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Revisiting the Role of Vitamin B12 in Anemia: Insights beyond Macrocytosis

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Abstract

Background: Anemia is a major global health concern, affecting approximately 22–25% of the world's population and a national prevalence of 53 – 55%, varying by age, sex, and geographic location. While iron deficiency remains the predominant cause, deficiencies of vitamin B12 (cobalamin) and folic acid are important contributors. Conventionally, vitamin B12 deficiency is associated with megaloblastic anemia characterized by macrocytosis. However, recent evidence suggests that vitamin B12 insufficiency may also exist in patients with microcytic or normocytic anemia, raising the need to reassess its diagnostic relevance beyond traditional morphological features.

Aims & Objectives: This study aimed to evaluate serum vitamin B12 levels in anemic patients, determine the prevalence of vitamin B12 deficiency in anemia without macrocytosis, compare vitamin B12 levels across anemia severity, and assess the association between vitamin B12 status and peripheral smear morphology.

Methods: A cross-sectional study was conducted on 154 anemic subjects (Hb <12 g/dL), aged 18–80 years. Patients on vitamin B12 supplementation or Metformin were excluded. Complete blood counts were performed using a 5-part hematology analyzer, and peripheral smears were reviewed by a pathologist. Participants were categorized into mild, moderate, and severe anemia based on WHO criteria. Fasting venous samples were analyzed for serum vitamin B12 and Ferritin levels using Chemiluminescent Immunoassay (CLIA) on Maglumi X2, with stringent quality control measures. Statistical analysis was performed using SPSS version 29.

Results: Serum vitamin B12 levels declined significantly with increasing severity of anemia, with median levels of 612 pg/mL, 318 pg/mL, and 148 pg/mL in mild, moderate, and severe anemia, respectively ($p < 0.05$). Vitamin B12 deficiency was more common among patients with moderate and severe anemia. A significant association was observed between peripheral smear findings and vitamin B12 status ($\chi^2 = 21.69$, $p < 0.001$). Among patients with microcytic hypochromic anemia, 43.2% exhibited suboptimal vitamin B12 status, including 13.5% with deficiency and 29.7% with borderline deficiency, despite the absence of macrocytosis.

Conclusion: Our findings highlight the importance of evaluating vitamin B12 levels in all anemic patients, irrespective of peripheral smear morphology, as deficiency may be present even in the absence of classical macrocytic features. Incorporating vitamin B12 estimation into routine anemia workup could facilitate earlier detection and appropriate management, thereby improving patient outcomes.

Keywords: Vitamin B12 deficiency, Anemia severity, Macrocytosis, Peripheral smear morphology.

Introduction

Anemia is a major global public health problem, affecting approximately 22–25% of the world's population**,** with a disproportionately higher burden in low- and middle-income countries. In India, the prevalence of anemia remains alarmingly high, with national surveys reporting rates of approximately 53–

55%**,** particularly among women of reproductive age, children, and the elderly [1,2]. Anemia is associated with significant morbidity, reduced work capacity, impaired cognitive performance, and increased maternal and perinatal mortality.

Iron deficiency remains the most common cause of anemia worldwide; however, deficienciesofvitamin B12 (cobalamin) and folic acid are increasinglyrecognized as important and often underdiagnosed contributors [3,4,6]. Vitamin B12 plays a critical role in DNA synthesis, erythroid maturation, and neurological function. Deficiency of vitamin B12 classically results in megaloblastic anemia, characterized by macrocytosis, hyper-segmented neutrophils, and ineffective erythropoiesis [3,4].

Traditionally, the presence of macrocytosis has been considered a key diagnostic indicator prompting evaluation for vitamin B12 deficiency. However, accumulating evidence suggests that this classical presentation represents only a subset of cases. Several studies have demonstrated that vitamin B12 deficiency may also present with normocytic or microcytic anemia, particularly in individuals with concurrent iron deficiency, anemia of chronic disease, chronic inflammation, or mixed nutritional deficiencies, wherein macrocytosis may be masked [5,7,10].

Indian studies have highlighted a high prevalence of subclinical and overt vitamin B12 deficiency, attributed largely to dietarypatterns, malabsorption, and socioeconomic factors [7,8,10]. Furthermore, the hematological manifestations of vitamin B12 deficiency may vary with the severity and duration of anemia, as well as the presence of coexisting nutritional deficiencies. Peripheral blood smear findings may range from classical megaloblastic features to nonspecific or mixed morphological patterns, thereby limiting the reliability of morphology alone for diagnosis [4,5,9].

Given the high burden of anemia and nutritional deficiencies in the Indian population, there is a compelling need to reassess the role of vitamin B12 in anemia beyond traditional morphological criteria. Systematic evaluation of vitamin B12 levels in anemic patients irrespective of red cell indices, and correlation with anemia severity and peripheral smear findings, may improve early diagnosis and prevent hematological and neurological complications.

To the best of our knowledge, only a few studies have evaluated vitamin B12 deficiency in anemia beyond classical macrocytic presentations, with existing reports showing contradictoryresults. No study has comprehensively examined the association between Vitamin B12 status, anemia severity, and peripheral smear findings irrespective of red cell indices. Therefore, this study focuses on evaluating the role of Vitamin B12 in anemia, with particular emphasis on non-macrocytic cases.

Aims & Objectives:

1. To evaluate vitamin B12 levels in patients with anemia.
2. To determine the prevalence of vitamin B12 deficiency in anemia without macrocytosis.
3. To compare vitamin B12 levels across varying severities of anemia.
4. To check the association between Vitamin B12 status and peripheral smear findings

Materials And Methods

Study Design, Setting and Duration:

This hospital-based cross-sectional observational study was conducted over a period of eight months in the Departments of Pathology, General Medicine, and Biochemistry at our institution, a tertiary care teaching hospital.

Study Population and Sampling

The study included patients diagnosed with anemia who attended the outpatient and inpatient servicesduring thestudyperiod. Atotalof154 participantswereenrolled. Thesample size was calculated based on a previous study by Asif M et al.[16], which reported an expected proportion ofvitaminB12 deficiencyof0.174. Thecalculation was performed with an absolute precision of 6% and a 5% level of significance using the formula:

$n > Z^2_{1-\alpha/2}\, p(1-p)/d^2$, where p represents the expected proportion, d denotes absolute precision and Z is the standard normal variate corresponding to the desired confidence level. Participants were selected using a convenient sampling technique.

Participant Classification

Patients aged above 18 years of either sex diagnosed with anemia were classified according to World

Volume 9, Issue 3; May-June 2026; Page No 605-613

Health Organization (WHO) criteria[6] based on hemoglobin levels into three groups:

Group I: Mild anemia (10.0–11.9 g/dL),
Group II: Moderate anemia (7.0–9.9 g/dL),
Group III: Severe anemia (<7.0 g/dL).

Inclusion Criteria

Individuals aged above 18 years of both sexes diagnosed with anemia and categorized as mild, moderate, or severe based on hemoglobin concentration were included in the study.

Exclusion Criteria

Participants were excluded if they had current or prior vitamin B12 supplementation, were using metformin, had received recent blood transfusions, were pregnant or lactating, had known hematological malignancies or bone marrow disorders, chronic systemic illnesses such as chronic kidney disease, chronic liver disease, or active infection, or were undergoing treatment for anemia.

Data Collection and Laboratory Investigations

Participants were identified based on peripheral blood smear findings suggestive of anemia. Complete Blood Count (CBC) reports and peripheral blood smear findings were obtained from the Department of Pathology. Demographic characteristics and detailed clinical history were recorded using a structured data collection form.

Venous blood samples were collected under standard aseptic precautions. Complete blood counts wereanalyzed using a five-part automated hematologyanalyzer. Peripheral blood smears were prepared, stained, and independently examined by a qualified pathologist for morphologicalassessment and classification ofanemia. SerumVitamin B12 and Ferritin levels were estimated using Chemiluminescent Immunoassay (CLIA) on the Maglumi X2 analyzer following the manufacturer's instructions and established internal quality control procedures. Based on the clinical presentation and laboratory findings, participants were advised appropriate treatment for anemia as per institutional treatment protocols and standard clinical guidelines. The treatment advice was provided independently of study participation and did not influence data collection or analysis.

Ethical Considerations

The study was conducted in accordance with ethical principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the Institutional Ethics Committee prior to commencement of the study. Written informed consent was obtained from all participants before enrollment.

Statistical Analysis

Data were analyzed using SPSS software version 29. Descriptive statistics were used to summarize demographic and clinical characteristics. Continuous variables were expressed as mean ± standard deviation (SD), while categorical variables were presented as frequencies and percentages. Comparative analyses (Kruskal–Wallis test) were performed to assess differences in vitamin B12 levels across anemia severity groups. Associations between vitamin B12 deficiency and peripheral smear morphologywere evaluated using appropriate statistical tests. A p-value of less than 0.05 was considered statistically significant.

Results

A total of 154 anemia patients participated in this study, females were more commonly affected than males, comprising 66.2% (n=102) of the study population, whereas males constituted 33.7% (n=52). Female predominance was consistently observed across all categories of anemia severity. In the mild anemia group, females represented 68.9% of cases, while males accounted for 31.1%. Among patients with moderate anemia, 63.2% were females and 36.8% weremales. Similarly, severe anemia was more frequently observed among females (69.7%) compared to males (30.3%) **(Table 1).**

The demographic and hematological characteristics of patients with mild, moderate, and severe anemia are presented as median with interquartile range (IQR). Intergroup comparisons were performed using the Kruskal–Wallis test followed by pairwise comparison analysis. A p-value <0.05 was considered statistically significant.

The median age increased progressively with anemia severity, from 34 years (IQR: 26, 43) in mild anemia to 47 years (IQR: 36,59) in severe anemia. Hemoglobin levels significantly declined across the groups, with median values of 11.2 g/dL, 7.6 g/dL, and

Volume 9, Issue 3; May-June 2026; Page No 605-613

4.9 g/dL in mild, moderate, and severe anemia respectively ($p < 0.05$).

RBC count, hematocrit, MCV, MCH, MCHC, reticulocyte count, platelet count, serum ferritin, and serum vitamin B12 levelsprogressivelydecreased with increasing anemiaseverity, indicating worsening microcytic hypochromic changes and depletion of iron stores. Serum ferritin levels declined from 26 ng/mL in mild anemia to 11 ng/mL in severe anemia, while serumvitamin B12 levelsdecreased from612 pg/mLto 148 pg/mLacrossthegroups($p < 0.05$). In contrast, WBC count showed a mild increase with worsening anemia severity. Pairwise comparison analysis demonstrated statistically significant differences between mild and moderate anemia, moderate and severe anemia, and mild and severe anemia for most hematological and biochemical parameters **(Table 2)**.

Vitamin B12 deficiency was more frequently observed among patients with dimorphic anemia, with a prevalence of 75%, compared to 43.2% among patients with microcytic hypochromic anemia. The presence of vitamin B12 deficiency even in patients without classical macrocytic morphology highlights the importance of evaluating vitamin B12 levels in all anemic patients irrespective of peripheral smear findings, to facilitate earlydiagnosis and appropriate management

Among patients with normal vitamin B12 levels, mild anemia was the most common presentation, observed in 48.4% of cases, while only 12.9% had severe anemia. In contrast, patients with borderline vitamin B12 deficiency predominantly presented with moderate anemia (57.4%), with mild and severe anemia accounting for 21.3% each.

Similarly, among patients with vitamin B12 deficiency, moderate anemia was the most common presentation (55.6%), followed by severe anemia (33.3%), whereas only 11.1% had mild anemia. These findings demonstrate an increasing proportion of moderate and severe anemia with declining vitamin B12 levels, suggesting an association between worsening anemia severity and vitamin B12 deficiency **(Table 3 & Fig.2)**.

Vitamin B12 deficiency was more frequently observed among patients with dimorphic anemia, with43.7% showing deficiencyand 31.3% showing borderline deficiency. In contrast, 56.8% of patients with microcytic hypochromic anemia had normal vitamin B12 levels, while 43.2% had suboptimal vitamin B12 status **(Fig.1)**, including 29.7% with borderline deficiency and 13.5% with deficiency, despite the absence of macrocytosis. A statistically significant association was observed between peripheral smear findings and vitamin B12 status ($\chi^2 = 21.69$, $p < 0.001$), with vitamin B12 deficiency being significantly more common in dimorphic anemia than in microcytic hypochromic anemia **(Table 4)**.

Discussion

The present hospital-based cross-sectional study evaluated serum Vitamin B12 levels among 154 adult patients withanemia and examined their association with anemia severityand peripheral smear findings. Female patients constituted the majority of the study population (66.2%), with female predominance observed across allgrades ofanemia. Similar observations have been reported in studies from developing countries, where nutritional deficiencies, menstrual blood loss, and increased physiological demands contribute to a higher burden of anemia among women [1,2]. The study demonstrated a progressive decline in serum Vitamin B12 levels with increasing severity of anemia and identified a significant association between Vitamin B12 deficiency and peripheral smear morphology. Importantly, Vitamin B12 deficiency was observed not only among patients with dimorphic anemia but also among those with microcytic hypochromic anemia without classical macrocytosis, highlighting the potential for underdiagnosis when evaluation is based solely on hematological indices or peripheral smear findings.

The present study was to evaluate serum Vitamin B12 levels among patients with anemia and compare Vitamin B12 status across varying severities of anemia. A significant decline in median serumVitamin B12 levels was observed with increasing severityofanemia, decreasing from 612 pg/mL in patients with mild anemia to 318 pg/mL in moderate anemia and 148 pg/mL in severe anemia ($p < 0.05$). This progressive reduction suggests that worsening anemia is associated with declining Vitamin B12 status and may reflect increasing impairment of erythropoiesis.

In addition to lower Vitamin B12 levels, patients with severe anemia demonstrated marked deterioration in

Volume 9, Issue 3; May-June 2026; Page No 605-613

several haematological parameters, including hemoglobin concentration, RBC count, hematocrit, MCV, MCH, and MCHC. Serum ferritin levels also declined significantly across the severity groups, indicating that iron deficiency may coexist with Vitamin B12 deficiency and contribute to the severityofanemia. These findings highlight the multifactorial nature of nutritional anemia and suggest that combined micronutrient deficiencies may play an important role in disease progression.

Further evidence of the relationship between Vitamin B12 status and anemia severity was provided by the distribution of anemia grades according to Vitamin B12 status. Patients with normal Vitamin B12 levels were predominantly represented in the mild anemia group, whereas moderate and severe anemia were more frequently observed among patients with Vitamin B12 deficiency. Notably, the proportion of severe anemia increased from 12.9% among patients with normal Vitamin B12 levels to 33.3% among those with Vitamin B12 deficiency. These findings indicate that lower Vitamin B12 levels are associated with a greater likelihood of severe anemia.

Similar observations have been reported in previous studies. O'Leary and Samman et al. described Vitamin B12 as an essential cofactor in DNA synthesis and erythropoiesis, with deficiency leading to ineffective red cell production and impaired maturation of erythroid precursors[4]. Likewise, Stabler et al. reported that progressive Vitamin B12 depletion adversely affects hematopoietic function and may contribute to worsening anemia when left untreated [15]. The findings of the present study are consistent with these observations and further support the association between declining Vitamin B12 levels and increasing severity of anemia.

Another important objective of the present study was to determine the prevalence of Vitamin B12 deficiency among anemic patients without macrocytosis and to assess its association with peripheral smear findings. A significant association was observed between peripheral smear morphology and Vitamin B12 status, with patients having dimorphic anemia demonstrating a substantially higher prevalence of suboptimal Vitamin B12 status (75%) compared with those having microcytic hypochromic anemia (43.2%) ($\chi^2 = 21.69$, $p < 0.001$). The high prevalence of Vitamin B12 deficiency in dimorphic anemia is expected and is consistent with the established association between dimorphic blood pictures and combined nutritional deficiencies, particularly iron and Vitamin B12 deficiency [9]. However, the most clinically significant finding of the present study was that Vitamin B12 deficiency was not confined to patients with dimorphic anemia or classical macrocytic morphology. Among patients with microcytic hypochromic anemia, 13.5% had Vitamin B12 deficiency and 29.7% had borderline deficiency, indicating that 43.2% had suboptimal Vitamin B12 status despite the absence of macrocytosis.

These findings are supported by previous studies demonstrating that coexisting iron deficiency may mask the macrocytosis typically associated with Vitamin B12 deficiency. Asif et al. reported that Vitamin B12 deficiency may remain undetected in patients with microcytic anemia because concurrent iron deficiency alters red cell indices[16]. Similarly, Lindenbaum et al. showed that a substantial proportion of patients with biochemical Vitamin B12 deficiency may not exhibit macrocytosis, therebylimiting the diagnostic utility of red cell indices alone[12]. Coexisting iron deficiency may normalize or reduce MCV values despite underlying Vitamin B12 deficiency[7,11,12]. Consequently, the characteristic macrocytosis of megaloblastic anemia may not be apparent, resulting in normocyticor microcytic presentations. This masking effect may contribute to delayed diagnosis and treatment of Vitamin B12 deficiency, particularly in populations where iron deficiency anemia is highly prevalent. The declining ferritin levels observed across the anemia severity groups in the present study further support the possibility of concurrent iron deficiency influencing the hematological manifestations of

Vitamin B12 deficiency.While the high prevalence of Vitamin B12 deficiency in dimorphic anemia reinforces existing knowledge, the finding that nearly half of the patients without macrocytosis also had suboptimal Vitamin B12 status carries greater clinical significance. This observation suggests that reliance solely on peripheral smear morphology or MCV values may lead to underdiagnosis of Vitamin B12 deficiency [7,11,12,13].

Clinical Implications

The findings of this study reinforce several important clinical considerations. Routine screening for Vitamin

Volume 9, Issue 3; May-June 2026; Page No 605-613

B12 should be performed in patients with anemia, particularly in moderate and severe cases. Borderline Vitamin B12 levels should not be ignored, as they may represent early deficiency. Moreover, Vitamin B12 deficiency may occur even in the absence of macrocytosis, limiting the utility of peripheral smear findings and red cell indices alone and suggesting that testing should not be restricted to macrocytic anemia.Early detection and appropriate treatment of Vitamin B12 deficiency may help prevent hematological complications and irreversible neurological sequelae. These observations support the importance of early identification and management of Vitamin B12 deficiency in patients with anemia.

Strengths of the Study

A major strength of the present study is the simultaneous evaluation of Vitamin B12 status across different grades of anemia severity and peripheral smear patterns in a well-defined cohort of adult anemic patients. The study also specifically addressed the prevalence of Vitamin B12 deficiency among patients without macrocytosis, a clinically important yet often underlooked subgroup. Furthermore, the use of chemiluminescent immunoassay for Vitamin B12 estimation and standardized hematological assessment enhanced the reliability of the findings.

Limitations And Future Directions:

The present study has certain limitations. Being a single-center, hospital-based cross-sectional study, the findings may not be generalizable to the wider population. Furthermore, the cross-sectional design precludes assessment of causal relationships between Vitamin B12 status and anemia severity. The relatively modest sample size may also limit external validity. Functional biomarkers suchas methylmalonic acid and homocysteine were not assessed, which could have improved the diagnostic accuracy of Vitamin B12 deficiency. In addition, dietary assessment and long-term clinical follow-up were not performed. Future studies with larger sample sizes, interventional prospective study, and comprehensive biochemical profiling are warranted to further validate these finding.

Conclusion

The present study demonstrated a significant decline in serum Vitamin B12 levels with increasing severity of anemia. Vitamin B12 deficiency was more common in moderate and severe anemia and was significantly associated with dimorphic anemia. Notably, Vitamin B12 deficiency was also observed in patients with microcytic hypochromic anemia without macrocytosis, highlighting the limitations of relying solely on red cell indices and peripheral smear findings for diagnosis. These findings support consideration of routine Vitamin B12 assessment in anemic patients, particularly those with moderate to severe anemia, to enable early detection and appropriate therapeutic intervention.

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Conflict of Interest: The authors have no competing interests to declare that are relevant to the content of this article.

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Volume 9, Issue 3; May-June 2026; Page No 605-613

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Table 1: Gender-wise Distribution among Severity of Anemia (n=154)

	Mild Anemia	Moderate Anemia	Severe Anemia	Total
Female	31 (68.9%)	48 (63.2%)	23 (69.7%)	102 (66.2%)
Male	14 (31.1%)	28 (36.8%)	10 (30.3%)	52 (33.7%)
Total	45(100%)	76(100%)	33(100%)	154 (100%)

Table 2: Demographic Data Distribution in each group and Comparison between the groups (n=154)

Parameters	G I- Mild Anemia (n=45) Median (IQR)	G II- Moderate Anemia (n=76) Median (IQR)	G-III Severe Anemia (n=33) Median (IQR)
Age	34 (26,43)	40 (30, 52)	47 (36,59)
Hb(g/dL)	11.2(10.9,11.9)	7.6(7,9.9)*	4.9(3.2, 6.6) #!
RBC 10^6/µL	4.98(4.39,5.25)	3.95(2.5,5)*	2.15(2.07, 4.09) #!
HCT (%)	35.9(34.4,37.4)	24.7(22.8,32.1)*	15.3(12.6, 24.1) #!
MCV(fL)	72(71.3,78.3)	66.4(56.8,90)*	58.9(58.7, 73.8) #!
MCH(pg)	22.6(21.9,25.5)	19.6(16.3,27.7)*	16.1(14.9, 23.9) #!
MCHC(g/dL)	31.7(30.4,32.5)	29.9(28.5,32.5)*	27.4(25.4, 32) #!
Reticulocyte count (%)	1.2 (0.8,1.8)	1.0 (0.6,1.5)*	0.8 (0.5, 1.2) #!
WBC (×10³/µL)	6.8 (5.5,8.2)	7.4 (5.8,9.1)*	8.1 (6.2, 10.5)#!
Platelet count (×10³/µL)	320 (250,410)	290 (220,380)*	260 (180, 340) #!
S.Ferritin (ng/mL)	26 (18,42)	16 (9,28)*	11 (5, 22) #!
S. Vitamin B12 (pg/mL)	612 (520,690)	318 (205,468)*	148 (96, 228) #!

p- Value <0.05 is statistically significant (Kruskal Wallis test).*significance when comparing G-I & G-II . #significance when comparing G-II & G-III . ! significance when comparing G-I & G-III.

Volume 9, Issue 3; May-June 2026; Page No 605-613

Table 3: Vitamin B12 Status among Study Participants (n = 154) based on Severity of Anemia

	G I Mild Anemia n (%)	G II Moderate Anemia n (%)	G III Severe Anemia n (%)	Total n (%)
Normal B12 levels (>300 pg/mL)	30 (48.4%)	24 (38.7%)	8 (12.9%)	62(100%)
Borderline deficiency (200-300 pg/mL)	10 (21.3%)	27 (57.4%)	10(21.3%)	47(100%)
B12 Deficiency(<200 pg/mL)	5 (11.1%)	25 (55.6%)	15 (33.3%)	45(100%)
Total	45(29.2%)	76 (49.4%)	33(21.4%)	154(100%)

Table 4: Association between Peripheral Smear Findings and Vitamin B12 Status (n = 154)

Peripheral smear findings	Normal B12 levels n (%)	Borderline deficiency n (%)	B12 Deficiency n (%)	Total	χ^2 value	p-value
Dimorphic Anemia	20(25.0%)	25(31.3%)	35(43.7%)	80		
Microcytic Hypochromic Anemia	42(56.8%)	22(29.7%)	10(13.5%)	74		
Total	62(40.3%)	47(30.5%)	45(29.2%)	154	21.69	<0.001*

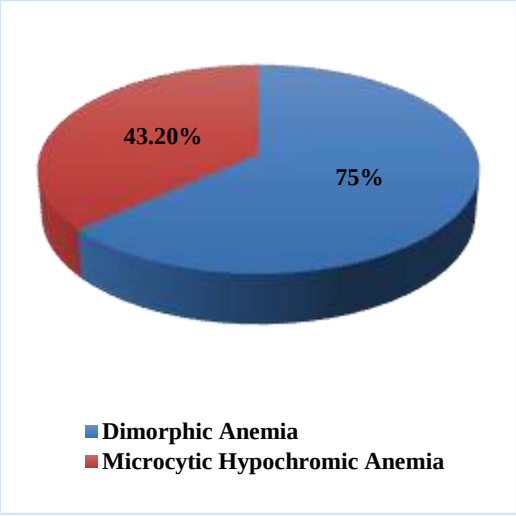

Fig 1: Vit.B12 deficiency among Anemia

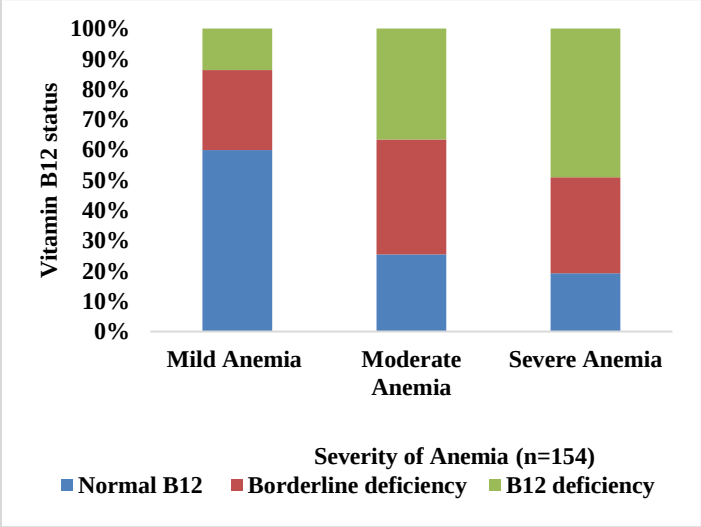

Fig 2: Vit.B12 Status among Study Participants based on Anemia Severity

Volume 9, Issue 3; May-June 2026; Page No 605-613

Volume 9, Issue 3; May-June 2026; Page No 605-613