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EFFECT OF FOUR-MONTHS ORAL FOLIC ACID AND VITAMIN B12 SUPPLEMENTATION ON ELEVATED HOMOCYSTEINE LEVELS: A COMPARATIVE STUDY

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Abstract: Hyperhomocysteinemia is a clinical condition characterized by a metabolic disorder with an increased risk of cardiovascular, neurological, and thromboembolic diseases. It was recently shown that both folic acid and vitamin B12 are crucial for proper homocysteine metabolism and combined supplementation should be recommended. This paper was designed to examine the effect of the oral administration of folic acid on its own in comparison with the simultaneous use of both folic acid and vitamin B12 on homocysteine levels in the plasma in a period of four months. Altogether, 120 patients were involved, all having plasma homocysteine levels above 15 µmol/L; 60 individuals received folic acid (5 mg/day) treatment and other 60 individuals received folic acid (5 mg/day) + vitamin B12 (500 µg/day) treatment. In the current research, there was a significant decrease in homocysteine levels ($p < 0.001$) observed in both groups, however, significantly more pronounced in the second group (9.8 ± 3.1 vs 6.2 ± 2.7 µmol/L, $p < 0.01$). The percentage of patients with normalized levels was also significantly higher in the second group (78% vs 52%).

Key words: Hyperhomocysteinemia; homocysteine; folic acid; vitamin B12; cardiovascular risk; vitamin deficiency

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INTRODUCTION

Homocysteine is one of the intermediary sulfur amino acids produced in the process of methionine metabolism. Metabolism of homocysteine relies heavily on cofactors such as folate, vitamin B12, and vitamin B6. The deficiency of these vitamins results in the buildup of homocysteine, leading to a condition known as hyperhomocysteinemia (Abbasi *et al*., 2025). New evidence confirms the strong relationship between homocysteine excess, endothelial dysfunction, oxidative stress, and an increased susceptibility to cardiovascular and cognitive diseases. In addition, clinical observations have shown a high occurrence of vitamin B12 deficiency (up to 56%) in subjects with high levels of homocysteine, emphasizing its nutritional pathogenesis (Ding *et al*., 2025; Abbasi *et al*., 2025). Conversion of homocysteine to methionine is dependent on folate and vitamin B12. Though supplementation of folic acid alone reduces homocysteine concentration, systematic review articles indicate better outcomes with combined supplementation (Omer *et al*., 2026; Preethi, *et al*., 2025). However, despite the available evidence, monotherapy with folic acid is being followed by clinicians. Hence, this study has been conducted to compare the two treatment strategies (Guo, *et al*., 2026; Yin *et al*., 2025).

MATERIALS AND METHODS

Study Design and Setting

This is a prospective, randomized comparative study (computer-generated randomization) carried out over a span of six months in a tertiary care hospital for the period Jan 2025 to Dec 2025 after receiving IRB approval (ERC-IRC-2024-2165). Out of all the patients enrolled in this study, 120 patients within an age range of 25–65 years with fasting plasma homocysteine concentration more than 15 µmol/L were selected for further study. The required sample size was determined using the WHO Sample Size Calculator, based on the expected effect size, a 95% confidence level, 80% statistical power, and a 5% level of significance. The calculation indicated that a minimum of 120 participants was

required to adequately detect a statistically meaningful difference between the study groups. Therefore, a total of 120 participants were enrolled in the study. Patients with other co-morbid disorders, pregnant females, and those who have taken vitamin supplements were excluded from this experiment in order to avoid any confounding factor. Two groups of patients (Group A and Group B) were formed equally. In Group A, patients took oral folic acid 5 mg/day. On the other hand, Group B comprised patients who took oral folic acid 5 mg/day and oral cyanocobalamin (vitamin B12) 500 µg/day.

Sample Collection and Biochemical Assays

Venous blood samples during fasting, obtained after an overnight fasting period of 8-12 hours, were collected using aseptic techniques. Homocysteine analysis required collection of blood samples in EDTA containing tubes, kept on ice, and centrifuged within 30 minutes to separate out plasma in order to prevent any in vitro alterations. Serum folate and vitamin B12 samples were obtained in normal tubes and were processed accordingly. Measurement of plasma homocysteine levels was performed using enzymatic cycling reaction by the Roche cobas c 501 chemistry analyzer as recommended by the company (Roche Diagnostics) (Rasmussen and Møller 2000). In this procedure, oxidation of homocysteine leads to generation of free homocysteine molecules which undergo conversion to S-adenosyl-homocysteine through enzymatic actions leading to hydrolysis and generation of cycling reaction. The end product of this reaction is detected photometrically where the absorbance is directly proportional to the homocysteine levels in the samples. The technique is known to be extremely sensitive, specific, and fit for clinical testing due to its automatization and low necessity of sample preparations (Roche Diagnostics, Homocysteine Enzymatic Assay for cobas systems). Folate and B12 serum concentrations were analyzed using an electrochemiluminescence immunoassay (ECLIA), in which the analyzer (Roche cobas e 801) uses ruthenium-labeled antibodies that produce a luminescent reaction proportionate to the analyte quantity (Guillerme, *et al*., 2024; Calderón *et al.*, 2018). Tablets of folic acid (5 mg) and vitamin B12 (500 µg) used in the test were prepared by two well-known pharmaceutical companies, Pfizer and Abbott, respectively.

Statistical analysis

Statistics was conducted using SPSS 25. Quantitative data was presented as mean ± standard deviation (SD). Comparison within groups before and after intervention was done by paired t-test, whereas comparison between groups was done using independent t-test. Chi-square test was employed for the proportion of subjects who had normalized homocysteine levels. Statistical significance level was set at a p-value < 0.05, implying significant differences among study groups.

RESULTS

There were no statistically significant differences in terms of demographics and biochemical parameters among the groups at the baseline ($p > 0.05$). There were a total of 120 subjects involved in the study; 60 subjects were assigned to each group. There was homogeneity between the two groups in terms of baseline characteristics, such as age, distribution in terms of gender, and biochemical parameters because there were no statistically significant differences ($p>0.05$) between the two groups. The average baseline plasma homocysteine levels in Groups A and B were 21.3 ± 4.5 µmol/L and 20.9 ± 4.2 µmol/L respectively. The baseline serum folate levels between the two groups were also comparable; Group A had an average value of 6.8 ± 2.1 ng/mL while Group B had an average of 6.6 ± 2.0 ng/mL (Table 1). However, the difference was not statistically significant ($p = 0.67$).

In the course of four months, both Groups A and B were found to have shown a significant reduction in the plasma concentration of homocysteine. Specifically, the mean plasma homocysteine levels among those who had only taken folic acid as supplementation decreased from 21.3 ± 4.5 µmol/L to 15.1 ± 3.8 µmol/L, with an average mean reduction of 6.2 ± 2.7 µmol/L (paired t-test, $p < 0.001$). On the other hand, in those who took both folic acid and vitamin B12 supplements, mean levels declined from 20.9 ± 4.2 µmol/L to 11.1±3.2 µmol/L, reflecting an average mean reduction of 9.8 ±3.1 µmol/L (paired t-test, $p < 0.001$).

In terms of the extent of reductions among patients, the difference in effectiveness between the two supplementations proved to be significant (independent t-test, $p<0.01$). Moreover, the percentage of patients that normalized their plasma homocysteine levels, which is less than 15 µmol/L, was found to be greater among the patients who took both folic acid and vitamin B12 supplements at 78% compared to just 52% among those who just took folic acid (Chi-square test, $p<0.01$). There were no side effects in either group, and compliance was more than 90% in both groups.

In summary, the study reveals that even though folic acid on its own is capable of decreasing homocysteine levels, the combined effect of folic acid with vitamin B12 produces much better results.

Table 1: Baseline Characteristics of Study Participants (n = 120)

Parameter	Group A	Group B	p-value
Age (years)	44.2 ± 10.1	45.6 ± 9.8	0.48
Homocysteine (µmol/L)	21.3 ± 4.5	20.9 ± 4.2	0.63
Vitamin B12 (pg/mL)	280 ± 90	275 ± 85	0.78
Serum Folate (ng/mL)	6.8 ± 2.1	6.6 ± 2.0	0.67

Note: Data shall be considered significant with $P < 0.05$

Table 2: Assessment of Homocysteine Reduction in group A and B after four months of oral supplementations of Vitamin B12 and Folic acid

Group	Baseline	4 Months	Mean Reduction	p-value
Group A	21.3 ± 4.5	15.1 ± 3.8	6.2 ± 2.7	<0.001
Group B	20.9 ± 4.2	11.1 ± 3.2	9.8 ± 3.1	<0.001

Comparison between group A and B showed significantly greater reduction in Group B ($p < 0.01$).

Table 3: Normalization (<15 µmol/L) of homocysteine levels after 4 months of oral supplementation of Folic acid and Vitamin B12

Group	Normalized	Percentage	p-value
Group A	31/60	52%	-
Group B	47/60	78%	<0.01

Note: Data shall be considered significant with $P < 0.05$

DISCUSSION

The present study demonstrates that the intake of folic acid alone has an effective influence on the decrease in homocysteine concentrations in the blood, a combination with vitamin B12 leads to better results. Moreover, this statement corresponds to numerous recent findings proving the advantage of combined B vitamins in terms of their efficacy compared to single supplements. According to recent meta-analyses conducted from 2025 to 2026, combined treatment by folic acid and vitamin B12 caused a significantly greater reduction in homocysteine concentrations in the blood, which was about −2.77 ($p < 0.0001$) (Smith and Refsum, 2016; Li and Jia, 2025; O'Leary and Samman, 2010). Under mechanistic reasoning, these results are biologically realistic and have substantial support from the currently established knowledge of one-carbon metabolism. The methylation of homocysteine into methionine, a step that takes place in the methionine cycle, depends on vitamins B12 and folate. Specifically, vitamins B12 acts as a co-factor of the enzyme called methionine synthase. Without sufficient levels of vitamin B12, folate is "locked" in its methylated version, 5-methyltetrahydrofolate, thus becoming metabolically inactive within this process. Therefore, although there can be enough folic acid available, its presence cannot bring about complete homocysteine metabolism without sufficient amounts of vitamin B12. This explains why supplementation with only folic acid leads to incomplete lowering of homocysteine while combined treatment has a stronger effect.

Clinical and epidemiological investigations have further corroborated these results in demonstrating that folic acid and vitamin B12 therapy, in addition to being effective in lowering homocysteine levels, also yields better clinical outcomes (Li *et al*., 2025). For instance, neurological problems that have been linked to elevated levels of homocysteine have yielded positive correlations with improvements in cognitive function following homocysteine lowering, while other metabolic problems such as diabetes and cardiovascular disease have indicated benefits in the reduction of vascular risks.

Another significant factor to consider here is the common presence of subclinical vitamin B12 deficiency, especially among individuals with hyperhomocysteinemia. Observational data published recently suggests that there is a large number of people who suffer from elevated homocysteine and vitamin B12 deficiency without being aware of this problem and presenting any obvious symptoms (Ren, *et al*., 2026; Green, *et al*., 2017). Ageing, diet, certain diseases affecting the digestive system, and drug intake (for instance, proton pump inhibitors or metformin) are factors leading to this condition. This rather widespread condition, which frequently remains unidentified, explains why folic acid is insufficiently effective when used as a single agent (Clarke, *et al*., 2010). The fact that there was a statistically significant difference among the treatment groups in the current investigation ($p<0.01$) is another factor that contributes to the clinical benefits of combined therapy. Indeed, this difference is statistically significant; however, it is also clinically significant, as even modest homocysteine-lowering effects have been shown to result in reduced risks of cardiovascular and neurologic complications according to previous studies. Overall, the results obtained indicate that the use of combined treatment including both folic acid and vitamin B12 supplementation is more effective for managing individuals with elevated homocysteine levels than folic acid alone. However, despite the demonstrated advantages of combined therapy, certain considerations should be taken into account. First, treatment response may vary across different patient populations because of differences in baseline nutritional status, disease characteristics, and the dosages administered. Second, evidence regarding long-term clinical outcomes remains limited, highlighting the need for well-designed randomized controlled trials to establish the optimal therapeutic dosage and duration of supplementation. Furthermore, a limitation of the present study is that only three biochemical parameters were assessed, which may not fully capture the complex metabolic and inflammatory pathways associated with homocysteine metabolism. Future studies should incorporate a broader panel of biochemical, inflammatory, and oxidative stress biomarkers to provide a more comprehensive understanding of the underlying biological mechanisms and to further validate the clinical benefits of combined vitamin supplementation.

CONCLUSION

In summary, this report further contributes to the growing body of knowledge on the effectiveness of the joint use of folic acid and vitamin B12, compared to only folic acid, on lowering homocysteine levels. This is a valid biological and clinical outcome, especially because of the current high-level scientific literature supporting such an approach.

Conflict of interest
Authors declare no conflict of interest.

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