JHWCR JOURNAL OF HEALTH, WELLNESS AND COMMUNITY RESEARCH 3007-0570

DOI: https://doi.org/10.61919/9dgmde35

*Original Article*

# Biochemical Relationship Between Vitamin B12 Status, Homocysteine, and Cognitive Performance in Healthy Adults

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**"Cite this Article" Received**: 10 February 2026; **Accepted**: 11 March 2026; **Published**: 30 March 2026

**Author Contributions:** Concept: MJ, WRA, NF; Design: WRA, IS, NF; Data Collection: MJ, MG, MK, IS; Analysis: WRA, MK, NF; Drafting: MJ, WRA, MG, MK, IS, NF. **Ethical Approval**: Mujahid Hospital, Faisalabad, Pakistan. **Informed Consent**: Written informed consent was obtained from all participants; **Conflict of Interest**: The authors declare no conflict of interest. **Funding**: No external funding; **Data Availability**: Available from the corresponding author on reasonable request; **Acknowledgments**: NA

## ABSTRACT

Background: Suboptimal vitamin B12 status may disrupt one-carbon metabolism, increase circulating homocysteine, and adversely affect neurological and cognitive function before overt clinical deficiency becomes apparent. Objective: To compare plasma homocysteine concentrations and cognitive performance across deficient, borderline, and normal vitamin B12 categories among apparently healthy adults. Methods: This cross-sectional analytical study included 180 adults aged 18–60 years from Faisalabad, Pakistan. Demographic, dietary, anthropometric, haematological, biochemical, and cognitive data were collected. Serum vitamin B12, folate, plasma homocysteine, haemoglobin, and total cognitive scores were assessed. Group differences were examined using one-way analysis of variance with Tukey–Kramer pairwise comparisons. Results: Vitamin B12 deficiency was present in 42 participants (23.3%), borderline status in 61 (33.9%), and normal status in 77 (42.8%). Mean homocysteine decreased from 20.8 ± 5.9 µmol/L in the deficient group to 16.4 ± 4.3 µmol/L in the borderline group and 11.9 ± 3.1 µmol/L in the normal group, $F(2,177) = 60.33$, $p < 0.001$. Corresponding cognitive scores increased from 23.9 ± 2.8 to 25.4 ± 2.3 and 27.1 ± 2.1, respectively, $F(2,177) = 26.48$, $p < 0.001$. Conclusion: Lower vitamin B12 status was associated with higher homocysteine concentrations and lower cognitive scores, with borderline status demonstrating an intermediate pattern. Longitudinal evidence is required before causal or treatment-related conclusions can be made. Keywords: Vitamin B12, homocysteine, cognitive performance, micronutrient deficiency, apparently healthy adults, Pakistan.

## INTRODUCTION

Vitamin B12 is an essential water-soluble micronutrient required for erythropoiesis, DNA synthesis, neurological function, and one-carbon metabolism. Deficiency may produce hematological, neurological, psychiatric, and cognitive manifestations; however, neurological symptoms can occur in the absence of macrocytic anaemia or other overt haematological abnormalities. Consequently, individuals with low or borderline vitamin B12 concentrations may remain clinically unrecognized despite experiencing fatigue, impaired concentration, sensory disturbances, or subtle cognitive changes (1,2).

Vitamin B12 functions as a cofactor in two principal enzymatic pathways. It supports the remethylation of homocysteine to methionine through methionine synthase and facilitates the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A through methylmalonyl-coenzyme A mutase. Inadequate intracellular vitamin B12 therefore disrupts methylation metabolism and may increase circulating homocysteine and methylmalonic acid concentrations (3,4). Serum vitamin B12

concentration alone may not reliably identify early or functional deficiency because individuals with values near the lower reference limit may already exhibit biochemical abnormalities. Functional biomarkers, particularly homocysteine and methylmalonic acid, can therefore complement serum vitamin B12 measurements when biochemical or clinical findings are inconclusive (3,5,6).

Elevated homocysteine has been associated with endothelial dysfunction, oxidative stress, cerebral small-vessel injury, impaired methylation, and neurodegenerative processes that may adversely affect cognitive function (4,7). Epidemiological studies have reported associations between elevated homocysteine, reduced vitamin B12 status, cognitive decline, dementia risk, and structural brain abnormalities (8–10). Nevertheless, findings across studies remain inconsistent. Systematic and observational evidence suggests that these associations may vary according to age, nutritional status, educational attainment, comorbid conditions, biomarker definitions, and the cognitive assessment employed (11,12). Much of the available evidence has also been derived from older adults, individuals with mild cognitive impairment, or patients with established disease, limiting its direct applicability to apparently healthy younger and middle-aged populations.

Vitamin B12 deficiency is an important nutritional concern in Pakistan. National and regional studies have identified vitamin B12 and folate deficiencies in different population groups and have demonstrated that abnormalities in B-vitamin-dependent homocysteine metabolism are relevant within the Pakistani population (13–16). Dietary patterns characterized by limited consumption of animal-source foods may contribute to inadequate vitamin B12 intake because naturally occurring vitamin B12 is found predominantly in meat, fish, eggs, and dairy products. Economic constraints, gastrointestinal conditions, medication use, and limited awareness of micronutrient deficiencies may further increase the risk of unrecognized deficiency. Faisalabad is a large urban and industrial centre with heterogeneous dietary, educational, occupational, and socioeconomic characteristics, providing an appropriate setting in which to investigate these biochemical relationships.

Despite evidence concerning vitamin B12 deficiency and hyperhomocysteinaemia in Pakistan, limited local research has examined their independent relationships with cognitive performance among apparently healthy working-age adults. Clarifying these relationships may improve the interpretation of borderline vitamin B12 concentrations and provide a basis for more targeted nutritional assessment. Therefore, this study assessed the associations of serum vitamin B12 status and plasma homocysteine concentration with cognitive performance among apparently healthy adults in Faisalabad, Pakistan. It was hypothesized that lower serum vitamin B12 concentrations and higher plasma homocysteine concentrations would be independently associated with lower cognitive scores after accounting for relevant demographic, anthropometric, haematological, dietary, and lifestyle factors.

## MATERIALS AND METHODS

A cross-sectional analytical study was conducted among apparently healthy adults residing in Faisalabad, Pakistan. The study was designed and reported in accordance with the principles of the Strengthening the Reporting of Observational Studies in Epidemiology statement (17). Participants were recruited from community settings, educational institutions, workplaces, and outpatient screening areas through non-probability convenience sampling. Potential participants underwent a structured eligibility interview before enrolment. The sample-size estimation was based on the anticipated association between vitamin B12 status, plasma homocysteine concentration, and cognitive score reported in previous research, using a 95% confidence level and 80% statistical power. Additional recruitment was planned to compensate for incomplete questionnaires, missing laboratory measurements, and unusable blood specimens. A total of 180 participants with complete information required for the principal analysis were included.

Men and women aged 18–60 years who were residents of Faisalabad, considered themselves generally healthy, could understand the cognitive-assessment instructions, and provided written informed consent were eligible. Individuals with a history of stroke, epilepsy, dementia, significant head injury, diagnosed

psychiatric illness, chronic kidney disease, chronic liver disease, thyroid disease, complicated diabetes mellitus, alcohol misuse, severe acute illness, or other major systemic disease were excluded. Pregnant women and individuals who had used vitamin B12, folic acid, or multivitamin supplements during the preceding three months were also excluded because supplementation could influence vitamin B12 and homocysteine concentrations.

Data were collected through a structured questionnaire administered by face-to-face interview. The questionnaire recorded age, sex, educational attainment, occupation, marital status, smoking status, physical activity, dietary pattern, frequency of animal-source food consumption, medical history, medication use, and recent supplement use. Face-to-face administration was used to improve comprehension and reduce missing responses. Completed forms were reviewed for completeness and internal consistency before data entry.

Anthropometric measurements were obtained using standardized procedures. Body weight was measured in kilograms and standing height in metres, and body mass index was calculated as weight divided by height squared and expressed in $kg/m^2$. Blood pressure was recorded after the participant had rested in a seated position. Measurements and questionnaire responses were obtained using uniform procedures to minimize variation between participants.

Approximately 5 mL of fasting venous blood was collected from each participant under aseptic conditions. Specimens were labelled with unique study codes rather than participant names to preserve confidentiality. Blood samples were processed promptly, and serum or plasma was separated according to the requirements of the respective biochemical tests. Serum vitamin B12 and folate concentrations were determined using routine immunoassay procedures, while plasma total homocysteine was measured using the routine analytical method employed by the laboratory. A complete blood count was performed to determine haemoglobin concentration and mean corpuscular volume because vitamin B12 deficiency may occur with or without overt anaemia and because haemoglobin status may influence cognitive performance.

Vitamin B12 status was classified as deficient, borderline, or normal according to the reference intervals applied by the testing laboratory. Plasma homocysteine was similarly categorized as normal or elevated using the laboratory reference range. Serum vitamin B12 and plasma homocysteine concentrations were also retained as continuous variables for correlation and regression analyses to avoid loss of information associated with categorical classification.

Cognitive performance was assessed individually in a quiet environment with minimal interruption. A trained researcher provided standardized instructions in language that each participant could understand and administered a brief standardized cognitive screening assessment. The assessment evaluated principal domains including memory, attention, orientation, language, calculation, and executive functioning. The total cognitive score was recorded as the primary outcome. Educational attainment was documented because performance on brief cognitive screening assessments may be influenced by formal schooling and was therefore included in the adjusted statistical analysis.

Questionnaire data, anthropometric measurements, laboratory findings, and cognitive scores were checked before entry and analysed using IBM SPSS Statistics version 26.0. Continuous variables were summarized as mean and standard deviation when their distributions were approximately normal and as median and interquartile range when distributional assumptions were not satisfied. Categorical variables were reported as frequencies and percentages. Participant characteristics and biochemical measurements were described for the complete sample, and vitamin B12 deficiency and borderline-status prevalence were calculated using the total number of analysed participants as the denominator.

Mean plasma homocysteine concentrations and cognitive scores were compared across the deficient, borderline, and normal vitamin B12 groups using one-way analysis of variance. Bivariate correlation

analysis was used to evaluate the relationships of serum vitamin B12 and plasma homocysteine with total cognitive score. Multiple linear regression analysis was performed with total cognitive score as the dependent variable and serum vitamin B12 and plasma homocysteine as the principal independent variables. The adjusted model included age, sex, educational attainment, body mass index, haemoglobin concentration, dietary pattern, and smoking status as potential confounding variables. Statistical tests were two-sided, and a p-value of less than 0.05 was considered statistically significant. Written informed consent was obtained before data collection, and participant confidentiality was maintained through coded questionnaires, coded specimens, and restricted use of study information.

## RESULTS

A total of 180 apparently healthy adults were included. Participants ranged in age from 18 to 60 years, with a mean age of 34.8 ± 10.6 years. The sample comprised 98 women (54.4%) and 82 men (45.6%). The mean body mass index was 24.9 ± 4.1 kg/m², and the mean haemoglobin concentration was 12.9 ± 1.3 g/dL. The recalculated overall mean serum vitamin B12 concentration was 301.7 ± 119.9 pg/mL, while the mean plasma homocysteine concentration was 15.5 ± 5.5 µmol/L. The mean total cognitive score was 25.8 ± 2.7.

*Table 1. Demographic, Anthropometric, and Biochemical Characteristics of Participants*

| Variable | Total Participants (n = 180) |
|---|---|
| **Age, years** | 34.8 ± 10.6 |
| **Male sex** | 82 (45.6%) |
| **Female sex** | 98 (54.4%) |
| **Body mass index, kg/m²** | 24.9 ± 4.1 |
| **Haemoglobin, g/dL** | 12.9 ± 1.3 |
| **Serum vitamin B12, pg/mL** | 301.7 ± 119.9 |
| **Serum folate, ng/mL** | 7.8 ± 2.6 |
| **Plasma homocysteine, µmol/L** | 15.5 ± 5.5 |
| **Total cognitive score** | 25.8 ± 2.7 |
| **Deficient vitamin B12 status** | 42 (23.3%) |
| **Borderline vitamin B12 status** | 61 (33.9%) |
| **Normal vitamin B12 status** | 77 (42.8%) |

Values are presented as mean ± standard deviation or n (%).

Forty-two participants had deficient vitamin B12 status, 61 had borderline status, and 77 had normal status. Thus, 103 participants, representing 57.2% of the sample, had either deficient or borderline vitamin B12 concentrations. Plasma homocysteine showed a graded reduction across the vitamin B12 categories, decreasing from 20.8 ± 5.9 µmol/L in the deficient group to 16.4 ± 4.3 µmol/L in the borderline group and 11.9 ± 3.1 µmol/L in the normal group. Conversely, the total cognitive score increased progressively from 23.9 ± 2.8 in the deficient group to 25.4 ± 2.3 in the borderline group and 27.1 ± 2.1 in the normal group.

*Table 2. Biochemical and Cognitive Measures According to Vitamin B12 Status*

| Variable | Deficient (n = 42) | Borderline (n = 61) | Normal (n = 77) | F (2,177) | p-Value | η² |
|---|---|---|---|---|---|---|
| **Serum vitamin B12, pg/mL** | 168.4 ± 31.7 | 251.6 ± 28.9 | 414.2 ± 89.5 | — | — | — |
| **Plasma homocysteine, µmol/L** | 20.8 ± 5.9 | 16.4 ± 4.3 | 11.9 ± 3.1 | 60.33 | <0.001 | 0.405 |
| **Total cognitive score** | 23.9 ± 2.8 | 25.4 ± 2.3 | 27.1 ± 2.1 | 26.48 | <0.001 | 0.230 |

Values are presented as mean ± standard deviation. F statistics were calculated using one-way analysis of variance. η²: eta squared.

The omnibus difference in plasma homocysteine among the three vitamin B12 categories was statistically significant, $F(2,177) = 60.33$, $p < 0.001$, with an eta-squared value of 0.405. Tukey–Kramer comparisons demonstrated that mean homocysteine was 4.40 µmol/L higher in the deficient group than in the borderline group, 8.90 µmol/L higher in the deficient group than in the normal group, and 4.50

µmol/L higher in the borderline group than in the normal group. All three pairwise differences were statistically significant.

Total cognitive scores also differed significantly across vitamin B12 categories, $F(2,177) = 26.48$, $p < 0.001$, $\eta^2 = 0.230$. The borderline group scored a mean of 1.50 points higher than the deficient group. The normal group scored 3.20 points higher than the deficient group and 1.70 points higher than the borderline group. Each pairwise difference remained statistically significant after Tukey–Kramer adjustment.

*Table 3. Pairwise Comparisons of Homocysteine Concentrations and Cognitive Scores Across Vitamin B12 Categories*

| Outcome | Comparison | Mean Difference | 95% CI | Adjusted p-Value |
|---|---|---|---|---|
| **Plasma homocysteine, µmol/L** | Deficient − Borderline | 4.40 | 2.36 to 6.44 | <0.001 |
| **Plasma homocysteine, µmol/L** | Deficient − Normal | 8.90 | 6.95 to 10.85 | <0.001 |
| **Plasma homocysteine, µmol/L** | Borderline − Normal | 4.50 | 2.76 to 6.24 | <0.001 |
| **Total cognitive score** | Borderline − Deficient | 1.50 | 0.39 to 2.61 | 0.005 |
| **Total cognitive score** | Normal − Deficient | 3.20 | 2.14 to 4.26 | <0.001 |
| **Total cognitive score** | Normal − Borderline | 1.70 | 0.75 to 2.65 | <0.001 |

CI: confidence interval. Pairwise comparisons were performed using the Tukey–Kramer procedure.

Overall, the findings demonstrated a consistent biochemical and cognitive gradient across vitamin B12 categories. Participants with deficient vitamin B12 status had the highest plasma homocysteine concentrations and the lowest cognitive scores, while those with normal vitamin B12 status had the lowest homocysteine concentrations and the highest cognitive scores. Participants with borderline vitamin B12 status occupied an intermediate position for both outcomes.

## DISCUSSION

This study demonstrated a graded relationship among vitamin B12 status, plasma homocysteine concentration, and cognitive performance in apparently healthy adults from Faisalabad. More than half of the participants had either deficient or borderline vitamin B12 status. Homocysteine concentrations were progressively lower and cognitive scores progressively higher across the deficient, borderline, and normal vitamin B12 categories. The magnitude of the group differences was substantial for homocysteine and notable for cognitive performance, although the cross-sectional design does not establish the temporal direction or causality of these relationships.

The finding that 57.2% of participants had deficient or borderline vitamin B12 status suggests that suboptimal vitamin B12 status may be frequent in this sample despite the absence of recognized major disease. Pakistani studies have previously documented vitamin B12 and folate deficiencies in women of reproductive age and other population groups (13,14). These studies differed from the present investigation in population composition and outcome assessment, but collectively indicate that micronutrient deficiencies remain relevant in Pakistan. The present findings extend this concern to an urban sample of apparently healthy adults, although convenience sampling prevents the observed proportions from being interpreted as population prevalence estimates.

The progressive elevation of homocysteine across lower vitamin B12 categories is biologically consistent with the role of vitamin B12 in one-carbon metabolism. Vitamin B12 serves as a cofactor for methionine synthase, which facilitates the remethylation of homocysteine to methionine. Inadequate vitamin B12 availability can impair this reaction and contribute to homocysteine accumulation (3,4). Elevated homocysteine may also reflect abnormalities involving folate, vitamin B6, renal function, medication use, and other metabolic factors; therefore, it should not be interpreted as a biomarker specific to vitamin B12 deficiency (4,7). Previous Pakistani studies have similarly demonstrated clinically relevant abnormalities in homocysteine metabolism, although they were conducted in populations with different exposures or established cardiovascular disease (15,16).

Cognitive performance also followed a graded pattern across vitamin B12 categories. Participants with normal vitamin B12 status scored higher than those with borderline or deficient status, while the borderline group performed better than the deficient group but less well than the normal group. This pattern is consistent with observational evidence linking lower vitamin B12 status and elevated homocysteine with poorer cognition, cognitive decline, dementia risk, and adverse brain imaging findings (8–10). Systematic and retrospective evidence, however, has not been entirely consistent, and associations may vary according to age, baseline nutritional status, comorbidity, cognitive instrument, biomarker definition, and study design (11,12). Much of the existing literature involves older adults or individuals with cognitive impairment, whereas the present sample included adults aged 18–60 years who were considered apparently healthy. Direct comparisons should therefore be made cautiously.

The intermediate biochemical and cognitive profile observed in the borderline vitamin B12 group is clinically relevant. Serum vitamin B12 values close to the lower reference limit may not reliably exclude intracellular or functional deficiency. Diagnostic approaches incorporating functional markers such as homocysteine or methylmalonic acid may improve interpretation when serum vitamin B12 concentrations are equivocal (3,5,6). Nevertheless, elevated homocysteine alone cannot confirm vitamin B12 deficiency, and biochemical findings should be interpreted alongside dietary history, haematological indicators, clinical features, medication use, and other potential causes.

Randomized trials in selected populations with mild cognitive impairment have suggested that B-vitamin supplementation can reduce homocysteine and may slow brain atrophy or influence some cognitive outcomes (18–20). These findings provide biological support for further investigation but should not be extrapolated directly to apparently healthy adults. The present study did not administer supplementation, assess treatment response, or measure longitudinal cognitive change. It therefore supports an association between biochemical status and cognitive performance rather than the effectiveness of screening or supplementation.

The findings have potential implications for nutritional and clinical assessment. Low or borderline vitamin B12 concentrations accompanied by elevated homocysteine may warrant closer evaluation, particularly in individuals with dietary risk factors, haematological abnormalities, neurological symptoms, or cognitive complaints. However, the results do not establish a threshold for population screening and cannot determine whether correction of vitamin B12 status would improve cognitive performance. Longitudinal studies incorporating standardized dietary assessment, validated cognitive measures, comprehensive biochemical testing, and repeated follow-up are required to clarify temporal relationships and clinical significance.

The study had several strengths, including the simultaneous evaluation of vitamin B12, folate, homocysteine, haematological variables, and cognitive performance in an apparently healthy adult sample. The inclusion of deficient, borderline, and normal vitamin B12 categories also permitted evaluation of a graded pattern rather than a simple deficient-versus-normal comparison. Important limitations should nevertheless be considered. The cross-sectional design precludes causal or temporal inference, and reverse causation cannot be excluded. Convenience sampling from a single city limits representativeness and generalizability. Dietary and lifestyle information was self-reported and may have been affected by recall or classification error. Residual confounding by socioeconomic circumstances, renal function, medication use, sleep, psychological status, physical activity, or other nutritional factors may also remain. Serum vitamin B12 and homocysteine do not provide complete diagnostic specificity, particularly in borderline cases. Finally, cognitive screening scores are influenced by educational, linguistic, and cultural factors and do not constitute a clinical diagnosis of cognitive impairment.

## CONCLUSION

Deficient and borderline vitamin B12 status were common in this sample of apparently healthy adults from Faisalabad and were associated with progressively higher plasma homocysteine concentrations and

lower cognitive scores. Participants with borderline vitamin B12 status demonstrated intermediate biochemical and cognitive findings between the deficient and normal groups. These results support the combined interpretation of vitamin B12 status, homocysteine, clinical characteristics, and dietary risk factors, while the cross-sectional design prevents conclusions regarding causality, disease progression, or the cognitive effects of vitamin supplementation.

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