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DNA Hypomethylation could be a Predictive Biomarker for B Vitamin Deficiencies Among Working Population

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Abstract

The methylation (CH_3) capacity is dependent on the availability of B vitamins in one carbon metabolism. This study evaluated B vitamin (B9 and B12) deficiency status and its impact on the Diagnostic Accuracy (DA) of DNA methylation among the working population. DNA methylation (5 methylcytosine) and B vitamins were quantified using the ELISA methods. Based on the cut-off values of B vitamins, the subjects were categorized into adequate, marginal, and deficiency status. Fagan's nomogram was used to assess the DA of DNA methylation in different conditions of B vitamin. DA metrics are used in this study as a Diagnostic Odd Ratio (DOR), a positive Likelihood Ratio (LR+), a negative Likelihood Ratio (LR-), and a posttest probability of true positive DNA methylation that is less than 2.0%. DNA methylation was significantly lower in marginal and deficient B-vitamin status as compared to adequate status. The DA metrics at 5 mC showed high sensitivity and modest specificity for marginal and deficient B vitamins. In vitamin B9 deficiency, LR (+) was 2.51 (95% CI: 0.99–6.39), DOR was 10.4 (95% CI: 2.06–51.2), and the post-test probability was 92% (95% CI: 83–97). In vitamin B12 deficiency, LR (+) was 1.58 (95% CI: 1.03–2.44), DOR was 3.16 (95% CI: 1.29–7.66), and the post-test probability was 86% (95% CI: 80–90). In B vitamin deficiency, DA metrics of DNA methylation: LR+, LR-, DOR, and post-test probability were more precisely predicted. DNA hypomethylation could be used as a predictive biomarker in B vitamin deficiency. The findings of the study help to prevent adverse health effects due to methylation capacity loss.

Keywords: B Vitamins, Diagnostic Accuracy, DNA Methylation, 5-Methylcytosine

1. Introduction

The combination of folate, methionine, and trans-sulphuration pathways are known as One Carbon Metabolism (OCM)[1]. These pathways are needed for redox state maintenance in the body[2]. B vitamin complex serves as a co-factor and substrate for various biological reactions[3]. The production of methyl group (CH_3) donors such as S-Adenosylmethionine (SAM) predominantly depends on the availability of B vitamins, i.e., riboflavin (B2), pyridoxine (B6), folic acid (B9), and cobalamin (B12)[4]. The deficiency of these vitamins affects the SAM synthesis and methylation capacity[5]. Vitamins B12 and B9 are essential for the maintenance of nerve cell integrity and the production of red blood cells, respectively. The availability of B vitamins in the body is also required

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for dihydrofolate reductase, Methylenetetrahydrofolate reductase, methionine synthase, and methyl-adenosyl transferase enzyme activities, which help in the production of the CH_3 group in OCM[3].

DNA methylation is the addition of a CH_3 group to the cytosine ring of DNA with the support of DNA methyltransferase activity[5]. It regulates the nucleosome placement, X chromosome inactivation, replication, repair, transcription, duplex stability, imprinting, and epigenetic memory[6]. In the repressed chromatin of mammals, 5mC was found abundantly. The 5 mC is represented as global or total genomic DNA methylation and serves as a prior screening tool when compared to gene-specific regions. DNA hypomethylation predicts the disease progression[7] and effects on intragenic and intergenic regions of DNA that create genomic instability and mutation events[8]. DNA hypomethylation conditions were evidenced in cancer,[9] chronic non-communicable diseases,[10] rheumatoid arthritis,[11] immune response sites,[12] and chemical pollutant exposure[13].

DA metrics differentiate between the target condition and health[14]. The DA metrics are sensitivity, specificity, positive and negative predictive values (PPV and NPV), Receiver Operating Characteristic (ROC) curve, Youden index, DOR, and Fagan nomogram, which provide the prior and post-test probabilities with likelihood ratio (LR+ and LR-). It is a graphical calculator and is used in evidence-based medicine and clinical epidemiological studies[15]. DA metrics of DNA methylation were evaluated in malignancies of the gastric,[16] head and neck,[17] colorectal,[18] and non-small lung[19]. A study by Gulvady *et al.*,[20] reported high incidences of vitamin B12 deficiency in executives in the company. DNA hypomethylation was reported in maternal diets with low levels of B9 and B12,[21] B12 deficiencies in gastrectomized rats[22] and colorectal cancer patients[23]. The availability of B vitamins in the body is defined as adequate, marginal, and deficient. The prevalence of marginal status was predominantly high when compared to deficient status, and is associated with cardiovascular disease,[24] a risk of fracture, impaired physical function, and a decline in cognitive function[25]. As per the review of the literature, we noted the gap in the identification of adequate, marginal, and deficiency status and predictive ability of DNA methylation in B vitamins (B9 and B12) deficiency conditions using gold standard parameters and Fagan's nomogram. It is a widely used diagnostic criterion.

2. Material and Methods

2.1 Study Design and Participants

This study design is diagnostic accuracy-comparative, and it was carried out during the year 2021-22. A total of 265 male working populations were enrolled from industrial establishments located in Karnataka, India. The study subjects were recruited based on contact between research and prospective participants. The working population, who were willing to participate and were 18 years of age or older, was included in the study. Subjects who were taking oral or injectable B vitamin supplementation continuously for more than 15 days during the previous year, had known cases of cardiovascular diseases, were diabetic with Metformin treatment, and recently donated or received blood were excluded from the study. The sample size was calculated based on the prevalence of vitamin B12 deficiency reported in workers[26]. The prevalence of vitamin B12 deficiency was 39 (π=0.39) with a relative precision of 15 % (ε=0.15) and a 95% CI was used to estimate the sample size. The estimated sample size was found to be 256 subjects. The written informed consent was obtained from each worker before participation in the study. The study protocol was approved by the institutional ethics committee (ROHCS/2019/14 dated 20th August 2019) by the National Ethical Guidelines for biomedical and health research involving human participants in India. A pre-structured questionnaire was used to collect individual data on age, BMI, smoking, and alcohol consumption habits. The worker's height was measured by centimetres using tape and their weight in kilograms with scales, while they wore lightweight clothes and without shoes. BMI was calculated by using subjective weight (kg) and height (meter squared) and expressed as kg/m^2.

2.2 Blood Collection

4 mL of venous whole blood sample was collected from each study subject and transferred in plain vacutainer tubes (Lab Tech Disposables, India). The serum was separated by centrifugation at a speed of 2000g for 10 minutes in a refrigerated centrifuge. The separated serum samples were stored at –80°C and were used for analysis of the B vitamins (B9 and B12) and DNA methylation within 3 months. The separation of serum, storage, and analysis time was followed using the Jansen and Beekhof protocol[27].

2.3 Vitamin B9 (Folic Acid)

Vitamin B9 levels were measured using the Enzyme-Linked Immunosorbent Assay (ELISA) kit method (BT Lab, cat. no. E3930Hu, Shanghai, China). The sample and standard absorbance were measured using a Lisa Scan EM microplate reader at 450 nm (Transasia Biomedical Limited, Mumbai, India). The LOD of the method is 0.03 ng/mL, and the detection range is 0.05-20 ng/mL. The intra and inter-assay coefficient variation of the method were found to be <8.0% and <10 %, respectively. The level of vitamin B9 units (ng/mL) was converted to SI units (nmol/L) using a conversion factor of 2.266. The results were expressed as nmol/L.

2.4 Vitamin B12 (Cobalamin)

The levels of vitamin B12 were measured using the ELISA kit method (CAL Biotech, cat. no. VB369B, El Cajon, CA, USA). The sample and standard absorbance were measured using a Lisa Scan EM microplate reader at 450 nm (Transasia Biomedical Limited, India). The LOD of the method is 41 pg/mL, and the detection range is 0-2000 pg/mL. The intra-assay of 210 pg/mL with 24 replicates and inter-assay of 201 pg/mL with 24 replicates found the CV is 6.23 and 6.25%, respectively. The level of vitamin B12 units (pg/mL) was converted to SI units (pmol/L) by using a conversion factor of 0.738. The results were expressed as pmol/L.

2.5 DNA Methylation (5 mC)

The measurement of DNA methylation (5 mC) was done as per the protocol of Kalahasthi *et al.*,[28] Serum DNA was isolated using the Fit Amp TM procedure (Epigentek, Cat. P-1004, USA). It allows the DNA measurement in the range of 0.1 to 2 µg. A Nanodrop Spectrophotometer (Thermo Scientific TM Nano Drop TM) was used to evaluate the yield and purity of DNA at a 260/280 nm ratio. The global methylation (5mC) of the isolated DNA was measured using the ELISA technique (Epigentek, cat. no. P 1030, USA). The methylated fraction of DNA is quantified using a Lisa Scan EM microplate reader at 450 nm (Transasia Biomedical Limited, India). The proportion of DNA methylation is relative to the intensity of absorbance measured. The detection limit of the method is 0.05% methylated DNA from 100 ng of input DNA.

2.6 Diagnostic Accuracy (DA) Metrics

DA metrics of DNA methylation in marginal and deficient B vitamin conditions of the working population were done using an online calculator:

https://www.medcalc.org/calc/diagnostic_test.php was used to calculate gold standard parameters such as sensitivity, specificity, and Positive and Negative Predictive Values (PPV and NPV)[29] and the online calculator http://araw.mede.uic.edu/cgi-bin/testcalc.pl was used to measure Likelihood Ratios (LR+ and LR-), as well as prior and posttest probabilities[30]. DOR serves as a global measure of the DA and efficacy of the test, and it was calculated using an online calculator: https://www.medcalc.org/calc/odds_ratio.php[31]. The median level of DNA methylation in B12 vitamin adequate status in the working population was true positive (<2%) and true negative (2%). The DA measures were constructed using 2x2 tables with true positive and true negative DNA methylation present and absent in marginal and deficient statuses of B vitamins. The distribution of adequate, marginal, and deficient B vitamin status was defined using the set limits of Allen *et al.*,[32] for vitamin B12 and Khan and Jilala[33] for vitamin B9. The cut-off values of vitamin B12 were used in this study as <148 pmol/L for deficiency, >148-221 for marginal, and >221 for adequate. The cut-off values of vitamin B9 were used as <4.5 nmol/L for deficiency, 4.5-9.1 for marginal, and >9.1 for adequate.

2.7 Statistical Analysis

The data analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 23 software (IBM Corp., Armonk, NY, USA). The demographic details of workers were presented as median, range, and percentage. The data distribution of DNA methylation among subjects was evaluated using the Shapiro-Walks test and found to be non-normal. The data of DNA methylation in B vitamin statuses were presented as mean, 95% CI and a mean rank score. The differences in DNA methylation between different statuses of B vitamins (B9 and B12) were compared using the Kruskal-Walli's test. The level of significance was considered as $p<0.05$.

3. Results

The demographic details of the working population are shown in Table 1. The median age and BMI of study subjects were 30 years and 21 kg/m^2, respectively. The

categorical variables, such as habits of smoking and alcohol consumption, were found to be 38% and 20% among study subjects, respectively.

Levels of DNA methylation in the working population with adequate, marginal, and deficient statuses of B vitamins are shown in Table 2. The Kruskal-Wallis test was used to find out the differences in DNA methylation in the working population with B vitamins statuses. Vitamin B9 statuses showed that there was a statistically significant decrease in DNA methylation: (χ^2) = 16.623, p = 0.001, with a mean rank score of 162 for adequate, 140 for marginal, and 90 for deficiency. Vitamin B12 statuses showed that there was a statistically significant difference in DNA methylation: (χ^2) = 13.513, p = 0.001, with a mean rank score of 149 for adequate, 146 for marginal, and 111 for deficiency.

Table 1. Demographic details among industrial workers

Continuous variables	n = 265	
	Median (range)	
Age (years)	30 (18-69)	
BMI (kg/m^2)	21 (15-36)	
Categorical variables	n	%
Alcohol consumption (Yes)	101	38
Smoking (Yes)	52	20

BMI = Body mass Index

The distribution of true positive and true negative cases of DNA methylation in the B vitamin (adequate, marginal, and deficient) status is shown in Table 3. The distribution of true positive and negative variables was utilized to assess the DA metrics by using 2x2 tables.

DA metrics of DNA methylation in workers with marginal and deficient B vitamins are reported in Table 4. The parameters of sensitivity, specificity, PPV, and NPV were evaluated. The sensitivity of DNA methylation was indicated as high, and the specificity was modest in B vitamin deficiencies. The DOR score has ranged from 0 to infinity. A DOR score above one indicates better performance, a score below one indicates incorrect performance and a value of exactly one indicates that the test has an equal chance of correctly predicting a favorable outcome and provides no information. The DOR score of DNA methylation among workers who were marginal and deficient in B vitamins was found to be greater than one, which indicates a better performance of DNA methylation in deficiency and marginal status. The LR (+) is greater than 1.0, was supports the true positive cases, and the LR (-) is less than 1, which supports the true negative cases. The DNA methylation in true positive cases LR (+) found > 1 in vitamin B9 and B12 marginal and deficiency status. The DNA methylation in true negative cases LR (-) was found to be < 1 in vitamin B9 and B12 marginal and deficiency status.

Table 2. Levels of DNA methylation (5 mC) in the working population with adequate, marginal and deficiency of B vitamins

B Vitamins	Category	Cut-off	n	DNA Methylation Mean (95% CI)	Mean rank
B9 (nmol/L)	Adequate	> 9.1	9	2.98 (0.82-5.15)	162
	Marginal	4.5-9.1	213	1.97 (1.82-2.13)	140
	Deficiency	< 4.5	43	1.07 (0.69-1.45)	90
B12 (pmol/L)	Adequate	> 221	26	2.07 (1.67-2.47)	149
	Marginal	148 – 221	139	2.10 (1.87-2.33)	146
	Deficiency	< 148	100	1.46 (1.24-1.68)	111

Table 3. Details of the true positive and true negative of DNA methylation in the working population

DNA methylation	Cut-off	Vitamin B9 (nmol/L)			Vitamin B12 (pmol/L)		
		A	M	D	A	M	D
True positive	< 2.0	3	124	36	12	78	73
True negative	> 2.0	6	89	7	14	61	27
Total		9	213	43	26	139	100

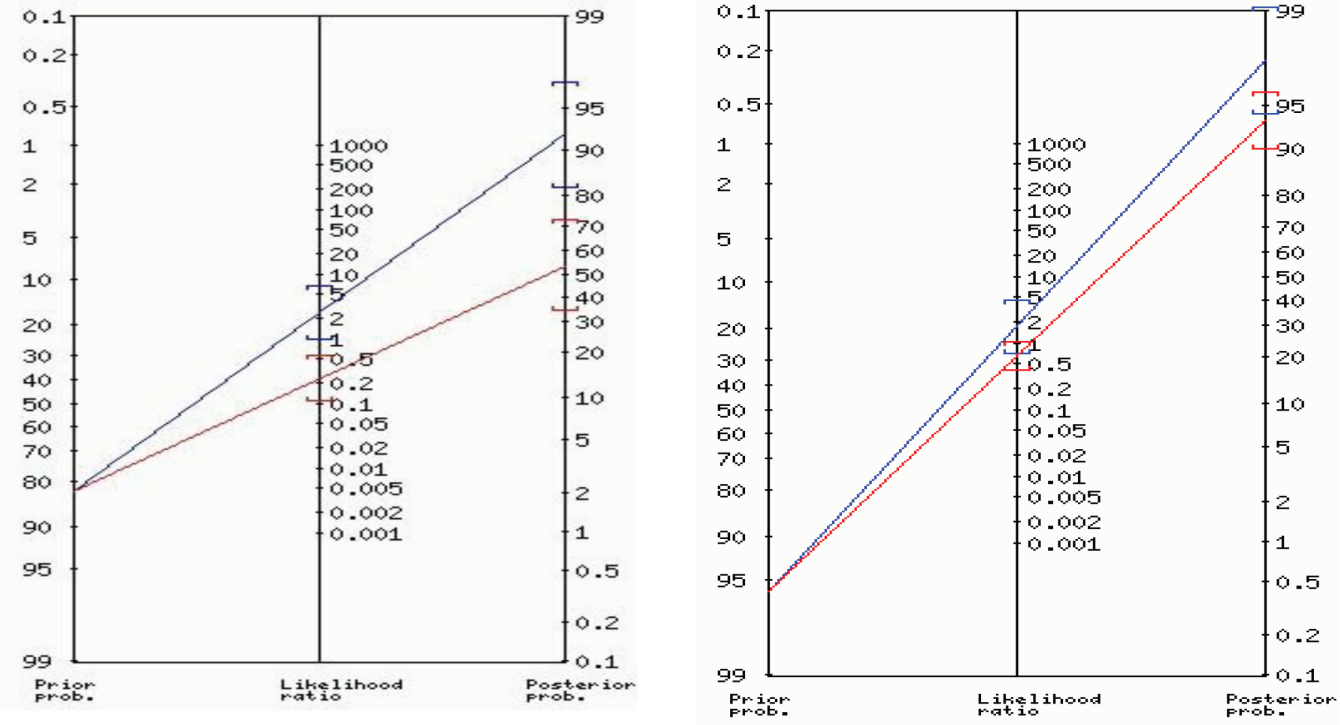
A = adequate, M = marginal status D = deficiency

Figure 1 (a) provides the details of the prior and post-test probability of DNA methylation in B9 deficiency. In the case of DNA methylation being true positive in B9 deficiency, we found prior and post-test probabilities of 83% and 92%, respectively, with an indication of 1 in 1.1 case detection. In the case of true negative DNA methylation in

Table 4. Diagnostic accuracy metrics of DNA methylation in marginal and deficiency status of B vitamins

Variables		Vitamin B9 % (95% CI)		Vitamin B12 % (95% CI)	
		Deficiency	Marginal	Deficiency	Marginal
Diagnostic accuracy	Sensitivity	84 (63-93)	58 (51.-65)	73 (63.-81)	56 (47-64)
	Specificity	67 (30-92)	67 (30-92)	54 (33-73)	54 (33-73)
	PPV	92 (70–92)	97 (26–37)	86 (80–90)	87 (80–91)
	NPV	46 (27–67)	6.3 (4–10)	34 (24-46)	19 (13–26)
	LR (+)	2.51 (0.99–6.39)	1.75 (0.7–4.4)	1.58 (1.03–2.44)	1.22 (0.78–1.89)
	LR (-)	0.24 (0.11–0.55)	0.98 (0.4–1.02)	0.5 (0.31–0.81)	0.82 (0.54–1.22)
	DOR	10.45 (2.06-51.2)	2.8 (0.68-11.4)	3.16 (1.29-7.66)	1.49 (0.64-3.45)
	Prevalence	83	96	79	84

PPV = Positive Predictive Value, NPV = Negative Predictive Values, LR^+ = Likelihood Ratio Positive, LR^- = Likelihood Ratio Negative, DOR = Diagnostic Odd Ratio



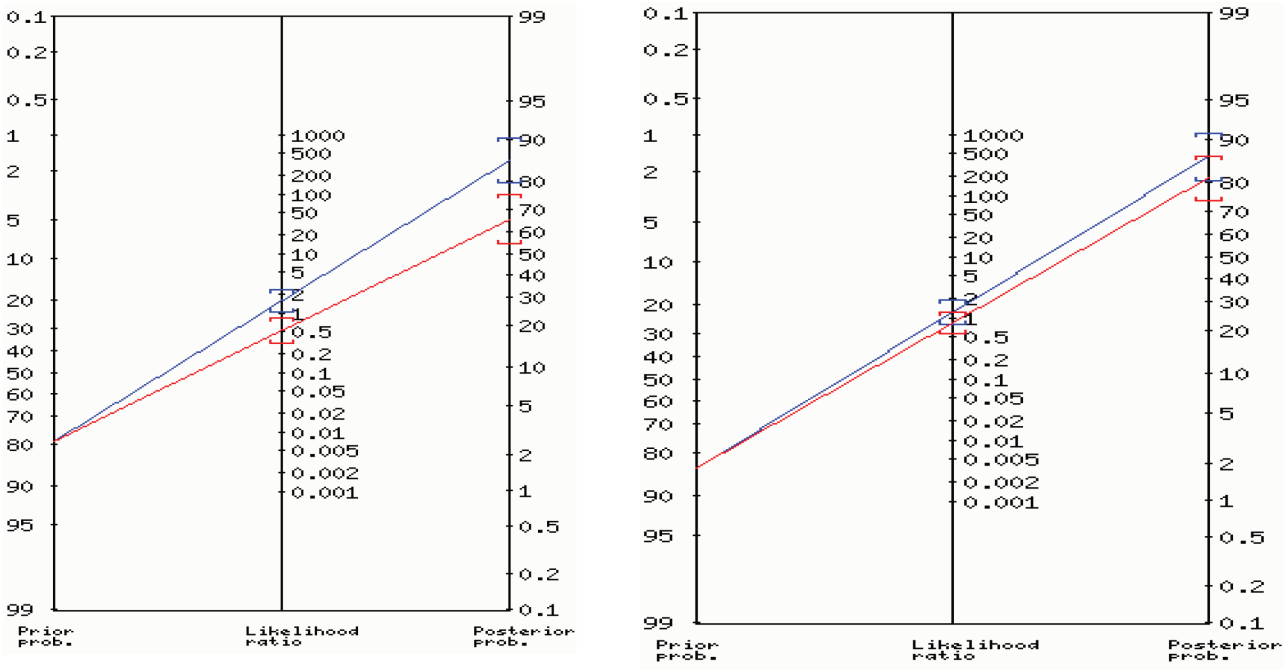
1(a) 1(b)

Figure 1: **(a)** Fagan nomogram with true positive (blue colour) and true negative (red colour) cases of DNA methylation in vitamin B9 deficient workers. **(b)** Fagan nomogram with true positive (blue colour) and true negative (red colour) cases of DNA methylation in vitamin B9 marginal status workers.

B9 deficiency, we noted the post-test probability was 53%, which indicates case detection 1 in 2.1.

Figure 1 (b) shows the details of the prior and post-test probability of DNA methylation in vitamin B9 marginal status. In the case of DNA methylation being true positive in the marginal status of B9, we found prior and post-test probabilities of 96% and 98%, respectively, with an indication of case detection of 1 in 1. In the case of true negative DNA methylation, it was noted that the post-test probability was 94%, which indicates 1 in 1.6 cases.

Figure 2 (a) provides the particulars of the prior and post-test probability of DNA methylation in vitamin B12 deficiency. In the case of DNA methylation being



2(a) 2(b)

Figure 2: **(a)** Fagan nomogram with true positive (blue colour) and true negative (red colour) cases of DNA methylation in vitamin B12 deficient status of workers. **(b)** Fagan nomogram with true positive (blue colour) and true negative (red colour) cases of DNA methylation in vitamin B12 marginal status of workers.

true positive in B12 deficiency, we found prior and post-test probabilities of 76% and 86 %, respectively, with an indication of 1 in 1.2 case detection. In the case of true negative DNA methylation in B12 deficiency, it was noted that the post-test probability was 66%, which indicates a 1 in 2.9 chance of detection.

Figure 2 (b) details the prior and post-test probability of DNA methylation in positive and negative B12 marginal status. DNA methylation is truly positive in marginal status B12; we found prior and post-test probabilities of 84% and 87%, respectively, with an indication of 1 in 1.2 case detection. In true negative DNA methylation in the marginal status of B12, it was noted that the post-test probability was 81%, which indicates a 1 in 5.4 case detection.

4. Discussion

In this study, we evaluated the degree of DNA methylation in B vitamin (B9 and B12) deficiency among the working population. Further, we assessed the predictive ability of DNA hypomethylation with B-vitamin deficiency. In the present study, we adopted the ELISA technique for the determination of serum B vitamins and DNA methylation. The analytical strategies of HPLC methods for B vitamins, found detection limits are outside the reference limits,[34] undetectable,[35] and lower than LOQ and need large volume of samples[36]. Regarding B-vitamin analysis in HPLC techniques to attain detection limits and sensitivity in biological materials, a lot of modifications are suggested. In this study, we used immunoassay methods for the estimation of B vitamins (B9 and B12) in the serum samples of the workers. These assays worked on the principle of antibody and antigen reactions and were characterized by high specificity, sensitivity, simplicity, and cost-effectiveness.

There are different methods available for the estimation of 5 mC, including ELISA, HPLC, and TLC mass spectrometry. The gold standard assay for DNA methylation is the HPLC-UV method with hydrolysed DNA samples. This procedure needed specialized equipment and a large amount of DNA (3-10 µg)[37]. Chromatography methods are costly, labour-intensive and produce subpar results. In this study, we employed the ELISA approach to assess 5mC, because it was rapid, easy to use, less expansive, requires less DNA input and detects significant changes in DNA methylation.

In the current study, we categorized the vitamin B12 deficiencies among workers using Allen *et al.*, criteria[32]

and found 37.7% had deficiency, 52.5% had marginal, and 9.8% of workers had adequate. A study from Gulvady et al found 65% of company executives had vitamin B12 deficiency.[20] In the present study, we noted that 37.7% had a deficiency and 52.5 per cent had marginal deficiency status. The deficiency status reported in this study was similar to Siva Prasad *et al.*, study[38]. Bandyopadhyay *et al.*,[39] assessed the prevalence of mild, moderate, and severe categories of vitamin B12 deficiency and found mild at 41% (>201 pmol/L), moderate at 43% (>150–200 pmol/L), and severe at 16% (<150 pmol/L). The prevalence of vitamin B12 deficiency noted in this study was higher due to different criteria applied. In a study from the USA, the adult age group of 20-60 years has reported that 3-6 % had deficiency (<148 pmol/L) and 15-20% had marginal status (>148-221)[40]. In the present study, we noted a higher prevalence of vitamin B12 deficiency in the same age group because the prevalence of B12 deficiency is more common in developing countries. In the present study, we evaluated vitamin B9 status using the Khan and Jilala criteria[33] and found 16.2% of workers had deficiency (<4.5 nmol/L), 80.4% had marginal (>4.5-9.1 nmol/L), and 3.4% had adequate (>9.1 nmol/L). In India, 17% of the population had a vitamin B9 deficiency[41]. The prevalence of B9 deficiency observed in this study is similar to the previous study.

The DA metrics of DNA methylation were evaluated in different cancers[16-19]. In the current work, we examined the DA metrics of DNA methylation in different categories of B vitamin deficiencies. The DA metrics used in this study are sensitivity, specificity, PPV and NPV as per the suggestion of Trevethan[42]. The results of this study found high sensitivity and modest specificity. The authors of previous work suggested that the DA metrics, such as sensitivity, specificity, PPV, and NPV, are influenced by the prevalence[43,44]. These authors suggested using Fagan's nomogram, which provides the LR (+), LR (-), and prior and post-test probabilities and is not dependent on the prevalence of the disease. In evidence-based medicine, LR+ and LR- and prior and post-test probability are important metrics for the prediction of test performance. Dujardin *et al.*,[45] also suggested that the LR improves the clinical decision-making.

In the present study, we evaluated the LR+ and LR- with prior and posttest probabilities of true positive and true negative of DNA methylation in different categories of B vitamin deficiencies (marginal and deficiency). In vitamin B9 deficiency and a true positive of DNA methylation, the posttest probability was found to be 92%, detecting 1 in 1.1 cases. In vitamin B12 deficiency and true positive DNA methylation noted that the posttest probability was 86% and indicated 1 in 1.2 cases. A DOR score above one indicates better performance. The DOR score of DNA methylation in B9 and B12 deficiencies was found to be more than one and indicated better performance.

5. Conclusion

Working population with B vitamins (B9 and B12) deficiency had considerably reduced DNA methylation was noted. The methylation capacity was reduced in the working population with marginal and deficient B vitamins as compared to adequate status. The DA metrics of DNA methylation demonstrated a high sensitivity and a moderate specificity in marginal and deficiency statuses of B vitamins. The DA metrics of LR+, LR-, DOR (LR+/LR-), and post-test probability were precisely predicted in the true positive condition of DNA methylation.

6. Limitations of the Study

The current study evaluated the DA metrics of total genomic DNA methylation (5 mC) in B vitamin (B9 and B12) deficiency. Further research is recommended to evaluate the DA metrics of DNA methylation in B vitamins (B2 and B6), as they are also implicated in the methylation process of OCM.

7. References

1. Lionaki E, Ploumi C, Tavernarakis N. One-Carbon Metabolism: Pulling the strings behind aging and neurodegeneration. Cells. 2022; 11(2). https://doi.org/10.3390/cells11020214 PMid:35053330 PMCid:PMC8773781
2. Ramakrishnan M, Papolu PK, Satish L, Vinod KK, Wei Q, Sharma A, *et al.* Redox status of the plant cell determines epigenetic modifications under abiotic stress conditions and during developmental processes. J Adv Res. 2022; 42:99-116. https://doi.org/10.1016/j.jare.2022.04.007 PMid:35690579 PMCid:PMC9788946
3. Lyon P, Strippoli V, Fang B, Cimmino L. B vitamins and one-carbon metabolism: Implications in human health and disease. Nutrients. 2020; 12(9). https://doi.org/10.3390/nu12092867 PMid:32961717 PMCid:PMC7551072

4. Vaccaro JA, Naser SA. The role of methyl donors of the methionine cycle in gastrointestinal infection and inflammation. Healthcare (Basel). 2021; 10(1). https://doi.org/10.3390/healthcare10010061 PMid:35052225 PMCid:PMC8775811
5. Robertson KD. DNA methylation and human disease. Nat Rev Genet. 2005; 6(8):597-610. https://doi.org/10.1038/nrg1655 PMid:16136652
6. Suzuki MM, Bird A. DNA methylation landscapes: Provocative insights from epigenomics. Nat Rev Genet. 2008; 9(6):465-76. https://doi.org/10.1038/nrg2341 PMid:18463664
7. Łoboś P, Regulska-Ilow B. Link between methyl nutrients and the DNA methylation process in the course of selected diseases in adults. RoczPanstwZaklHig. 2021; 72(2):123-36. https://doi.org/10.32394/rpzh.2021.0157 PMid:34114759
8. Wilson AS, Power BE, Molloy PL. DNA hypomethylation and human diseases. BBA - Rev Cancer. 2007; 1775(1):138-62. https://doi.org/10.1016/j.bbcan.2006.08.007 PMid:17045745
9. Ehrlich M. DNA hypomethylation in cancer cells. Epigenomics. 2009; 1(2):239-59. https://doi.org/10.2217/epi.09.33 PMid:20495664 PMCid:PMC2873040
10. Hanson M, Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD. Developmental plasticity and developmental origins of non-communicable disease: Theoretical considerations and epigenetic mechanisms. Prog Biophys Mol Biol. 2011; 106(1):272-80. https://doi.org/10.1016/j.pbiomolbio.2010.12.008 PMid:21219925
11. Ciechomska M, Roszkowski L, Maslinski W. DNA Methylation as a future therapeutic and diagnostic target in rheumatoid arthritis. Cells. 2019; 8(9). https://doi.org/10.3390/cells8090953 PMid:31443448 PMCid:PMC6770174
12. Deaton AM, Webb S, Kerr AR, Illingworth RS, Guy J, Andrews R, *et al*. Cell type-specific DNA methylation at intragenic CpG islands in the immune system. Genome Res. 2011; 21(7):1074-86. https://doi.org/10.1101/gr.118703.110 PMid:21628449 PMCid:PMC3129250
13. Lee DH, Jacobs DR Jr, Porta M. Hypothesis: A unifying mechanism for nutrition and chemicals as lifelong modulators of DNA hypomethylation. Environ Health Perspect. 2009; 117(12):1799-802. https://doi.org/10.1289/ehp.0900741 PMid:20049195 PMCid:PMC2799450
14. Eusebi P. Diagnostic accuracy measures. Cerebrovasc Dis. 2013; 36(4):267-72. https://doi.org/10.1159/000353863 PMid:24135733
15. Caraguel CG, Vanderstichel R. The two-step Fagan's nomogram: Ad hoc interpretation of a diagnostic test result without calculation. Evid Based Med. 2013; 18(4):125-8. https://doi.org/10.1136/eb-2013-101243 PMid:23468201
16. Hu W, Zheng W, Liu Q, Chu H, Chen S, Kim JJ, *et al*. Diagnostic accuracy of DNA methylation in detection of gastric cancer: A meta-analysis. Oncotarget. 2017; 8(68):113142-113152. https://doi.org/10.18632/oncotarget.22613 PMid:29348893 PMCid:PMC5762578
17. Ji X, Guan C, Jiang X, Li H. Diagnostic accuracy of DNA methylation for head and neck cancer varies by sample type and number of markers tested. Oncotarget. 2016; 7(48):80019-32. https://doi.org/10.18632/oncotarget.12219 PMid:27683120 PMCid:PMC5346768
18. Li M, Gao F, Xia Y, Tang Y, Zhao W, Jin C, *et al*. Filtrating colorectal cancer associated genes by integrated analyses of global DNA methylation and hydroxymethylation in cancer and normal tissue. Sci Rep. 2016; 6. https://doi.org/10.1038/srep31826 PMid:27546520 PMCid:PMC4992821
19. Liu B, RicarteFilho J, Mallisetty A, Villani C, Kottorou A, Rodgers K, *et al*. Detection of promoter DNA methylation in urine and plasma aids the detection of non-small cell lung cancer. Clin Cancer Res. 2020; 26(16):4339-48. https://doi.org/10.1158/1078-0432.CCR-19-2896 PMid:32430478 PMCid:PMC7442601
20. Gulvady C, Pingle S, Shanbhag S. Incidence of vitamin B12/D3 deficiency among company executives. Indian J Occup Environ Med. 2007; 11(2):83-5. https://doi.org/10.4103/0019-5278.34535 PMid:21938222 PMCid:PMC3168103
21. Mahajan A, Sapehia D, Thakur S, Mohanraj PS, Bagga R, Kaur J. Effect of imbalance in folate and vitamin B12 in maternal/parental diet on global methylation and regulatory miRNAs. Sci Rep. 2019; 9(1). https://doi.org/10.1038/s41598-019-54070-9 PMid:31772242 PMCid:PMC6879517
22. Brunaud L, Alberto JM, Ayav A, Gérard P, Namour F, Antunes L, *et al*. Vitamin B12 is a strong determinant of low methionine synthase activity and DNA hypomethylation in gastrectomized rats. Digestion. 2003; 68(2-3):133-40. https://doi.org/10.1159/000075307 PMid:14646334
23. Boughanem H, Hernandez-Alonso P, Tinahones A, Babio N, Salas-Salvadó J, Tinahones FJ, *et al*. Association between serum Vitamin B12 and global DNA methylation in colorectal cancer patients. Nutrients. 2020; 12(11). https://doi.org/10.3390/nu12113567 PMid:33233812 PMCid:PMC7709022
24. Zhu Y, Minović I, Dekker LH, Eggersdorfer ML, van Zon SKR, Reijneveld SA, *et al*. Vitamin status and diet in elderly with low and high socioeconomic status: The lifelines-minuthe study. Nutrients. 2020; 12(9). https://doi.org/10.3390/nu12092659 PMid:32878227 PMCid:PMC7551314
25. Tanaka K, Ao M, Kuwabara A. Insufficiency of B vitamins with its possible clinical implications. J Clin Biochem Nutr. 2020; 67(1):19-25. https://doi.org/10.3164/jcbn.20-56 PMid:32801464 PMCid:PMC7417807
26. Chahal JS, Raina SK, Sharma KK, Kaur N. How common is Vitamin B12 deficiency-A report on deficiency among

healthy adults from a medical college in rural area of North-West India. Int J Nutr Pharmacol Neurol Dis. 2014; 4(4):241-5. https://doi.org/10.4103/2231-0738.139406
27. Jansen EHJM, Beekhof PK. Stability of folate and Vitamin B12 in human serum after long-term storage: A follow-up after 13 years. J Nutr Metab. 2018; 2018. https://doi.org/10.1155/2018/9834181 PMid:29725544 PMCid:PMC5872588
28. Kalahasthi R, Adepu VK, Nagaraju R. Assessment of diagnostic accuracy and clinical utility of DNA methylation (5-mC) in detecting severity of occupational lead exposure. Indian J Clin Biochem. 2023:1-7. https://doi.org/10.1007/s12291-023-01138-z
29. MedCalc Software Ltd. Diagnostic test evaluation calculator. Available from: https://www.medcalc.org/calc/diagnostic_test.php
30. Diagnostic test calculator. Available from: http://araw.mede.uic.edu/cgi-bin/testcalc.pl?DT=&Dt=&dT=&dt=&2x2=Compute((version 2010042101)
31. MedCalc Software Ltd. Odds ratio calculator. https://www.medcalc.org/calc/odds_ratio.php (Version 22.017).
32. Allen LH. How common is vitamin B-12 deficiency? Am J Clin Nutr. 2009; 89(2):693S-6S. https://doi.org/10.3945/ajcn.2008.26947A PMid:19116323
33. Khan KM, Jialal I. Folic acid deficiency. Treasure Island(FL): StatPearls Publishing; 2023.
34. Giorgi MG, Howland K, Martin C, Bonner AB. A novel HPLC method for the concurrent analysis and quantitation of seven water-soluble vitamins in biological fluids (plasma and urine): A validation study and application. Sci World J. 2012: 1-8. https://doi.org/10.1100/2012/359721 PMid:22536136 PMCid:PMC3317597
35. Chatzimichalakis PF, Samanidou VF, Verpoorte R, Papadoyannis IN. Development of a validated HPLC method for the determination of B-complex vitamins in pharmaceuticals and biological fluids after solid phase extraction. J Sep Sci. 2004; 27(14):1181-88. https://doi.org/10.1002/jssc.200401858 PMid:15537074
36. Shaik MM, Gan SH. Rapid resolution liquid chromatography method development and validation for simultaneous determination of homocysteine, Vitamins B6, B9, and B12 in human serum. Indian J Pharmacol. 2013; 45(2). https://doi.org/10.4103/0253-7613.108303 PMid:23716893 PMCid:PMC3660929
37. Martisova A, Holcakova J, Izadi N, Sebuyoya R, Hrstka R, Bartosik M. DNA methylation in solid tumors: Functions and methods of detection. Int J Mol Sci. 2021; 22(8). https://doi.org/10.3390/ijms22084247 PMid:33921911 PMCid:PMC8073724
38. Sivaprasad M, Shalini T, Reddy PY, Seshacharyulu M, Madhavi G, Kumar BN, *et al*. Prevalence of vitamin deficiencies in an apparently healthy urban adult population: Assessed by subclinical status and dietary intakes. Nutrition. 2019; 63-64:106-113. https://doi.org/10.1016/j.nut.2019.01.017 PMid:30954757
39. Bandyopadhyay D, Choudhury JR, Mukherjee K. Vitamin B12 deficiency in Eastern India: A hospital based cross-sectional study. J Clin Diagn Res. 2021; 15(4);1-4. https://doi.org/10.7860/JCDR/2021/46642.14685
40. Shipton MJ, Thachil J. Vitamin B12 deficiency - A 21st century perspective. Clin Med (Lond). 2015; 15(2):145-50. https://doi.org/10.7861/clinmedicine.15-2-145 PMid:25824066 PMCid:PMC4953733
41. Khanduri U, Sharma A, Joshi A. Occult cobalamin and folate deficiency in Indians. Natl Med J India. 2005; 18(4):182-3.
42. Trevethan R. Sensitivity, specificity, and predictive values: Foundations, pliabilities, and pitfalls in research and practice. Front Public Health. 2017; 5. https://doi.org/10.3389/fpubh.2017.00307 PMid:29209603 PMCid:PMC5701930
43. Brena RM, Huang TH, Plass C. Quantitative assessment of DNA methylation: Potential applications for disease diagnosis, classification, and prognosis in clinical settings. J Mol Med (Berl). 2006; 84(5):365-77. https://doi.org/10.1007/s00109-005-0034-0 PMid:16416310
44. Safari S, Baratloo A, Elfil M, Negida A. Evidence based emergency medicine; Part 5 Receiver operating curve and area under the curve. Emerg (Tehran). 2016; 4(2):111-3.
45. Dujardin B, Van den Ende J, Van Gompel A, Unger JP, Van der Stuyft P. Likelihood ratios: A real improvement for clinical decision making? Eur J Epidemiol. 1994; 10(1):29-36. https://doi.org/10.1007/BF01717448 PMid:7957786