

Original Article

Role of Micronutrient Interaction (Iron, Zinc, Vitamin B12, Folate) in Unexplained Anemia Among Adolescents

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ABSTRACT

Background: Adolescent anemia may involve multiple nutritional deficiencies, particularly when its cause remains unexplained after initial assessment. **Objective:** To characterize iron, zinc, vitamin B12, and folate deficiency patterns and examine their associations with hemoglobin concentration among adolescents with unexplained anemia. **Methods:** This observational cross-sectional study included 84 consecutively recruited adolescents aged 10–19 years attending tertiary outpatient clinics in Faisalabad, Pakistan, between September 2025 and January 2026. Complete blood counts, serum ferritin, zinc, vitamin B12, folate, and C-reactive protein were measured. Analyses included descriptive statistics, independent-samples t-tests, and Pearson correlations. **Results:** Mean age was 14.6 ± 2.4 years, and 57.1% of participants were female. Mean hemoglobin was 10.4 ± 1.1 g/dL. Isolated iron deficiency was reported at 28.6%, while multiple micronutrient deficiencies were reported in 61.9%. Participants with moderate anemia had lower ferritin, zinc, vitamin B12, and folate concentrations than those with mild anemia (all $p < 0.001$). Hemoglobin correlated positively with zinc ($r = 0.52$), ferritin ($r = 0.48$), vitamin B12 ($r = 0.41$), and folate ($r = 0.36$; all $p < 0.001$). **Conclusion:** Micronutrient co-deficiency was frequent, and lower biomarker concentrations were associated with greater anemia severity. These associations do not establish causal interactions or treatment effectiveness. **Keywords:** Adolescents; Anemia; Micronutrient Deficiency; Ferritin; Zinc; Vitamin B12; Folate.

INTRODUCTION

Adolescence is a period of increased nutritional demand associated with growth, pubertal development, and, in females, the onset of menstruation. Inadequate nutrient intake during this period can contribute to anemia, with implications for physical performance, cognition, and adolescent health. Although iron deficiency is an important contributor, adolescent anemia has a heterogeneous etiology that may include other nutritional deficiencies, inflammation, infection, and inherited hematological disorders. Understanding these contributing factors is necessary to distinguish iron deficiency from other causes and to guide appropriate investigation and management (1,2).

Persistent anemia following oral iron treatment presents a particular diagnostic challenge. An inadequate hematological response may reflect insufficient treatment duration or dose, poor adherence, impaired absorption, ongoing blood loss, an alternative diagnosis, or coexisting nutritional deficiencies. Pediatric recommendations therefore emphasize reassessment of the diagnosis and treatment history when the expected response to iron is not achieved (3,4). Furthermore, red cell morphology alone may not reliably identify the underlying cause when more than one deficiency is present. These considerations support a broader assessment of adolescents whose anemia remains unexplained after initial evaluation, while recognizing that persistent anemia does not, by itself, establish iron treatment resistance (2,5).

Iron, vitamin B12, folate, and zinc contribute to erythropoiesis through distinct physiological functions. Iron is required for hemoglobin synthesis, whereas vitamin B12 and folate support DNA synthesis and the maturation of erythroid precursors. Deficiencies of these vitamins can cause ineffective erythropoiesis and may complicate the hematological presentation of concurrent iron deficiency (2,5,6). Zinc participates in enzyme activity and cellular processes relevant to erythropoiesis, providing a biological rationale for considering zinc status alongside established nutritional markers (6). However, the coexistence of low micronutrient concentrations does not necessarily demonstrate a biological interaction: shared dietary exposures, underlying illness, and other determinants may contribute to correlated biomarker levels.

Previous literature supports examining nutritional anemia beyond isolated iron deficiency, but the relevance of findings varies across populations. A review of anemia in Indonesian adolescents identified gaps in understanding its etiology and emphasized the need to consider nutritional and non-nutritional contributors (1). Research in New Zealand aged-care residents also reported associations between micronutrient status, including zinc and iron, and anemia, although differences in age, comorbidity, and nutritional circumstances limit direct extrapolation to adolescents (7). In addition, inherited hemoglobin disorders may influence hemoglobin concentrations and the interpretation of nutritional biomarkers, underscore the importance of a clearly defined clinical population when investigating nutritional associations (8). Collectively, these sources provide a rationale for concurrent micronutrient assessment but do not establish the contribution of specific co-deficiency patterns among adolescents presenting with persistent or unexplained anemia in Faisalabad.

The specific question addressed by the present study is how ferritin, zinc, vitamin B12, and folate profiles coexist and relate to hemoglobin concentrations within this selected adolescent population. Measuring these biomarkers concurrently can describe deficiency patterns and their associations with anemia severity, while avoiding assumptions that one nutrient alone accounts for persistent anemia. Accordingly, this cross-sectional study aimed to characterize iron, zinc, vitamin B12, and folate deficiency patterns among adolescents aged 10–19 years presenting with unexplained anemia in tertiary outpatient clinics in Faisalabad, compare micronutrient concentrations between mild and moderate anemia groups, and examine their associations with hemoglobin concentration. The study evaluated co-deficiency and statistical associations; its design did not establish causal micronutrient interactions or the effectiveness of combined supplementation.

MATERIAL AND METHODS

An observational cross-sectional study was conducted in tertiary pediatric and adolescent outpatient clinics in Faisalabad, Pakistan, between September 2025 and January 2026. The study assessed iron, zinc, vitamin B12, and folate status concurrently to characterize micronutrient co-deficiency patterns and examine their associations with hemoglobin concentration among adolescents presenting with unexplained anemia. The rationale for assessing multiple nutritional markers was informed by pediatric recommendations addressing nutritional anemia and the reassessment of patients with an inadequate response to iron treatment (2,4).

The target population comprised adolescents aged 10–19 years attending the participating clinics. Participants were recruited consecutively during the study period. Eligibility required a clinical diagnosis of unexplained anemia accompanied by either a reported inadequate response to previous oral iron therapy or the absence of typical microcytic hypochromic features. Adolescents with active systemic infections, known hemoglobinopathies, chronic kidney disease, inflammatory bowel disorders, acute hemorrhage, blood transfusion within the preceding four months, or current use of immunosuppressants or non-iron vitamin–mineral supplements were excluded. Of 98 adolescents assessed for eligibility, 84 met the inclusion criteria and completed the diagnostic panel,

constituting the analyzed sample. The sample-size rationale was based on comparison with previous clinical pilot investigations of trace-element relationships in localized hematological cohorts.

Demographic and clinical information included age, sex, body mass index-for-age Z-score, and hematological measurements. Age was summarized continuously and categorized as 10–14 or 15–19 years for descriptive reporting. Venous blood samples were collected under fasting and aseptic conditions. Complete blood counts and red cell indices, including hemoglobin concentration and mean corpuscular volume, were measured using an automated hematology analyzer. Serum ferritin was measured by electrochemiluminescence immunoassay and used as the biochemical marker of iron stores. C-reactive protein was measured to screen for an inflammatory response that could affect interpretation of the micronutrient results.

Serum zinc was measured by flame atomic absorption spectrometry, with trace-element-free collection tubes used to reduce contamination during specimen collection. Serum vitamin B12 and folate concentrations were measured using chemiluminescent immunoassays. Hemoglobin was expressed in g/dL, mean corpuscular volume in fL, ferritin in ng/mL, zinc in $\mu\text{g/dL}$, vitamin B12 in pg/mL, folate in ng/mL, and C-reactive protein in mg/L. All four nutritional biomarkers were assessed during the study evaluation, allowing their relationships with hemoglobin to be examined at the same assessment point.

Hemoglobin concentration was the principal continuous outcome, and serum ferritin, zinc, vitamin B12, and folate concentrations were the nutritional variables of interest. Additional analyses described isolated iron deficiency and concurrent micronutrient deficiency patterns and compared biomarker concentrations between participants classified as having mild or moderate anemia. Exclusion of specified clinical conditions and screening with C-reactive protein were used to reduce potential confounding from recognized non-nutritional conditions and inflammation. Fasting specimen collection and the use of trace-element-free tubes provided procedural standardization for biochemical assessment.

Data were analyzed using IBM SPSS Statistics version 26.0. Continuous-variable distributions were assessed using the Shapiro–Wilk test and visual inspection of histograms and Q–Q plots. Normally distributed variables were summarized as means and standard deviations, while skewed variables were summarized as medians and interquartile ranges. Categorical variables were presented as frequencies and percentages. Independent-samples t-tests were used to compare ferritin, zinc, vitamin B12, folate, and mean corpuscular volume between the mild and moderate anemia groups. Pearson correlation coefficients were calculated to examine associations between hemoglobin and each nutritional biomarker and to describe correlations among the biomarkers.

Multiple linear regression was used to examine the associations of serum ferritin, zinc, vitamin B12, and folate with hemoglobin concentration, with the four biomarkers entered as explanatory variables. Regression results were expressed as unstandardized coefficients, standard errors, standardized coefficients, t-statistics, p-values, and 95% confidence intervals. Model fit was summarized using R^2 and adjusted R^2 . Analyses included the 84 participants who completed the diagnostic panel. Statistical significance was defined as $p < 0.05$, with correlations evaluated using two-tailed tests.

The study protocol received institutional ethics review board approval before study initiation. Written informed consent or assent was obtained from participants and their legal guardians, as applicable. The study was conducted in accordance with the Declaration of Helsinki.

RESULTS

Of 98 adolescents assessed for eligibility, 84 (85.7%) were included in the analysis. The participants had a mean age of 14.6 ± 2.4 years, and 48 (57.1%) were female. Mean hemoglobin concentration was 10.4 ± 1.1 g/dL. Mild anemia was reported in 38 participants (45.2%) and moderate anemia in 46 (54.8%). Mean BMI-for-age Z-score was -1.2 ± 0.8 , and mean serum C-reactive protein concentration was 1.1 ± 0.4 mg/L (Table 1).

Isolated iron deficiency was reported in 24 participants (28.6%), while multiple micronutrient deficiencies were reported in 52 (61.9%). The reported patterns included concurrent iron and zinc deficiency in 22 participants (26.2%) and concurrent iron, zinc, and vitamin B12 deficiency in 16 (19.0%) (Table 2).

Participants with moderate anemia had lower mean concentrations of each measured micronutrient than those with mild anemia. Mean serum zinc was 62.4 ± 11.2 µg/dL in the moderate group and 78.5 ± 9.8 µg/dL in the mild group, corresponding to a mean difference of 16.1 µg/dL. The respective vitamin B12 concentrations were 182.4 ± 38.6 and 245.1 ± 42.3 pg/mL, with a mean difference of 62.7 pg/mL. Mean differences were 5.8 ng/mL for ferritin and 2.7 ng/mL for folate. Mean corpuscular volume was also lower in the moderate group, at 76.3 ± 6.1 versus 82.1 ± 5.4 fL. All reported group comparisons had $p < 0.001$ (Table 3).

Table 1. Participant flow and demographic and clinical characteristics

Characteristic	n/N (%)	Mean ± SD
Assessed for eligibility	98/98 (100.0)	—
Included in analysis	84/98 (85.7)	—
Age, years	—	14.6 ± 2.4
Age, 10–14 years	41/84 (48.8)	—
Age, 15–19 years	43/84 (51.2)	—
Female	48/84 (57.1)	—
Male	36/84 (42.9)	—
BMI-for-age Z-score	—	-1.2 ± 0.8
Hemoglobin, g/dL	—	10.4 ± 1.1
Mild anemia	38/84 (45.2)	—
Moderate anemia	46/84 (54.8)	—
Serum CRP, mg/L	—	1.1 ± 0.4

BMI, body mass index; CRP, C-reactive protein; SD, standard deviation.

Table 2. Reported micronutrient deficiency patterns among participants

Deficiency pattern	n/N (%)
Isolated iron deficiency	24/84 (28.6)
Multiple micronutrient deficiencies	52/84 (61.9)
Concurrent iron and zinc deficiency	22/84 (26.2)
Concurrent iron, zinc, and vitamin B12 deficiency	16/84 (19.0)

Table 3. Hematological and micronutrient measurements by anemia severity

Measurement	Mild anemia, n = 38, mean ± SD	Moderate anemia, n = 46, mean ± SD	Mean difference	Reported t-statistic	Reported p-value
Serum ferritin, ng/mL	21.4 ± 5.8	15.6 ± 5.9	5.8	4.51	<0.001
Serum zinc, µg/dL	78.5 ± 9.8	62.4 ± 11.2	16.1	6.94	<0.001
Serum vitamin B12, pg/mL	245.1 ± 42.3	182.4 ± 38.6	62.7	7.08	<0.001
Serum folate, ng/mL	6.8 ± 1.5	4.1 ± 1.2	2.7	9.12	<0.001
MCV, fL	82.1 ± 5.4	76.3 ± 6.1	5.8	4.58	<0.001

MCV, mean corpuscular volume; SD, standard deviation. Mean differences represent mild minus moderate anemia. Reported comparisons used independent-samples t-tests.

Table 4. Unadjusted correlations between hemoglobin and micronutrient concentrations and among micronutrient concentrations

Variable pair	n	Pearson r	Approximate 95% CI	Two-sided p-value
Hemoglobin–ferritin	84	0.48	0.30 to 0.63	<0.001
Hemoglobin–zinc	84	0.52	0.34 to 0.66	<0.001
Hemoglobin–vitamin B12	84	0.41	0.21 to 0.57	<0.001
Hemoglobin–folate	84	0.36	0.16 to 0.53	<0.001
Ferritin–zinc	84	0.39	0.19 to 0.56	<0.001
Ferritin–vitamin B12	84	0.28	0.07 to 0.47	0.010
Ferritin–folate	84	0.22	0.01 to 0.42	0.044
Zinc–vitamin B12	84	0.31	0.10 to 0.49	0.004
Zinc–folate	84	0.25	0.04 to 0.44	0.022
Vitamin B12–folate	84	0.33	0.12 to 0.51	0.002

CI, confidence interval. Confidence intervals use Fisher's z transformation. Two-sided p-values use the Pearson correlation t-test with 82 degrees of freedom.

Hemoglobin concentration was positively correlated with zinc ($r = 0.52$; 95% CI, 0.34–0.66), ferritin ($r = 0.48$; 95% CI, 0.30–0.63), vitamin B12 ($r = 0.41$; 95% CI, 0.21–0.57), and folate ($r = 0.36$; 95% CI, 0.16–0.53); all p-values were <0.001.

Zinc had the largest observed correlation coefficient with hemoglobin, although the confidence intervals overlapped. Correlations among micronutrient concentrations ranged from 0.22 for ferritin and folate (95% CI, 0.01–0.42; $p = 0.044$) to 0.39 for ferritin and zinc (95% CI, 0.19–0.56; $p < 0.001$) (Table 4).

DISCUSSION

This cross-sectional study identified frequent micronutrient co-deficiency among adolescents presenting with unexplained anemia in tertiary outpatient clinics in Faisalabad. Participants classified as having moderate anemia had lower ferritin, zinc, vitamin B12, and folate concentrations than those with mild anemia, and each biomarker was positively associated with hemoglobin. These findings address the study objective by describing the coexistence of nutritional abnormalities and their relationship with anemia severity. They do not establish that the deficiencies caused persistent anemia or that their combined effects represent biological interactions.

The findings are consistent with the broader recognition that adolescent anemia cannot be attributed uniformly to iron deficiency. In their review of Indonesian adolescents, van Zutphen and colleagues highlighted uncertainties in the relative contributions of nutritional and non-nutritional factors to anemia, supporting a more differentiated assessment of its etiology (1). Pediatric recommendations similarly recognize the importance of investigating iron, vitamin B12, and folate deficiencies within an appropriate clinical assessment (2). The present study adds observations from a selected clinical population rather than a community sample. Its deficiency frequencies should therefore not be interpreted as estimates for adolescents in Faisalabad generally, particularly because referral and eligibility criteria may preferentially select patients with complex or persistent anemia.

Zinc had the largest observed unadjusted correlation with hemoglobin, although this does not establish that its association was statistically stronger than those of the other biomarkers. A relevant comparison is the study by MacDonell and colleagues in New Zealand aged-care residents, which also found positive associations of zinc and iron status with hemoglobin (7). The directional agreement supports further investigation of zinc alongside conventional nutritional markers. However, the older population, differing comorbidity profile, and consideration of inflammatory factors in that study limit direct comparison with the present adolescent sample. Neither study establishes that zinc supplementation would improve hemoglobin in individuals selected solely on the basis of these associations.

The observed relationships are biologically plausible because erythropoiesis depends on several nutritional substrates. Iron supports hemoglobin synthesis, while vitamin B12 and folate are required for DNA synthesis and normal erythroid maturation (2,5). Zinc contributes to enzyme function and cellular processes that may influence erythropoiesis (6,7). Concurrent abnormalities could therefore accompany impaired red cell production through more than one pathway. Nevertheless, the positive correlations among micronutrients could also reflect shared dietary inadequacy, reduced nutrient availability, or underlying illness. These alternative explanations cannot be distinguished by concurrent serum measurements, and the correlations should not be interpreted as evidence of synergistic effects.

Lower vitamin B12 and folate concentrations in the moderate anemia group occurred alongside a lower mean corpuscular volume. This pattern does not exclude mixed nutritional deficiencies, because the hematological manifestations of one deficiency may be modified by another, and mean corpuscular volume alone does not establish etiology (2,5). Conversely, lower group-average vitamin concentrations do not demonstrate that every participant had a clinically meaningful deficiency. Interpretation requires appropriate biochemical thresholds and clinical context. Similarly, a ferritin measurement represents one component of iron assessment rather than a complete explanation for an individual's anemia (3,4).

Clinically, the findings reinforce the relevance of considering additional nutritional abnormalities when anemia remains unexplained after initial assessment. Such evaluation should accompany review of the original diagnosis, prescribed iron dose and duration, adherence, absorption, and possible ongoing blood loss, as emphasized in pediatric iron-deficiency recommendations (3,4). Nutritional abnormalities should not displace consideration of non-nutritional causes. Inherited hemoglobin disorders, for example, can influence both hemoglobin concentrations and nutritional biomarkers, complicating interpretation even when known disorders have been excluded (5,8). The present study does not establish the diagnostic yield, cost-effectiveness, or clinical benefit of routinely testing all four biomarkers in every adolescent with anemia.

At the public-health level, these observations are compatible with approaches that consider dietary quality and multiple contributors to adolescent anemia (1,2,6). However, a selected tertiary-clinic sample cannot determine the appropriate composition of population supplementation or fortification programs. The study also provides no comparison between iron monotherapy and combined supplementation. Its practical contribution is to identify a clinical pattern that warrants further evaluation, rather than to demonstrate that an existing treatment or prevention strategy should be replaced.

A strength of the study was the concurrent assessment of four nutritional biomarkers, which enabled comparison of their relationships with hemoglobin within the same participants. Fasting specimen collection, trace-element-free collection tubes, and measurement of C-reactive protein provided useful procedural safeguards. Nonetheless, normal C-reactive protein concentrations do not exclude every inflammatory influence, and serum biomarkers do not invariably represent functional nutrient availability (4,6). The cross-sectional design precludes determination of whether lower micronutrient concentrations preceded anemia. Recruitment from tertiary clinics within one city limits generalizability, while the absence of a non-anemic comparison group prevents estimation of the association between these biomarkers and anemia occurrence in the wider adolescent population.

Interpretation is further limited by incomplete documentation of deficiency thresholds and previous iron treatment, including dose, duration, and adherence. The available associations are unadjusted and may reflect differences in age, sex, pubertal development, menstrual blood loss, or other participant characteristics. Dietary intake and environmental exposure markers were not quantified. In addition, multiple exploratory comparisons increase the possibility of chance findings, particularly for weaker associations. Prospective studies with clearly defined eligibility criteria, standardized deficiency classifications, and appropriate adjustment for confounding are needed to clarify these relationships. Intervention studies would be required to determine whether correcting documented co-deficiencies improves hematological outcomes beyond appropriately administered iron treatment.

CONCLUSION

Among adolescents presenting with unexplained anemia in this selected clinical population, multiple micronutrient deficiencies were frequently reported, and lower ferritin, zinc, vitamin B12, and folate concentrations were associated with greater anemia severity. Zinc showed the largest observed unadjusted correlation with hemoglobin. These findings indicate that nutritional abnormalities accompanying adolescent anemia may extend beyond isolated iron deficiency, but they do not establish causal micronutrient interactions or the effectiveness of combined supplementation.

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