hair, with anemia being a common finding, and typically affects children 6-24 months of age [1,2]. It occurs more often in breast-fed babies from vegetarian mothers lacking vitamin B12 in their vegetarian diet. Regression is the loss of previously attained developmental milestones following a period of normal development, suggestive of an underlying neurological or metabolic disorder. Pre-ITS is defined as an early or subclinical stage of infantile tremor syndrome characterized by features of ITS without overt tremors. Recent studies have reframed ITS as neurocutaneous infantile B12 deficiency (NIB) syndrome, emphasizing its pathophysiological basis in functional cobalamin deficiency [3,4].

Vitamin B12 is vital for one-carbon metabolism and neurological development. It is a cofactor for

methionine synthase, an enzyme that converts homocysteine to methionine. Vitamin B12 deficiency disrupts this conversion process, increasing levels of homocysteine and interrupting biochemical pathways requiring methylation for DNA synthesis, the production of neurotransmitters, and myelination [5]. Thus, raised levels of homocysteine in serum are indicative of functional B12 deficiency and can be used as a sensitive biochemical indicator in ITS.

It is also known that hyperhomocysteinemia is associated with oxidative stress, neuronal injury, endothelial dysfunction, and impaired myelination, which can lead to neurodevelopmental abnormalities in affected infants [6]. Vitamin B12 deficiency is crucial as a diagnostic and monitoring marker. Early diagnosis and treatment may lead to a quick improvement in clinical symptoms and levels of homocysteine in the bloodstream [3,7-9].

Although hyperhomocysteinemia has been reported in children with ITS, evidence regarding its diagnostic discrimination and relationship with different clinical presentations across the ITS spectrum remains limited [10,11]. Serum vitamin B12 also has limitations as a marker of functional cobalamin status because circulating concentrations may not always reflect intracellular cobalamin availability [5]. Therefore, the present study aimed to evaluate the ability of serum homocysteine to discriminate children with ITS/pre-ITS from independently recruited, apparently healthy, unmatched controls and to compare its discriminatory performance with serum vitamin B12. Additionally, the study aimed to identify an exploratory receiver operating characteristic (ROC)-derived homocysteine threshold and examine differences in homocysteine concentrations between established ITS and pre-ITS. The ROC-derived threshold and multivariable analyses were considered preliminary and hypothesis-generating rather than confirmatory diagnostic assessments.

Materials And Methods

Study design and setting

This observational unmatched case-control study was conducted in the Department of Pediatrics of Gandhi Medical College, Bhopal, Madhya Pradesh, in collaboration with the Central Institutional Biochemistry Laboratory, from June 2024 to December 2025. A total of 80 children were enrolled, comprising 40 children with infantile tremor syndrome (ITS)/pre-ITS and 40 independently recruited apparently healthy controls.

Study participants and clinical classification

Children aged 6-24 months with clinical features suggestive of ITS or pre-ITS were enrolled as cases. The characteristic clinical features considered for ITS included coarse tremors, developmental delay or regression, pallor with anemia, and neurocutaneous changes such as hyperpigmented knuckles and sparse brown hair [6].

For the present study, predefined operational criteria were applied consistently for clinical classification. ITS was defined as the presence of at least three of four characteristic features: (1) coarse tremors, (2) developmental delay or regression, (3) pallor with anemia, and (4) neurocutaneous changes, including hyperpigmented knuckles and sparse brown hair, with tremors required for classification as established ITS. Pre-ITS was operationally defined as the presence of neurocutaneous manifestations, together with developmental delay or regression in the absence of overt tremors. These definitions were used as study-specific operational criteria for the consistent classification of participants. Of the 40 cases, 21 were classified as ITS and 19 as pre-ITS.

The control group consisted of apparently healthy children attending the pediatric outpatient department for routine immunization or well-child visits. Controls were recruited independently and were not individually matched to cases by age, sex, or other characteristics.

Children with sequelae of hypoxic-ischemic encephalopathy or other significant neurological disorders, a history of significant neonatal hyperbilirubinemia, congenital malformations, endocrine abnormalities, or suspected inborn errors of metabolism were excluded.

Data collection and clinical assessment

Data were collected using a prestructured study proforma. Demographic characteristics and detailed clinical histories were recorded, including gestational history, maternal dietary history, breastfeeding practices, complementary feeding, nutritional history, and developmental milestones.

All participants underwent comprehensive clinical examination and anthropometric assessment. Anthropometric measurements included weight, length/height, head circumference, and mid-upper arm circumference. General examination included assessment for pallor and characteristic skin and hair changes. Neurological and other systemic examinations were performed.

Developmental status was assessed clinically by a pediatrician using age-appropriate developmental milestones across relevant developmental domains. Findings were documented in the structured study

proforma. Developmental status was classified as developmental delay, developmental regression, or age-appropriate development, as applicable. No standardized developmental assessment instrument or instrument-derived developmental scores were used in the statistical analysis. Consequently, developmental classification relied on clinical assessment and may have been subject to observer variability.

Laboratory investigations

Venous blood samples were collected from all participants under aseptic precautions. Samples collected in serum separator tubes were used for the estimation of serum homocysteine, vitamin B12, folate, and electrolytes, whereas ethylenediaminetetraacetic acid (EDTA) samples were used for complete blood counts and peripheral blood smear examination.

Serum homocysteine was quantitatively measured by chemiluminescence immunoassay using the Atellica platform (Siemens Healthineers, Erlangen, Germany). The institutional laboratory reference range was 3.7-13.9 µmol/L. For categorical analysis, a serum homocysteine concentration of ⩾15 µmol/L was considered hyperhomocysteinemia based on the predefined literature-based biochemical threshold [9].

Serum vitamin B12 and folate concentrations were measured by chemiluminescence immunoassay using the Atellica platform. The institutional laboratory reference ranges were 211-911 pg/mL for vitamin B12 and 4.6-18.7 ng/mL for folate.

Serum sodium, potassium, and calcium concentrations were measured using the ion-selective electrode method. Hematological evaluation included complete blood count and peripheral blood smear examination.

Sample size calculation

The original sample size was estimated using the conventional single-proportion formula:

$$n = 4pq/d^2$$

where n represents the estimated sample size, p represents the anticipated percentage of hyperhomocysteinemia, q = 100 - p, and d represents the allowable absolute error.

Based on the study by Ljungblad et al., the anticipated proportion of hyperhomocysteinemia was taken as 46%, with p = 46, q = 54, and an allowable absolute error of 16% [9].

Accordingly, $n = (4 \times 46 \times 54)/16^2 = 38.81.$

The estimated sample size was rounded to 40 cases. An additional 40 apparently healthy controls were independently recruited at a 1:1 case-to-control ratio, resulting in a total sample of 80 participants.

However, the single-proportion formula was not specifically designed to estimate the sample size required for comparing diagnostic discrimination or ROC areas in an unmatched case-control study. Consequently, the present diagnostic analyses should be considered exploratory rather than adequately powered confirmatory assessments. Larger studies with sample sizes calculated specifically for diagnostic accuracy comparisons are required to validate these findings.

Statistical analysis

Data were entered into Microsoft Excel (Microsoft Corp., Redmond, WA) and analyzed using IBM SPSS Statistics version 20.0 (IBM Corp., Armonk, NY). Continuous variables were summarized as mean ± standard deviation (SD) when approximately normally distributed and as median with interquartile range (IQR) for skewed distributions. Categorical variables were presented as frequencies and percentages.

Between-group comparisons of continuous variables were performed using the independent-samples Student's t-test or Welch's t-test for parametric data, as appropriate, and the Mann-Whitney U test for non-parametric data. Categorical variables were compared using the chi-square test, Fisher's exact test, or Fisher-Freeman-Halton exact test for contingency tables with more than two categories, as appropriate. The statistical test used for each comparison and the corresponding test statistic (t, U, or χ^2, as applicable) were reported with the p-value in the relevant tables or legends. For exact tests, the exact p-value was reported.

Receiver operating characteristic (ROC) curve analysis was performed to assess the discrimination of serum homocysteine, vitamin B12, and other laboratory parameters between ITS/pre-ITS cases and healthy controls. Areas under the ROC curve (areas under the curve {AUCs}) were reported with 95% confidence intervals (CIs). Exploratory ROC-derived thresholds were selected using Youden's index. Sensitivity and specificity were reported with exact Clopper-Pearson 95% CIs. Pairwise comparisons of AUCs were performed using the paired DeLong method because the biomarkers being compared were measured in the

same participants.

Two distinct serum homocysteine thresholds were used for different purposes. A concentration of ≥15 µmol/L was used as the predefined literature-based biochemical threshold for hyperhomocysteinemia [9]. In contrast, ≥19.2 µmol/L was identified from the ROC analysis of the present dataset as an exploratory threshold for discriminating ITS/pre-ITS cases from healthy controls. The ROC-derived threshold was not considered an externally validated diagnostic cutoff.

For selected non-parametric comparisons, percentile bootstrap 95% CIs were obtained using 20,000 resamples. Because sparse cells and near separation were present in the case-control dataset, an exploratory multivariable analysis was performed using Firth penalized logistic regression. The model included serum homocysteine (per 10 µmol/L increase), vitamin B12 (per 100 pg/mL increase), folate (per 1 ng/mL increase), nutritional status (severe acute malnutrition {SAM}/moderate acute malnutrition {MAM} versus normal), age (per month increase), and sex (male versus female). Adjusted odds ratios (ORs) with profile penalized likelihood 95% CIs were reported, and p-values were obtained using penalized likelihood ratio tests. Continuous predictors were modeled linearly on the log-odds scale. Given the relatively small sample size, sparse cells, and near separation, the multivariable estimates were considered exploratory.

The source dataset contained laboratory measurements reported beyond the laboratory reporting boundaries using ">X" or "<X" notation. For statistical analysis, these observations were assigned their corresponding reported boundary values. For example, measurements reported as >1,500 were analyzed as 1,500, while measurements reported as <50 were analyzed as 50. This approach affected serum potassium, vitamin B12, and folate measurements. Boundary-value substitution may have influenced the observed variability, distributional summaries, ranking of observations, and statistical estimates involving these parameters. Therefore, the corresponding analyses were interpreted cautiously. No model-based imputation was performed for missing observations, and analyses otherwise used the available recorded values for each variable.

All statistical tests were two-sided, and a p-value of <0.05 was considered statistically significant.

Ethical considerations

The study protocol was approved by the Institutional Ethics Committee (IEC) of Gandhi Medical College, Bhopal (IEC protocol number, 54/IEC/2024; approval date, March 27, 2024). Written informed consent was obtained from the parent or legal guardian of each participant before enrollment. Participant confidentiality was maintained throughout data collection, analysis, and reporting.

Results

Age and sex distributions did not differ significantly between cases and controls (p = 0.165 and p = 1.000, respectively). Severe acute malnutrition was recorded in 72.5% of cases and moderate acute malnutrition in 12.5%, whereas all controls had normal nutritional status. Developmental delay or regression was present in all cases and formed part of their clinical selection criteria. Of the 40 cases, 21 (52.5%) had ITS, and 19 (47.5%) had pre-ITS (Table *1*).

Characteristic	Cases (n = 40)	Controls (n = 40)	Test statistic	P-value
Age, months, median (Q1-Q3)	10.00 (9.00-12.25)	12.00 (9.75-15.75)	U = 656.0	0.165[a]
Age 6-12 months	30 (75.0%)	25 (62.5%)	-	-
Age >12-24 months	10 (25.0%)	15 (37.5%)	-	-
Male	23 (57.5%)	22 (55.0%)	-	1.000[b]
Female	17 (42.5%)	18 (45.0%)	-	-
Normal nutritional status	6 (15.0%)	40 (100.0%)	-	<0.001[c]
Severe acute malnutrition	29 (72.5%)	0 (0.0%)	-	-
Moderate acute malnutrition	5 (12.5%)	0 (0.0%)	-	-
Developmental regression	17 (42.5%)	0 (0.0%)	-	-
Developmental delay	23 (57.5%)	0 (0.0%)	-	-
Normal development	0 (0.0%)	40 (100.0%)	-	-
ITS	21 (52.5%)	Not applicable	-	-
Pre-ITS	19 (47.5%)	Not applicable	-	-

TABLE 1: Demographic and clinical characteristics of cases and controls

Values are n (%) unless otherwise specified. For exact tests, the exact p-value is reported rather than a conventional test statistic. Developmental features used in case selection were not subjected to significance testing

[a]Mann-Whitney U test

[b]Two-sided Fisher's exact test for overall sex distribution

[c]Fisher-Freeman-Halton exact test for the overall three-category nutritional distribution

ITS: infantile tremor syndrome

Cases had significantly lower hemoglobin, platelet counts, calcium, and folate than controls. Homocysteine was significantly higher, with median concentrations of 35.70 versus 5.52 µmol/L (U = 1592.0; p < 0.001). Potassium was also significantly higher (U = 1036.5; p = 0.023). Vitamin B12 did not differ significantly between groups (U = 633.5; p = 0.110). Mean corpuscular volume (MCV), total leukocyte count (TLC), lymphocytes, granulocytes, and sodium showed no statistically significant differences (Table *2*).

Parameter	Cases (n = 40)	Controls (n = 40)	Difference (95% CI)	Test statistic	P-value
Hemoglobin (g/dL)	7.14 ± 1.75	11.74 ± 1.00	-4.60 (-5.23 to -3.96)	t = -14.399	<0.001[a]
MCV (fL)	78.80 (73.47-101.88)	84.00 (78.60-86.08)	-5.20 (-8.20 to 14.70)	U = 726.0	0.479[b]
TLC (/mm³)	7,665 (5,700-10,755)	8,300 (6,550-9,800)	-635 (-2,170 to 1,675)	U = 767.5	0.758[b]
Lymphocytes (%)	47.69 ± 16.49	49.55 ± 12.27	-1.86 (-8.34 to 4.62)	t = -0.572	0.569[a]
Granulocytes (%)	47.55 ± 16.36	46.55 ± 12.79	1.00 (-5.54 to 7.54)	t = 0.304	0.762[a]
Platelets (/mm³)	180,000 (115,750-235,000)	272,500 (207,500-310,000)	-92,500 (-165,500 to -25,000)	U = 488.0	0.003[b]
Sodium (mEq/L)	136.74 ± 4.06	137.26 ± 3.00	-0.52 (-2.11 to 1.06)	t = -0.658	0.512[a]
Potassium (mEq/L)†	4.80 (4.07-6.10)	4.35 (4.00-4.60)	0.45 (0.05 to 0.90)	U = 1036.5	0.023[b]
Calcium (mg/dL)	8.15 (7.38-9.00)	9.40 (9.00-9.80)	-1.25 (-1.80 to -0.80)	U = 224.0	<0.001[b]
Homocysteine (µmol/L)	35.70 (30.53-42.92)	5.52 (4.70-6.20)	30.19 (27.55 to 33.90)	U = 1592.0	<0.001[b]
Vitamin B12 (pg/mL)†	137.30 (111.05-1,347.25)	447.50 (385.50-531.25)	-310.20 (-364.40 to 64.95)	U = 633.5	0.110[b]
Folate (ng/mL)†	5.10 (3.68-12.30)	11.10 (10.15-14.60)	-6.00 (-7.60 to -4.10)	U = 426.5	<0.001[b]

TABLE 2: Comparison of hematological and biochemical parameters between cases and controls

Values are mean ± SD or median (Q1-Q3), as appropriate. Differences are cases minus controls

[a]Welch's t-test; mean difference with Welch 95% CI

[b]Mann-Whitney U test; median difference with percentile bootstrap 95% CI using 20,000 resamples. Median-difference CIs and Mann-Whitney tests assess different summaries

†Laboratory values reported as >X or <X were assigned the corresponding reporting-boundary value X for statistical analysis; this applied to potassium, vitamin B12, and folate measurements

MCV, mean corpuscular volume; TLC, total leukocyte count; CI, confidence interval; SD, standard deviation

Using the predefined biochemical threshold of ⩾15 µmol/L, hyperhomocysteinemia was recorded in all cases compared to 12.5% of controls. Hypocalcemia occurred in 60.0% versus 7.5% and anemia in 97.5% versus 17.5%, respectively. All three differences were statistically significant ($p < 0.001$). The anemia comparison requires caution because anemia contributes to the clinical case phenotype (Table *3*).

Laboratory abnormality	Cases (n = 40)	Controls (n = 40)	Statistical test	P-value
Hyperhomocysteinemia, ≥15 µmol/L	40 (100.0%)	5 (12.5%)	Fisher's exact test	<0.001
Hypocalcemia, <8.5 mg/dL	24 (60.0%)	3 (7.5%)	Fisher's exact test	<0.001
Anemia, Hb < 11 g/dL	39 (97.5%)	7 (17.5%)	Fisher's exact test	<0.001

TABLE 3: Comparison of categorical laboratory abnormalities

Values are n (%). Two-sided Fisher's exact tests were used. For Fisher's exact test, the exact p-value is reported rather than a conventional test statistic. The predefined biochemical threshold of ≥15 µmol/L used to define hyperhomocysteinemia is distinct from the exploratory ROC-derived threshold of ≥19.2 µmol/L

Hb, hemoglobin; ROC, receiver operating characteristic

Mean homocysteine was significantly higher in ITS than in pre-ITS: 41.61 ± 9.64 versus 32.82 ± 8.05 µmol/L.

The mean difference was 8.79 µmol/L (95% CI, 3.12-14.46; t = 3.140; p = 0.003). Vitamin B12 and folate did not differ significantly between subgroups (p = 0.776 and p = 0.569, respectively). This cross-sectional difference does not establish disease progression (Table 4).

Parameter	ITS (n = 21)	Pre-ITS (n = 19)	Difference (95% CI)	Test statistic	P-value
Homocysteine (µmol/L)	41.61 ± 9.64	32.82 ± 8.05	8.79 (3.12 to 14.46)	t = 3.140	0.003[a]
Vitamin B12 (pg/mL)†	129.40 (114.60-1,500.00)	144.00 (106.80-1,048.75)	-14.60 (-676.90 to 1,028.40)	U = 210.5	0.776[b]
Folate (ng/mL)†	5.20 (3.80-15.60)	5.00 (3.54-9.47)	0.20 (-2.55 to 5.83)	U = 221.0	0.569[b]

TABLE 4: Comparison of micronutrient parameters between ITS and pre-ITS cases

Differences are ITS minus pre-ITS

[a]Welch's t-test with mean-difference 95% CI

[b]Mann-Whitney U test with percentile bootstrap median-difference 95% CI

†Laboratory values reported as >X or <X were assigned the corresponding reporting-boundary value X for statistical analysis

CI, confidence interval; ITS, infantile tremor syndrome

Homocysteine showed high apparent discrimination between ITS/pre-ITS cases and healthy controls, with an AUC of 0.995 (95% CI: 0.987-1.000). ROC analysis identified an exploratory threshold of ⩾19.2 µmol/L, with sensitivity of 100.0% and specificity of 92.5%. This ROC-derived threshold is distinct from the predefined biochemical threshold of ⩾15 µmol/L used to define hyperhomocysteinemia. Hemoglobin also showed high discrimination, with an AUC of 0.992. Calcium, folate, platelets, and vitamin B12 had lower AUCs. These cutoffs were derived and evaluated in the same sample and have not undergone independent validation (Table 5).

Parameter	AUC (95% CI)	ROC-derived cutoff	Sensitivity percentage (95% CI)	Specificity percentage (95% CI)
Homocysteine (µmol/L)	0.995 (0.987-1.000)	≥19.2	100.0 (91.2-100.0)	92.5 (79.6-98.4)
Hemoglobin (g/dL)	0.992 (0.979-1.000)	≤9.6	97.5 (86.8-99.9)	97.5 (86.8-99.9)
Platelets (/mm³)	0.695 (0.573-0.817)	≤220,000	72.5 (56.1-85.4)	65.0 (48.3-79.4)
Calcium (mg/dL)	0.860 (0.780-0.940)	≤8.5	67.5 (50.9-81.4)	90.0 (76.3-97.2)
Folate (ng/mL)†	0.733 (0.603-0.864)	≤6.1	65.0 (48.3-79.4)	97.5 (86.8-99.9)
Vitamin B12 (pg/mL)†	0.604 (0.464-0.744)	≤218.6	60.0 (43.3-75.1)	87.5 (73.2-95.8)

TABLE 5: Exploratory ROC analysis of laboratory parameters for discriminating ITS/pre-ITS cases from healthy controls

AUC CIs use the DeLong variance with a normal approximation bounded at 0-1. Sensitivity and specificity CIs are exact Clopper-Pearson intervals. Cutoffs maximize Youden's index in the same dataset. All analyses include 40 cases and 40 controls

†Laboratory values reported beyond the reporting boundary were assigned the corresponding boundary value for statistical analysis. The ≥19.2 µmol/L homocysteine threshold is exploratory and ROC-derived and should not be confused with the predefined ≥15 µmol/L biochemical threshold for hyperhomocysteinemia

AUC, area under the curve; ROC, receiver operating characteristic; ITS, infantile tremor syndrome; CI, confidence interval

Homocysteine had a significantly higher AUC than vitamin B12, with an AUC difference of 0.391 (95% CI, 0.250-0.531; z = 5.45; p < 0.001). Its AUC did not differ significantly from hemoglobin (difference, 0.003; 95% CI, -0.012 to 0.018; z = 0.41; p = 0.686). These comparisons describe discrimination within this selected sample and do not establish general clinical superiority (Table *6*).

Comparison	AUC difference	95% CI	Z statistic	P-value
Homocysteine versus vitamin B12†	0.391	0.250 to 0.531	5.45	<0.001
Homocysteine versus hemoglobin	0.003	-0.012 to 0.018	0.41	0.686

TABLE 6: Formal comparison of ROC areas using DeLong's test

Differences are homocysteine AUC minus comparator AUC. The paired DeLong test compares biomarkers measured in the same children; "paired" refers to the biomarkers and does not indicate case-control matching

†Vitamin B12 values reported beyond the laboratory reporting boundary were assigned the corresponding boundary value for statistical analysis

AUC, area under the curve; ROC, receiver operating characteristic; CI, confidence interval

In the exploratory adjusted model, each 10 µmol/L increase in homocysteine was associated with higher odds of case status (adjusted OR, 7.45; 95% CI, 2.25-210.55; p < 0.001). Vitamin B12, folate, nutritional status, age, and sex were not statistically significant. The wide confidence intervals and the absence of malnourished controls indicate substantial uncertainty; therefore, these adjusted estimates should be considered exploratory (Table *7*).

Predictor	Adjusted odds ratio	95% CI	P-value
Homocysteine, per 10 µmol/L increase	7.45	2.25 to 210.55	<0.001
Vitamin B12, per 100 pg/mL increase†	0.92	0.43 to 2.42	0.819
Folate, per 1 ng/mL increase†	0.93	0.33 to 1.42	0.610
SAM/MAM versus normal nutritional status	12.46	0.94 to 9.17 × 10^{8}	0.055
Age, per month increase	0.85	0.47 to 1.49	0.811
Male versus female	0.18	2.98 × 10^{-8} to 10.74	0.578

TABLE 7: Exploratory multivariable analysis of factors associated with case status

Firth penalized logistic regression was used because of sparse cells and near separation. Adjusted ORs are presented with profile penalized likelihood 95% CIs, and p-values were obtained using penalized likelihood ratio tests. Continuous predictors were modeled linearly on the log-odds scale. The very wide confidence intervals reflect sparse data and substantial model uncertainty; therefore, this analysis should be considered exploratory

†Laboratory values reported beyond the reporting boundary were assigned the corresponding boundary value for statistical analysis

CI, confidence interval; SAM, severe acute malnutrition; MAM, moderate acute malnutrition; OR, odds ratio

Discussion

The present study demonstrated marked hyperhomocysteinemia among children with ITS/pre-ITS, together with a high burden of malnutrition, anemia, and neurodevelopmental impairment. Serum homocysteine showed significantly greater discrimination between cases and healthy controls than serum vitamin B12, supporting its potential role as an adjunct biochemical marker in this study population. Most affected children were aged 6-12 months, consistent with the classical epidemiological profile of ITS reported in Indian literature [6]. This period of infancy coincides with declining vitamin B12 stores and increasing dependence on complementary feeding, particularly among exclusively breastfed infants born to mothers consuming predominantly vegetarian diets [6,10]. Gowda et al. (2018) [10] reported a mean age of 13.2 months among children with ITS, while Francis et al. (2020) [11] reported a mean age of 11.5 ± 3.5 months.

No significant difference in sex distribution was observed between cases and controls, indicating the comparability of the study groups with respect to sex rather than establishing equal susceptibility to ITS among males and females. Gowda et al. (2018) [10] similarly reported an almost balanced sex distribution among affected children, whereas Francis et al. (2020) [11] observed a slight male predominance. The present study did not evaluate whether sociocultural factors or differences in healthcare-seeking behavior influenced the observed sex distribution.

A substantial burden of malnutrition was observed among children in the combined ITS/pre-ITS group. Severe acute malnutrition (SAM) was present in 72.5% of cases, while an additional 12.5% had moderate acute malnutrition (MAM), indicating that 85% of cases had some degree of acute malnutrition, compared with normal nutritional status among controls. These findings are consistent with the recognized nutritional-metabolic context of ITS [6]. Gowda et al. reported protein-energy malnutrition in all children with ITS [10]. Francis et al. (2020) reported malnutrition in 95.7% (88/92) of children with ITS and described frequent hyperhomocysteinemia and hematological abnormalities [11]. Bajaj et al. (2022) reported moderate-to-severe malnutrition in 69.2% of affected children [12]. However, the marked imbalance in nutritional status between cases and controls represents an important potential confounding factor and limits the ability to determine whether the observed biochemical differences were specific to ITS/pre-ITS or partly reflected underlying nutritional differences between the groups.

The substantial burden of malnutrition among affected children may be influenced by several nutritional and sociocultural factors, including inadequate complementary feeding, limited dietary diversity, maternal micronutrient deficiencies, household food insecurity, and the delayed recognition of nutritional problems. These factors may contribute to nutritional deficiencies during infancy and potentially influence homocysteine metabolism. Although maternal dietary history, breastfeeding practices, and complementary feeding information were recorded, their individual associations with nutritional status and serum homocysteine concentrations were not systematically analyzed. Therefore, the contribution of these factors remains uncertain, and further studies incorporating detailed dietary and socioeconomic assessments are warranted.

Developmental delay or regression was present in all cases; developmental delay occurred in 57.5%, whereas regression occurred in 42.5%. These manifestations formed part of the operational clinical criteria used for

case selection and therefore cannot be interpreted as independently demonstrated associations with ITS in the present study. Gowda et al. (2018) reported developmental delay in 91% and developmental regression in 35% of children with ITS [10]. John et al. (2020) demonstrated adaptive-cognitive impairment in 80% of infants with nutritional vitamin B12 deficiency, while gross motor and fine motor developmental delays were observed in 93% and 87%, respectively [13]. Developmental regression has also been recognized as an important clinical manifestation of ITS [6]. Neurological manifestations associated with vitamin B12 deficiency have been discussed in relation to disturbances in myelination, neurotransmitter synthesis, and methylation pathways [5,14].

Cases had significantly lower hemoglobin concentrations than controls (7.14 ± 1.75 versus 11.74 ± 1.00 g/dL; $p < 0.001$). Anemia was present in 97.5% of cases compared to 17.5% of controls, indicating a substantial hematological burden in the case group. Bajaj et al. (2022) reported severe anemia in 84.6% of children with ITS [12]. Similarly, Francis et al. (2020) reported frequent hyperhomocysteinemia accompanied by anemia and vitamin B12 deficiency among children with ITS [11]. MCV did not differ significantly between cases and controls in the present study ($p = 0.479$), suggesting that macrocytosis was not a consistent hematological feature. Gautam et al. (2017) described a dimorphic anemia pattern in children with ITS, consistent with the possibility of concurrent nutritional deficiencies [15]. Cases also had significantly lower platelet counts than controls ($p = 0.003$). These findings demonstrate accompanying hematological abnormalities, although the present analysis was not designed to establish their specific nutritional causes.

The principal biochemical finding was the marked elevation of serum homocysteine among children with ITS/pre-ITS. Median serum homocysteine was 35.70 µmol/L in cases compared to 5.52 µmol/L in controls ($p < 0.001$). Using the predefined literature-based biochemical threshold of ⩾15 µmol/L, hyperhomocysteinemia was present in all cases and 12.5% of controls. Thus, hyperhomocysteinemia was strongly associated with case status in this study population but was not exclusive to affected children. Francis et al. (2020) [11] reported hyperhomocysteinemia in 96% of children with ITS, while Gowda et al. (2018) [10] identified elevated homocysteine as an important biochemical finding associated with vitamin B12 deficiency in ITS. The universal occurrence of hyperhomocysteinemia among cases in the present study supports its potential usefulness in biochemical assessment; however, the findings do not establish specificity for vitamin B12 deficiency or its utility for monitoring nutritional recovery. Other studies evaluating nutritional deficiencies during infancy have similarly demonstrated elevated homocysteine in children with hematinic deficiencies [16]. Ljungblad et al. (2021) also reported an association between elevated homocysteine and neurological manifestations during infancy [9].

The markedly elevated serum homocysteine concentrations observed among children with ITS/pre-ITS may be explained by disturbances in one-carbon metabolism associated with nutritional deficiencies. Vitamin B12 acts as an essential cofactor for methionine synthase, which facilitates the conversion of homocysteine to methionine. Inadequate vitamin B12 availability may impair this pathway, resulting in homocysteine accumulation [5]. Folate deficiency may further contribute to impaired homocysteine remethylation. In the present study, significantly lower folate concentrations and the high burden of acute malnutrition among affected children provide a plausible nutritional context for the observed hyperhomocysteinemia. However, the specific contribution of functional vitamin B12 deficiency could not be established because methylmalonic acid and other functional markers were not evaluated.

Interestingly, hyperhomocysteinemia was also observed in 12.5% (5/40) of apparently healthy controls, indicating that elevated serum homocysteine is not specific to ITS/pre-ITS. Possible explanations include subclinical vitamin B12 or folate insufficiency, variations in dietary intake, and other physiological or metabolic factors affecting homocysteine metabolism. However, the individual causes of elevated homocysteine concentrations among these five children were not investigated. Therefore, these explanations remain speculative. This observation highlights the importance of interpreting serum homocysteine in conjunction with clinical findings and other biochemical investigations rather than considering it an isolated diagnostic marker.

Serum folate was significantly lower in cases than controls ($p < 0.001$), whereas serum vitamin B12 did not differ significantly between groups ($p = 0.110$), despite a lower median concentration among cases. Serum vitamin B12 demonstrated limited discrimination, with an AUC of 0.604 (95% CI: 0.464-0.744), compared with 0.995 (95% CI: 0.987-1.000) for homocysteine. Pairwise DeLong analysis demonstrated a significantly greater AUC for homocysteine than vitamin B12, with an AUC difference of 0.391 (95% CI, 0.250-0.531; $p < 0.001$). Thus, serum homocysteine demonstrated greater case-control discrimination than serum vitamin B12 in the present sample. Francis et al. similarly reported homocysteine as a more consistent biochemical indicator of cobalamin deficiency than serum vitamin B12 among children with ITS [11]. Nevertheless, the present findings do not independently establish functional intracellular cobalamin deficiency in every affected child [5].

The comparison of ITS and pre-ITS cases demonstrated significantly higher serum homocysteine concentrations among children with established ITS. Mean homocysteine concentrations were 41.61 ± 9.64 µmol/L in ITS and 32.82 ± 8.05 µmol/L in pre-ITS, corresponding to a mean difference of 8.79 µmol/L (95% CI, 3.12-14.46; $p = 0.003$). In contrast, serum folate and vitamin B12 concentrations did not differ significantly between the two groups ($p = 0.569$ and $p = 0.776$, respectively). Ljungblad et al. (2021) [9]

reported an association between elevated homocysteine concentrations and neurological abnormalities during infancy, while Francis et al. (2020) [11] reported hyperhomocysteinemia in 96% of children with ITS. The present subgroup findings suggest an association between higher homocysteine concentrations and the established ITS clinical phenotype. However, because ITS and pre-ITS were compared cross-sectionally at a single time point, these findings do not demonstrate temporal progression from pre-ITS to ITS or establish homocysteine as a marker of disease severity. Longitudinal studies using standardized measures of neurological severity are required to investigate this relationship.

Serum homocysteine demonstrated excellent apparent discrimination between ITS/pre-ITS cases and healthy controls, with an AUC of 0.995 (95% CI: 0.987-1.000). ROC analysis identified an exploratory threshold of ≥19.2 µmol/L, which yielded 100% sensitivity and 92.5% specificity. In comparison, serum vitamin B12 demonstrated an AUC of 0.604 (95% CI: 0.464-0.744), with 60.0% sensitivity and 87.5% specificity at an ROC-derived threshold of ≤218.6 pg/mL. Importantly, the study-derived homocysteine threshold of ≥19.2 µmol/L should be distinguished from the predefined literature-based biochemical threshold of ≥15 µmol/L used to define hyperhomocysteinemia. Previous studies by Gowda et al. (2018) [10] and Francis et al. (2020) [11] support the frequent occurrence of hyperhomocysteinemia among children with ITS. The present study extends these observations by evaluating ROC-based case-control discrimination and formally comparing homocysteine with serum vitamin B12. However, the ROC-derived thresholds were developed and evaluated in the same study population and have not undergone independent validation. Their diagnostic performance therefore requires confirmation in external populations before routine clinical application.

An important consideration in interpreting the ROC findings is the substantial difference in nutritional status between cases and controls. Severe acute malnutrition was present in 72.5% of cases, whereas all controls had normal nutritional status. This imbalance may have contributed to the observed biochemical differences and potentially inflated the apparent diagnostic discrimination of serum homocysteine. Furthermore, the use of apparently healthy controls rather than children presenting with comparable nutritional or neurological conditions introduces spectrum bias and limits the generalizability of the findings to routine clinical settings. Therefore, the exceptionally high AUC of 0.995 should be interpreted as apparent discrimination within this selected study population rather than definitive evidence of diagnostic accuracy among clinically heterogeneous children.

Hemoglobin also demonstrated high discriminatory performance, with an AUC of 0.992. However, reduced hemoglobin is a nonspecific finding that occurs in anemia of multiple etiologies. Furthermore, pallor with anemia contributed to the operational clinical phenotype used to classify ITS cases, which may partly explain the high discriminatory performance of hemoglobin in this selected population. Although the AUC for homocysteine was numerically higher than that for hemoglobin, the difference was not statistically significant (AUC difference, 0.003; $p = 0.686$). In contrast, homocysteine demonstrated significantly greater discrimination than serum vitamin B12. Therefore, the present findings do not establish the superiority of homocysteine over hemoglobin but support the further evaluation of serum homocysteine as an adjunct biochemical marker in children with suspected ITS/pre-ITS.

In the exploratory multivariable analysis, each 10 µmol/L increase in serum homocysteine was associated with higher odds of case status after adjustment for vitamin B12, folate, nutritional status, age, and sex (adjusted OR, 7.45; 95% CI, 2.25-210.55; $p < 0.001$). The other variables included in the model were not statistically significant. However, the very wide confidence intervals, small sample size, sparse cells, near separation, and marked nutritional imbalance between cases and controls substantially limit the precision and interpretation of these adjusted estimates. Consequently, the exploratory model does not permit firm conclusions regarding an independent disease-specific association. In addition, the use of reporting-boundary values for some vitamin B12 and folate measurements introduces uncertainty into their estimated associations.

Overall, hyperhomocysteinemia emerged as a prominent biochemical abnormality associated with ITS/pre-ITS in the present study, occurring alongside malnutrition, anemia, and neurodevelopmental impairment. These observations are compatible with nutritional and one-carbon metabolic disturbances described in ITS [5,6,11]; however, the case-control design does not establish a causal relationship between elevated homocysteine and the syndrome. Serum homocysteine demonstrated significantly greater case-control discrimination than serum vitamin B12, while higher concentrations among children with established ITS than pre-ITS suggest an association with the clinical phenotype. Further independent diagnostic validation and longitudinal studies are required to establish its clinical utility and determine whether serum homocysteine is associated with disease severity.

Limitations

This study has several limitations. First, it was a single-center study with a relatively small sample size, which may limit the generalizability of the findings. Second, the case-control and cross-sectional nature of the subgroup comparisons precluded the assessment of temporal changes in serum homocysteine or progression from pre-ITS to established ITS. Cases and controls also differed substantially in nutritional status, introducing potential confounding and limiting the ability to determine whether the observed

biochemical differences were specific to ITS/pre-ITS or partly attributable to underlying malnutrition. In addition, developmental abnormalities and pallor with anemia contributed to the operational clinical criteria used for case classification, which may have influenced comparisons involving these characteristics and hemoglobin. The ROC-derived homocysteine threshold of ⩾19.2 µmol/L was derived and evaluated in the same dataset and therefore requires independent external validation. Methylmalonic acid and other functional markers of intracellular vitamin B12 status were not assessed. Finally, the exploratory multivariable analysis was limited by the small sample size, sparse cells, near separation, and wide confidence intervals, while reporting-boundary substitutions for some laboratory measurements introduced additional uncertainty. Larger multicenter prospective studies with nutritionally comparable control groups and longitudinal follow-up are required to validate these findings.

Conclusions

Serum homocysteine was markedly elevated among children with ITS/pre-ITS and demonstrated significantly greater discrimination between cases and apparently healthy controls than serum vitamin B12. Higher homocysteine concentrations among children with established ITS compared with pre-ITS suggest a possible association with the clinical phenotype. However, substantial nutritional differences between cases and controls, together with the use of healthy rather than disease-matched controls, limit the generalizability of these findings. The exploratory ROC-derived threshold of ⩾19.2 µmol/L requires independent validation in larger, clinically representative cohorts before routine clinical application. Serum homocysteine may serve as a potential adjunct biochemical marker, but its independent diagnostic utility remains to be established.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

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Disclosures

Human subjects: Informed consent for treatment and open access publication was obtained or waived by all participants in this study. The Institutional Ethics Committee of Gandhi Medical College, Bhopal, issued approval 54/IEC/2024. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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