



# Assessment of RBC Folate and Serum Vitamin B<sub>12</sub> Levels in Pakistani Women of Reproductive Age Group: An Approach towards Prevention of Adverse Pregnancy Outcomes

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## Authors' contributions

This work was carried out in collaboration among all authors. Authors IN and MW drew the conception and design, data acquisition, data analysis and interpretation and manuscript draft. The manuscript was critically revised by authors SN, MR and SSN and the final version was approved. All of the authors accept responsibility for the article that was published.

## Article Information

DOI: 10.9734/JPRI/2022/v34i28B36033

### Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/84882>

Original Research Article

Received 20 January 2022

Accepted 26 March 2022

Published 02 April 2022

## ABSTRACT

**Background:** Folate and Vitamin B<sub>12</sub> are important micronutrients necessary for fetal growth and development. Deficiencies of these vitamins in women of reproductive age results in adverse pregnancy outcome.

**Objective:** To determine red blood cell folate and serum Vitamin B<sub>12</sub> levels in apparently healthy women of reproductive age group.

**Methodology:** The study was conducted in Baqai medical university, Karachi. Apparently healthy volunteer women (N=196) between ages of 15 and 49 years residing in different areas of Karachi were selected after fulfilling the inclusion and exclusion criteria. The cut off values for defining Vitamin B<sub>12</sub> deficiency, marginal insufficiency and folate insufficiency were <203 pg/mL, 203-299

pg/mL, and <400 ng/mL, respectively. Analysis of red blood cell folate and serum vitamin B<sub>12</sub> levels was done after complete blood count and peripheral smear morphology.

**Results:** The mean serum vitamin B<sub>12</sub> and red blood cell folate levels were 233.69 ± 54.10 pg/mL and 540.34 ± 54.04 ng/mL. Percent values of Vitamin B<sub>12</sub> deficiency and marginal insufficiency were 42% and 46%. All of the study participants had red blood cell folate levels >400 pg/mL. A significant negative association was present between age and serum vitamin B<sub>12</sub> levels.

**Conclusion:** Low serum levels of vitamin B<sub>12</sub> in women of reproductive age group can be considered an important etiologic factor for adverse pregnancy outcomes especially neural tube defects. The need is to assess pre-conceptual level of vitamin B<sub>12</sub> and to implement public health program especially food fortification to improve vitamin B<sub>12</sub> status.

*Keywords: RBC folate; serum vitamin B<sub>12</sub>; women of reproductive age group.*

## 1. INTRODUCTION

The value of micronutrients in our daily lives cannot be underestimated. They play an important role in growth and development. A single micronutrient deficiency can have a negative impact on the human body [1]. Among the various micronutrients folate (Vitamin B<sub>9</sub>) and cobalamin (Vitamin B<sub>12</sub>) are utmost important. Folate is abundant in green leafy vegetables and fruits. Folate functions as a co-enzyme in the transfer of single carbon units from one compound to another, and thus plays a critical role in DNA synthesis, cell division, and proliferation. Biologically active form of folate is tetrahydrofolate [1,2]. Cobalamin is synthesized solely by microorganisms. The main source of Vitamin B<sub>12</sub> is food derived from animal origin (meat, milk, eggs, fish, and shellfish) [3,4].

Functions of vitamin B<sub>12</sub> and folate are interlinked as they both involved in many common metabolic pathways especially the formation of S-adenosyl methionine (SAM) [3]. This reaction is nearly irreversible and is first step in the pathway by which methyl - THF which enters the cell is converted into all the intracellular folate co-enzymes [3]. Methylene-tetrahydrofolate reductase (MTHFR) converts 5, 10-me-THF produced by the folate cycle into 5-methyl-THF (5-me-THF) within the cytoplasm. The methyl group of 5-me-THF is then donated to remethylate homocysteine (HCY) to form methionine [5]. This reaction connects 1CM to the methionine cycle, which occurs when methionine is adenylated to S-adenosyl-methionine (SAM) by an enzyme called methionine adenosyl-transferase (MAT). SAM is a universal methyl unit carrier and donor, as well as an enzymatic cofactor involved in epigenetic maintenance, polyamine biosynthesis, creatine and phosphatidylcholine biosynthesis, and sulphur metabolism [5,6].

Vitamin B<sub>12</sub> deficiency can adversely impact this process and prevent the use of the storage form of folate when it is required, resulting in functional folate deficiency. These findings suggest that vitamin B<sub>12</sub> deficiency causes a nuclear 5-methylTHF trap, which suppresses de novo dTMP biosynthesis and causes DNA damage, explaining the pathophysiology of megaloblastic anaemia seen in vitamin B<sub>12</sub> and folate deficiency [7].

A lack of folate and vitamin B<sub>12</sub> may also result in an increase in homocysteine levels. Hyperhomocysteinemia has both vasotoxic and vasoocclusive properties. Hcy has the ability to spontaneously self-oxidize, producing reactive oxygen species (ROS) that damage the vascular endothelium and initiate thrombotic events [8]. Therefore, in pregnancy hyperhomocysteinemia can result in faulty placental functions, intrauterine growth retardation, and early pregnancy loss [9,10].

Vitamin B<sub>12</sub> (adenosyl cobalamin) functions as a cofactor of methylmalonyl-coA mutase in the conversion of methylmalonyl-coA to succinyl-coA, a key molecule in the tricarboxylic cycle. Therefore, Vitamin B<sub>12</sub> deficiency causes elevated methylmalonic acid levels in the blood, indicating vitamin B<sub>12</sub> status. The MTHFR enzyme regulates the availability of 5-MTHF for homocysteine remethylation. Several polymorphisms in the MTHFR gene have been identified, with C677T being the most common. This results in a decrease in folate levels and hyperhomocysteinemia, both of which are important risk factors for neural tube defects [9-11].

Women of reproductive age (WRA) are more vulnerable to folate and vitamin B<sub>12</sub> deficiency. Both vitamins are more important during pregnancy because they are required for foetal

growth, development, and metabolic needs. Multiple pregnancies, poor diet, lactation, and infections may all increase the need for folate and vitamin B<sub>12</sub> in WRA [2,6]. A recent review by Torheim et al. revealed that WRA in low and middle income countries, as well as in South and South East Asia, are taking several micronutrients, including folate and vitamin B<sub>12</sub>, at levels lower than the WHO/FAO Estimated Average Requirement [7]. There are multiple and interrelating elements known to cause birth defects out of which micronutrient deficiencies cannot be left unnoticed. Neural tube defects are common and major birth defects. NTDs are birth defects of the brain and spinal cord. Anencephaly, spina bifida, and encephalocele all result in significant mortality and morbidity [5]. The importance of folate and vitamin B<sub>12</sub> in embryogenesis is well established. Deficiencies in both vitamins during the periconceptual period are risk factors for recurrent miscarriages, low birth weight, abruptio placentae, stillbirth, preterm birth, and neural tube defects [2,3,8]. The neural tube closes by 6 weeks of gestation, and the majority of pregnancies go unnoticed [5].

According to a systematic literature review conducted by Zaganjor et al., the prevalence of NTD varies greatly across the country. Spina bifida was found to be the most common anomaly among anencephaly and encephalocele. Various studies in Pakistan revealed an increasing prevalence of neural tube defect (38.6 and 124.1 per 10,000 births) [9]. Previous clinical trials reported a decrease in the prevalence of NTD in countries following folic acid fortification programs [10].

The purpose of this study was to determine the status of serum vitamin B<sub>12</sub> and red blood cell (RBC) folate in apparently healthy Pakistani women of reproductive age and to calculate the deficiencies of these vitamins in order to observe their importance in the prevention of adverse events during pregnancy.

## 2. MATERIALS AND METHODS

A cross-sectional study was conducted at the Baqai Institute of Hematology, Baqai Medical University in Karachi, between May 2017 and April 2018. Sample size was calculated using the following formula.

$$n = \frac{z^2(p)(1-p)}{c^2}$$

z=1.96; p=0.05; c=0.07; n=196

z=value corresponding to a given confidence interval

p=% frequency of the outcome factor

c=standard error

n=sample size

The research population (n=196) was made up of healthy volunteer women between the ages of 15 and 49 who lived in various areas of Karachi. After discussing the study protocol in detail, participants were asked to sign a written informed consent form. A complete history of the subjects' personal, medical, surgical, medication, and dietary habits was obtained. A quick general physical examination was performed, and weight and height were measured in kilograms and feet-inches, respectively. BMI (kg/m<sup>2</sup>) was measured. Participants with a BMI less than 18.5 kg/m<sup>2</sup>, pregnancy, lactation, a strict vegetarian diet, a recent transfusion history, renal disease, diabetes, smoking, liver disease, malabsorption, any previous gastrointestinal surgery, any chronic illness, use of vitamin supplements, and proton pump inhibitors were excluded. One pen-and-paper form food frequency questionnaire was used to collect a complete dietary history [12]. It was modified to correspond to cultural standards. The participants were asked to recollect the type, frequency, and amount of food and drinks they had consumed.

Following aseptic procedures, fasting venous blood samples (10 ml) were taken. For RBC folate and serum vitamin B<sub>12</sub> analysis, an equal amount of blood was transferred into EDTA (Ethylenediaminetetraacetic acid) and gel-containing vacutainers. Specimens were kept cold and light-protected. Within 1 hour after collection, serum was extracted by centrifugation (2000 rpm for 10 minutes) and transferred to aliquotes, which were refrigerated at - 20°C. Whole blood samples were stored at 4°C and tested within 2 hours after collection. All specimens were transferred to the laboratory on dry ice. The levels of folate and vitamin B<sub>12</sub> in red blood cells were determined using a Roche Diagnostics Cobas electrochemiluminescence e 411 analyzer in Mannheim, Germany. Hemolysate was generated for RBC folate assay by combining 3ml of 0.2 percent ascorbic acid solution and 100µl of whole blood and incubating it for 90 minutes at 20-25°C away from light. The complete blood count (CBC) was performed on a Sysmex XP100 in Tokyo, Japan, and

haemoglobin levels more than 12g/dl [13] were acceptable. Leishman's stain was used to complete peripheral smear morphology, and subjects with normocytic normochromic blood images and no hyper segmented neutrophils were included.

Red blood cell folate levels less than 400 ng/mL (906 nmol/L) were considered insufficient [14]. The blood vitamin B<sub>12</sub> cutoff limit for vitamin B<sub>12</sub> deficiency and marginal insufficiency was <150 pmol/L (203 pg/mL) and 150–221 pmol/L (203–299 pg/mL) respectively [15,16].

### 2.1 Statistical Analysis

The statistical analysis was done by using Statistical Package for Social Sciences (SPSS)

version 23.0. Data was arranged as mean ± standard deviation, range and median. Association of parameters was determined by Pearson correlation coefficient. P value of <0.05 was considered significant for all of the analyses.

### 3. RESULTS

Table 1 shows the baseline characteristics of participants (N=196). Females had a mean age of 27.79 ± 6.70 years. The average Hb of the participants was 13.3 ± 1.0, while the average Hct was 42.1 ± 3.1. The female participant's MCV was 79.97 ± 4.53. 40% of females belonged to the low income class [Rs. 13,000 (US\$120), 41% to the lower middle class (Rs. 13,000 – 25,000), and 19% to the upper middle class (>Rs25, 000).

**Table 1. Baseline characteristics of study population (N=196)**

Variables	Parameters	Values	Frequency (%)
Age (years)	Mean ± SD§	27.79 ± 6.70	--
	Median	28.00	--
	Range	15- 49	--
	Percentile 25	25	--
	Percentile 50	28	--
	Percentile 75	32	--
BMI $\bar{p}$ (kg/m <sup>2</sup> )	Mean ± SD	21.3 ± 3.8	--
Hb $\bar{q}$ (g/dl)	Mean ± SD	13.3 ± 1.0	--
	Median	12.8	--
	Range	12.8 – 15.5	--
	Percentile 25	12.5	--
	Percentile 50	12.8	--
	Percentile 75	13.8	--
Hct * (%)	Mean ± SD	42.1 ± 3.1	--
	Median	40.8	--
	Range	36.2 – 55.3	--
	Percentile 25	40.2	--
	Percentile 50	40.8	--
	Percentile 75	43.9	--
MCV $\dagger$ (fL)	Mean ± SD	79.97 ± 4.53	--
	Median	78.4	--
	Range	72.1 – 93.3	--
	Percentile 25	76.65	--
	Percentile 50	78.40	--
	Percentile 75	81.60	--
Income	Low income class	--	79 (40)
	Lower middle class	--	80 (41)
	Upper middle class	--	37 (19)
Education level	No education	--	30 (15)
	Middle (grade 8)	--	49 (25)
	Matric (grade 10)	--	60 (31)
	College & University	--	57 (29)

§ sample size; § standard deviation;  $\bar{p}$  body mass index;  $\bar{q}$  haemoglobin; \* haematocrit;  $\dagger$  mean cell volume

**Table 2. Serum vitamin B<sub>12</sub> and Red cell folate levels in women of reproductive age group (N=196)**

Variables	Parameters	Values	Frequency (%)
Serum Vitamin B <sub>12</sub> (pg/mL)	Mean ± SD§	233.69 ± 54.10	--
	95% CI†† for mean		--
	Lower bound	226.07	--
	Upper bound	241.31	--
	Median	210.21	--
	Range	168.45 – 392.30	--
	Percentile 25	190.67	--
	Percentile 50	210.21	--
	Percentile 75	264.89	--
	Vitamin B <sub>12</sub> deficiency (< 203 pg/mL)	--	--
Marginal vitamin B <sub>12</sub> levels (203 – 299 pg/mL)	--	--	90 (46)
Adequate vitamin B <sub>12</sub> levels (> 299 pg/mL)	--	--	23 (12)
RBC folate (ng/mL)	Mean ± SD	540.34 ± 54.04	--
	95% CI for mean		--
	Lower bound	532.73	--
	Upper bound	541.48	--
	Median	540.76	--
	Range	468.34 – 634.89	--
	Percentile 25	498.13	--
	Percentile 50	540.76	--
Folate insufficiency (< 400 ng/mL)	--	--	0 (0)

‡ sample size; § standard deviation; †† confidence interval

Participants in the research had an average blood vitamin B<sub>12</sub> level of 233.69 ± 54.10 pg/mL as mentioned in Table 2. Serum vitamin B<sub>12</sub> levels were highly inversely associated to age (r = - 0.768, p < 0.001). Vitamin B<sub>12</sub> deficiency (203 pg/mL) was found in 42% of the women, with moderate insufficiency (203-299pg/mL) prevalent in 46% of the individuals. Only 12% of the participants in the research had acceptable vitamin B<sub>12</sub> levels (>299pg/mL). The study population's mean RBC folate level was 540.59 ± 54.04ng/mL. RBC folate levels in all of the women were greater than 400ng/mL. RBC folate was not shown to be substantially related to age. There was no significant relationship between serum vitamin B<sub>12</sub> levels and red cell folate levels.

#### 4. DISCUSSION

Neural tube defects (NTD) are classified into complex and diverse group of congenital anomalies of brain and spinal cord [17]. The closure of neural tube is very sensitive, highly systematic and organized mechanism. This involves interaction of different genes with each other and is modified by the environmental factors. It is widely known that numerous environmental variables may play a role in epigenetic alteration of DNA, influencing gene expression, as demonstrated in the majority of neural tube instances. The nutritional status of

the mother also reflect fetal development and well-being. Among the several nutrients, folate and vitamin B<sub>12</sub> are the most significant [11].

According to the findings of our study, vitamin B<sub>12</sub> deficiency, particularly marginal insufficiency, is developing as a severe health concern in WRA. However, the mean red cell folate level in our research sample was 540.34 ± 54.04ng/ml, which was significantly higher than 400 ng/ml (WHO criteria). The high folate levels might be attributed to the simple availability, affordability, and accessibility of folate-rich diets vs. those originating from animal sources. Fresh green leafy vegetables, legumes, and fruits are high in folate [1,14]. According to WHO recommendations, the ideal red cell folate level in women of reproductive age to avoid NTD-affected pregnancies should be more than 400 ng/mL. [14]. Our research participants had an average blood vitamin B<sub>12</sub> level of 233.69 ± 54.10 pg/mL. According to the findings of our study, 42 percent of women with vitamin B<sub>12</sub> deficiency had levels less than 203 pg/mL, while 46 percent had levels between 203-299 pg/mL. (Marginal insufficiency). All of our research participants had red cell folate levels above the recommended level (>400ng/ml), as established by WHO for neural tube defect prevention.

The key strength of our study was that we were able to assess RBC folate levels. The majority of previous investigations on WRA in Pakistan [15,18,19] and other country [20] investigated serum folate levels, however there is very little data on RBC folate status of WRA. Because serum folate represents current folate consumption and is impacted by several factors, such as a brief drop in folate intake, a single result cannot accurately depict chronic deficiency conditions. Frequent assessments are advised to identify folate status in an individual [21]. Red blood cell folate has a long-term folate status because it is concentrated in the erythrocyte during erythrocyte formation in the bone marrow and remains there for 120 days. Dietary changes have a gradual effect on it [14,21]. According to WHO's current recommendations on assessing the risk of NTD in the community, RBC folate is a more sensitive biomarker than serum folate [14].

There is scarcity of data regarding RBC folate levels in WRA. The use of different cut off points to define folate and vitamin B<sub>12</sub> deficiency and insufficiency; difference in detection techniques makes it challenging to compare our results [20,21]. A recent study conducted by Yasmin et al. in Karachi, Pakistan on women (mean age=27.6 ± 5.9 years) determined that mean red blood cell folate level at baseline was 623.6 ± 406.6 [22]. A case control study reported by Nauman et al. estimated that 84% control mothers had red blood cell folate levels more than 150ng/ml [23]. Shamah-Levy et al. found a very low prevalence of folate deficiency in Mexican women aged 20 to 49 years old [24]. Karabulut et al. also found a very low prevalence of folate deficiency (0.3 percent) in Mediterranean women aged 18-45 years [25].

Previous research on the potential causes of vitamin B<sub>12</sub> deficiency has identified dietary insufficiency, socioeconomic status, malabsorption, parasitic infestation, pernicious anaemia, and gastrointestinal pathologies as the leading causes [26-30]. A cross sectional study conducted in North Iran revealed high prevalence of vitamin B<sub>12</sub> deficiency (22.7%) in women of reproductive age group (aged 15-49 years). The reasons behind owing to dietary insufficiency, decreased intake of animal derived foods because of high cost and socioeconomic inequality [27]. Another study performed on women of childbearing group (aged 19-39 years) in United Kingdom, reported 22.95% women had decreased serum vitamin B<sub>12</sub> levels (≤150 pmol/L) and agreed that vitamin B<sub>12</sub> should be measured during antenatal visits. Sukumar N et

al. concluded from a systematic literature review that vitamin B<sub>12</sub> levels decreases during pregnancy. They estimated 20-30% vitamin B<sub>12</sub> deficiency which was more prevalent in India, Eastern Mediterranean and South American regions [28]. Balci YI et al. in a study on pregnant women and their neonates demonstrated 70.8% vitamin B<sub>12</sub> deficiency in mothers. They observed that lack of consumption of food from animal source was the basis of high prevalence of vitamin B<sub>12</sub> deficiency [26]. A study conducted in Bangalore, India found a link between low maternal vitamin B<sub>12</sub> levels and small for gestation babies, supporting the use of vitamin B<sub>12</sub> supplements in pregnant women [29]. Garcia et al. reported 36.99% and 61.34% vitamin B<sub>12</sub> deficiency (200 pg / ml) in adolescent (aged 15 years) and pregnant mothers (aged 14-24+ years) in Venezuela, respectively, with socioeconomic catastrophe being a major contributor [30].

A study conducted in India by Refsum et al. revealed a high prevalence of vitamin B<sub>12</sub> deficiency (56 percent). In addition to the vegetarian diet, malabsorption was found to be an important underlying factor in the increased deficiency of vitamin B<sub>12</sub> [31]. A cross-sectional study on South Asian women in Auckland by Gammon CS et al. discovered lower levels of serum vitamin B<sub>12</sub> in vegetarians than in non-vegetarians. Vitamin B<sub>12</sub> deficiency (150 pmol/L) was found in 24% of vegetarians and 9% of non-vegetarians [32]. A cross-sectional study on Indian adolescents conducted by Kapil et al. revealed an overall 73.5 percent prevalence of vitamin B<sub>12</sub> deficiency, with 87.5 percent, 80.7 percent, and 47.1 percent belonging to the low-income, middle-income, and high-income groups, respectively. They hypothesised that the high prevalence of deficiency was due to a low dietary intake of animal-derived foods [33]. A national survey of Belizean women of reproductive age (aged 15-49 years) found 17.2 percent and 33.2 percent of them to be vitamin B<sub>12</sub> deficient or insufficient, respectively. Differences in socioeconomic group, lifestyle, geographical background, and inaccessibility and unaffordability of vitamin B<sub>12</sub>-enriched foods were all significant contributors to vitamin B<sub>12</sub> deficiency [34]. Moreover, Dang et al. found 45.5% and 25% vitamin B<sub>12</sub> deficiency and marginal deficiency (200-299 pg/mL) in women aged 10 to 49 years in a study on Chinese women from northwest China. They concluded that geographical and climatic effects, interregional lifestyle differences, and the availability and affordability of

micronutrients in rural and urban areas were the primary causes of the difference in vitamin B<sub>12</sub> levels [35].

Previous research has also revealed the role of folate in NTDs and the importance of folic acid supplementation in improving folate status and reducing poor pregnancy outcomes in women of reproductive age [36,37]. However, the role of vitamin B<sub>12</sub> in NTDS cannot be overlooked [10]. There is mounting evidence that maternal vitamin B<sub>12</sub> deficiency and marginal insufficiency are linked to adverse pregnancy outcomes such as NTD [37,38]. Groenen et al. discovered in a case control study that case mothers (those who had children with spina bifida) had 21 percent lower vitamin B<sub>12</sub> levels than control mothers, and that vitamin B<sub>12</sub> levels of 185 pmol/L were associated with a 3.5 fold increase in spina bifida risk [39]. Gaber et al. reported a case control study and documented that median serum vitamin B<sub>12</sub> levels were significantly lower in the women with a history of or having NTD affected pregnancy compared to the controls. They recommended pre-conception vitamin B<sub>12</sub> and folic acid supplementations as a preventive measure to reduce the occurrence of neural tube defects [40].

The low vitamin B<sub>12</sub> levels and high prevalence of vitamin B<sub>12</sub> deficiency in our study population could be attributed to dietary insufficiency. Food derived from animals is the primary source of vitamin B<sub>12</sub>. Meat, fish, chicken, and dairy products are common sources of vitamin B<sub>12</sub>. Pakistan, as a developing country, faces numerous challenges in maternal and child health care. The majority of our participants (40%) were low-income or lower-middle-class (41 percent). Poverty, inability to afford, dietary habits, cultural norms, malabsorption, and parasitic infestation are all major causes of vitamin B<sub>12</sub> deficiency. The study's main limitation is the small sample size. Large-scale population-based studies would be required to assess the prevalence and magnitude of vitamin B<sub>12</sub> deficiency.

## 5. CONCLUSION

It is therefore concluded that having a mean folate level above 400ng/ml indicates good folate status, which also meets the WHO criteria for preventing neural tube defects in a population. Vitamin B<sub>12</sub> deficiency, on the other hand, is extremely common in women of reproductive age. According to previous research, vitamin B<sub>12</sub> deficiency, particularly marginal insufficiency, may pose more serious threats to maternal and

child well-being. It is suggested that measures be taken to improve the vitamin B<sub>12</sub> status of women of reproductive age through a mass food fortification programme. Furthermore, the inclusion of synthetic vitamin B<sub>12</sub> in folic acid tablets for use during the periconceptional period should be considered to prevent NTD.

## CONSENT

As per international standard or university standard, Participants' written consent has been collected and preserved by the author(s).

## ETHICAL APPROVAL

The Ethics Committee of Baqai Medical University in Karachi approved the study (Reference No. BMU-EC/2017-04).

## DATA SHARING STATEMENT

The data sets generated or analyzed during the present study are available from the corresponding author on reasonable request.

## ACKNOWLEDGEMENT

All technical staff of Baqai Institute of Hematology.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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