Folic Acid and Vitamin B12 Interplay in Pregnant women with Hypothyroidism: Its Effects on Thyroid Hormones and Placental DNA Methylation

Dr. Srilatha Bashetti 1* , Dr. Aparna Varma 2 , Dr. Vijaya Raghavan 3 , Dr. Neetha Kundoor 4 , Dr. Raghavendra Rao M.V. 5

1*Associate Professor Department of Biochemistry, Apollo Institute of Medical Sciences & Research, Hyderabad, Telangana
2 Professor, Department of Biochemistry, All India Institute of Medical Sciences, Bibinagar, Telangana, India.
3 Professor, Department of Research, Saveetha Institute of Medical Sciences, Tamil Nadu, India.
4 Professor, Department of Biochemistry, Apollo Institute of Medical Sciences & Research, Hyderabad, Telangana
5 Professor and Vice-President, World Academy of Medical Sciences, Netherlands (Europe)

Corresponding Author:
Dr. Srilatha Bashetti

Abstract
Background: Adequate folate and vitamin B12 intake during pregnancy is essential to prevent long-term maternal and fetal outcomes. Emerging evidence suggests that excessive folic acid combined with vitamin B12 deficiency may lead to epigenetic modifications, potentially affecting newborn development and increasing the risk of chronic diseases. Materials and Methods : Women aged 21–45 years attending antenatal clinics from the first trimester or upon pregnancy confirmation were enrolled. Serum levels of FT3, FT4, TSH, folic acid, and vitamin B12 were measured across all trimesters and at delivery, along with cord blood sampling. DNA methylation analysis of MTHFR and TCN2 genes was performed on 16 placental samples. Statistical analysis was conducted using SigmaPlot 13, including ANOVA, Pearson's and Spearman's correlation, and ROC curve analysis. Results: TSH levels were significantly elevated in subclinical and overt hypothyroid groups across all trimesters (p < 0.001). In cord blood, the folic acid/B12 ratio negatively correlated with FT3 and FT4 in control and subclinical hypothyroid (SCH) groups, and positively with TSH in the SCH group (p < 0.033). Methylation analysis revealed low CpG methylation in MTHFR and TCN2, methylation at promoter site in one sample. Conclusion: Balanced folic acid and B12 intake is critical in pregnancy. High folic acid with B12 deficiency may disrupt thyroid hormone levels and induce subtle epigenetic effects, recommending broader studies on global methylation and gene expression.

Keywords: Vitamin B12, Folic acid, Micronutrients, Pregnancy, Methylation.

Introduction

Folic acid, a water-soluble B vitamin, plays an essential role in one-carbon metabolism by transferring single-carbon units through tetrahydrofolate (THF), specifically via its N5 and N10 positions. These processes are vital for DNA synthesis, amino acid metabolism, and cellular growth. During pregnancy, the demand for folate significantly increases—often up to five times—making dietary sources insufficient. As a result, folic acid supplementation, especially in the first trimester, is strongly recommended to prevent neural tube defects (NTDs), which affect the brain and spinal cord.[1]Vitamin B12 (cobalamin), another crucial water-soluble vitamin, is necessary for converting homocysteine to methionine and regenerating THF from methyl-THF. This process, catalyzed by homocysteine methyltransferase, is essential for nucleotide synthesis. A deficiency in vitamin B12 can lead to the "methyl-folate trap," where methyl-THF accumulates, reducing the availability of folate derivatives required for DNA synthesis. Thus, B12 deficiency can indirectly cause functional folate deficiency.Vitamin B12 deficiency is also linked to neurological damage, including peripheral neuropathy and central nervous system impairment. One proposed mechanism involves the accumulation of homocysteine, which may inhibit methylation processes in neural tissue. Cobalamin deficiency during pregnancy has been associated with impaired cognitive development in children, as shown in studies such as Bonilla et al. (2012), which reported lower cognitive test scores in children born to mothers with low B12 intake[2].

Thyroid hormones are vital during pregnancy for fetal growth and brain development. Hypothyroidism, including subclinical forms, can cause menstrual irregularities, infertility, miscarriage, and fetal growth retardation[3]. Thyroid hormones also influence gene expression and DNA methylation, suggesting a possible interaction between thyroid status and folate/Vitamin B12 metabolism.While folic acid supplementation reduces the risk of NTDs, excessive intake without adequate B12 may disrupt thyroid function. Animal studies indicate that high folate levels may suppress thyroid hormone production[4]. Moreover, a high folate-to-B12 ratio has been linked to global DNA hypomethylation and developmental abnormalities in animal models. In breastfeeding mothers, B12 deficiency can lead to neurological delays in infants[5,6].Folate can mask the hematological symptoms of B12 deficiency, potentially delaying diagnosis and worsening neurological outcomes[7]. Balancing folate and B12 intake is essential to avoid long-term maternal and fetal health risks. As few studies had stated that excess folic acid with low vitamin B12 lead to epigenetic changes which may further have imprinting effect in the new born, expressed in their later life causing certain chronic diseases [8,9]. These study explores folate and B12 status in pregnancy, their influence on thyroid hormones, and placental DNA methylation in key genes such as *MTHFR* and *TCN2*.

Methodology

The study was conducted at the Apollo general hospital, Department of Obstetrics and Gynecology and Department of Biochemistry, Apollo Institute of Medical Sciences and Research (AIMSR), Jubilee Hills, Hyderabad, Telangana. The study was approved by the Ethics Committee. Participants were recruited after taking the written informed consent. This study included women of reproductive age; 21-45 years. Pregnant women attending antenatal clinic for a routine antenatal care from 1st trimester or at the time of confirmation of pregnancy done by ultrasound report were included in this study. Screening was done using structured interview questionnaire. The pregnant women satisfying both inclusion and exclusion criteria were included in the study. The patients were assured of data confidentiality. Participants were recruited after taking the written informed consent. The patient information sheet and consent form were prepared in three languages (English, Telugu, Urdu) Permission was also obtained from the hospital authority for the sample and data collection.The total number of participants were 245 among them 68 (27.75%) were controls, 92 (37.55%) were subclinical hypothyroid and 85 (34.69%) were overt hypothyroid group. The distribution of participants into groups was done based on the biochemical values of FT3, FT4 and TSH. For the study of Placental DNA methylation the placenta was collected immediately after delivery from all the participants, transported to the laboratory in ice packs and stores at -80°C in normal saline until DNA isolation.

All the pregnant women satisfying the inclusion criteria were included in the study. The study period was approximately 9 months for each participant, from the time of pregnancy confirmation or from first trimester to till the time of delivery. A structured interview questionnaire was used to collect information on maternal socio-demographic, age, residence, health history, smoking habit, medical and reproductive life style, characteristics and food frequency including alcohol intake, habit and knowledge of consuming iodized salt. Fasting venous sample was collected from the participant for the biochemical analysis of thyroid hormones (FT3, FT4 and TSH), Folic acid and Vitamin B_{12} thrice, during first, second and third trimester respectively. After delivery cord blood is collected and processed for vitamin B12 and Folic acid. First trimester samples were processed immediately, whereas second and third trimester samples were processed in batch. Based on the biochemical results of thyroid hormones the participants are grouped as controls, subclinical hypothyroid and hypothyroid subjects. All pregnancies were dated according to ultrasonographic measurement of the foetal growth - rump length during the first trimester till the time of delivery.

SAMPLING: 5ml each of early morning fasting venous blood specimens was collected from all the subjects in sterile silicon coated glass tube. The serum sample is appropriately labelled and stored at-80°C until batch analysis for FT_3, FT_4, TSH, Folate and Vitamin B_{12} is done. The sample was collected thrice from each individual-once in first trimester, second trimester and in third trimester or at the time of delivery respectively.

Cord blood: 3ml of cord blood was drawn into disposable plain polystyrene tubes which was collected after delivery, was immediately processed for folic acid and vitamin B12 parameters.

Placental DNA Methylation: Placenta was collected immediately after delivery, transported to the laboratory in ice packs. 10g of the placental tissue from the fetal side is separated and washed and stored in normal saline (0.9% Sodium chloride) at -80°C for DNA isolation. Only 16 randomly selected placental samples of hypothyroid women were taken for methylation study. Estimated parameters are serum FT3, FT4, and TSH, were estimated on SEIMENS ADVIA centaur CP chemiluminescence immune assay (CLIA). Vitamin $B_{12}$and Folic acid were estimated on Elecsys and Cobase electrochemiluminescence immunoassay (ECLIA).

DNA Extraction- QIAamp DNA Mini Kit (QIAGEN, cat. no. 51304) is used**.**

Estimation of DNA: The DNA was studied qualitatively and quantitatively before using it for molecular analyses. Nucleic acid (DNA) has maximum Absorbance at 206nm. One OD value (standard) corresponds approximately 50 mg/ml of double standard DNA. The estimation of DNA was done with the help of spectrophotometer.

PCR was used to amplify TCN_2 gene and MTHFR gene fragments of isolated DNA. 4 sets of primers consisting of forward and reverse reading frames were used.

Bisulfite Sequencing: Bisulfiteconversionisthegold-standardmethodformethylation detection at single base resolution.TreatinggenomicDNAwithbisulfitecauses the deamination of unmethylated cytosinestouracil, while their methylated counter parts remain resistant to the effects of the reagent. The basic principle behind this sequencing technique is that bisulfite treatment converts cytosine to uracil and when the single stranded DNA is subsequently amplified via PCR and sequenced, the uracil residue is recognized as thymine; conversely 5-methylcytosine is not affected by bisulfite and read as cytosine only. EZ DNA Methylation™ Kit was used.

Data Analysis:

Data was analyzed by using SigmaPlot 13.0 (Systat software, USA). Normally distributed data was represented as mean ± standard error and categorical variables as number (percentages). Comparison of FT3, FT4, TSH, folic acid and vitamin B_{12} within the group was estimated by using unpaired one-way ANOVA and between the groups by using paired one way repeated measures of ANOVA. To study the correlation between folic acid and FT3, FT4 and TSH Pearson correlation was used for the normally distributed data. Non-parametric statistics was used for the ratio analysis of folic acid and vitamin B_{12} with thyroid hormones in all the three trimesters and all the three groups by Spearman's evaluation. The association of the parameters with ratio p <0.05 was considered as significant. To consider the test results between two populations (control with subclinical hypothyroid pregnant women) and (control with overt hypothyroid pregnant group) we used receiver operating characteristic (ROC) curve analysis. p<0.05 was considered to be significant, it explains the area under the ROC curve is evident that the tests values obtained does have the ability to distinguish between two groups and helps in the diagnosis of subclinical hypothyroidism and overt hypothyroidism in pregnant women with respect to the ratio of vitamin B_{12} and folic acid.

Results:

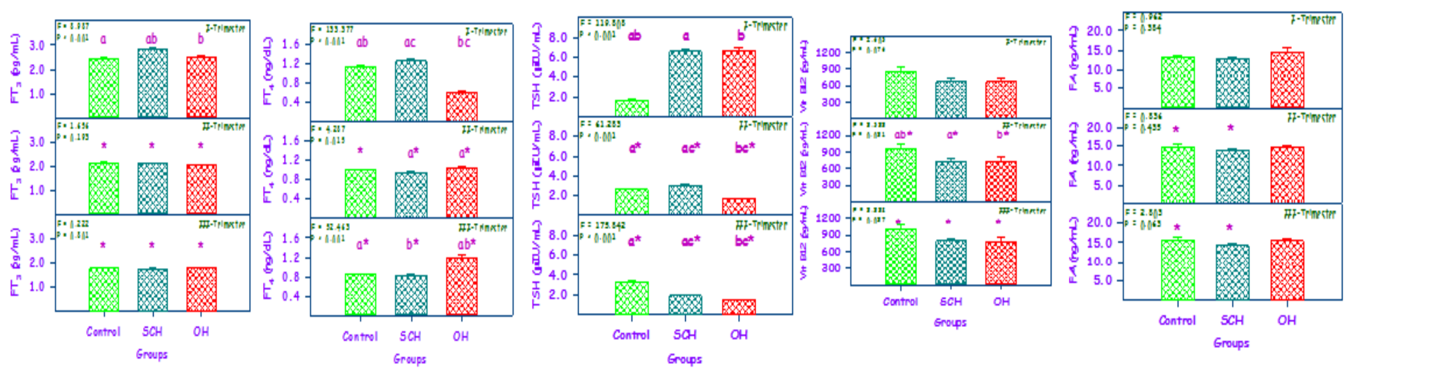
The study assessed FT3, FT4, TSH, folic acid, and vitamin B12 in pregnant women with subclinical (SCH) and overt hypothyroidism (OH) across the 3 trimesters. FT3 levels showed significant variation between control verses SCH and OH verses SCH in the first trimester ($p < 0.001$; Table1, Figure 1), while intra-group differences were significant across all trimesters ($p < 0.001$; Figure 1). FT4 levels varied significantly between all groups in the first and third trimesters ($p < 0.001$; Table 1, Figure 1), with significant intra-group changes across trimesters (Figure 1). TSH levels were significantly elevated in SCH and OH groups across all trimesters compared to controls ($p < 0.001$; Table 1, Figure 2), with significant within-group changes across trimesters (Figure1).

Table 1 Correlation of serum FT3 with TSH, FT4 with TSH and FT3 with FT4 in I, II and III trimester of Control, Subclinical Hypothyroidism (SCH) and Overt hypothyroidism (OH)

Trimester	FT3 with TSH			FT4 with TSH			FT3 with FT4		
	Control	SCH	OH	Control	SCH	OH	Control	SCH	OH
I Trimester	r= 0.105 p=0.393	r= 0.080 p=0.446	r= 0.214 p=0.049	r= -0.006 p=0.964	r= -0.159 p=0.029	r= -0.376 p < 0.001	r= 0.118 p=0.339	r= 0.317 p=0.002	r= -0.030 p=0.783
II Trimester	r= 0.176 p=0.151	r= 0.090 p=0.407	r= 0.099 p=0.369	r= 0.064 p=0.605	r= -0.186 p=0.083	r= 0.006 p=0.960	r= 0.086 p=0.483	r= 0.194 p=0.071	r= 0.144 p=0.187
III Trimester	r= -0.232 p=0.057	r= -0.080 p=0.459	r= 0.320 p=0.003	r= -0.060 p=0.628	r= -0.248 p=0.020	r= 0.075 p=0.494	r= -0.150 p=0.223	r= -0.138 p=0.198	r= 0.035 p=0.747

SCH (Subclinical hypothyroid); OH (overt Hypothyroid)

Figure 1 : The levels of FT3, FT4, TSH, Vitamin B12 & Folic acid in control, subclinical and overt Hypothyroid patients of I, II & III trimesters.



Mean $\pm$ SE (n – control = 65; SCH = 88; OH = 95). The 'F' and 'P' values are comparing control, SCH and OH of the same trimester by one way ANOVA. Same alphabetical characters are mutually significant. The FT3, FT4, TSH, Vitamin B12 and Folic acid levels were compared for I, II and III trimester by one way repeated measures ANOVA. *Significantly different from I trimester;

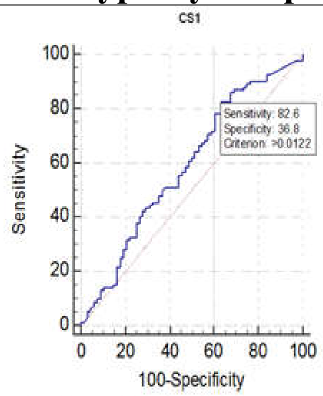
Folic acid and vitamin B12 levels showed no significant differences between groups in most trimesters (Tables 2; Figures 1), although both showed significant intra-group changes in control and SCH groups ($p < 0.001$), but not in OH. Vitamin B12 was significantly reduced in SCH and OH during the second trimester ($p < 0.031$; Table 2, Figure 1). Spearman correlation analyses revealed significant associations between maternal FT3/FT4 and the folic acid-vitamin B12 ratio in various groups and trimesters (Tables 2), especially in SCH and OH ($p < 0.001$).

Table 2. Pearson Correlation and Spearman Correlation of the ratio of folic acid and vitamin B12 of I, II and III trimesters with serum free T3, free T4 and TSH in I, II and III trimester of Control, Subclinical Hypothyroidism (SCH) and Overt hypothyroidism (OH)

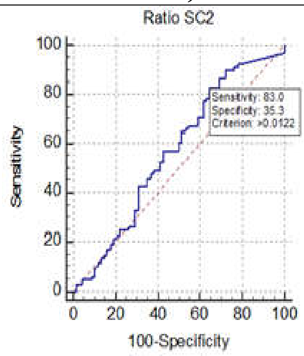
	I trimester			II trimester			III trimester		
Parameter	**Control (n=68)**	**SCH (n=92)**	**OH (n=85)**	**Control (n=68)**	**SCH (n=88)**	**OH (n=85)**	**Control (n=68)**	**SCH (n=88)**	**OH (n=85)**
Pearson correlation									
Ratio of FA& B12 with FT3	r= -0.223 *p*=0.068	r= -0.340 *p*<0.001	r= -0.309 *p*<0.004	r= -0.325 p <0.007	r= -0.055 p = 0.614	r= -0.375 *p*<0.001	r= -0.399 *p* <0.001	r= -0.264 *p*<0.013	r= 0.006 *p*=0.957
Ratio of FA & B12 withFT4	r= -0.137 *p*=0.267	r = -0.123 p = 0.245	r= 0.066 *p*=0.550	r= -0.307 p=0.011	r = -0.009 p = 0.933	r= -0.081 *p*=0.459	r= -0.416 *p <0.001*	r = -0.187 p = 0.081	r= -0.088 *p*=0.422
Ratio of FA & B12 withTSH	r= 0.104 *p*=0.399	r= 0.173 *p*=0.099	r= 0.174 *p*=0.112	r= 0.011 *p*=0.932	r= 0.025 *p*=0.817	r= 0.138 *p*=0.209	r= -0.069 *p*=0.578	r= 0.207 *p*=0.053	r= 0.061 *p*=0.581
Spearman correlation									
Ratio of FA& B12 with FT3	r= -0.230 *p*=0.059	r= -0.411 *p*<0.001	r= -0.579 *p*<0.001	r= -0.332 *p*=0.006	r= -0.483 *p*<0.001	r= -0.577 *p*<0.001	r= -0.511 *p <0.001*	r= -0.474 *p*<0.001	r= -0.624 *p*<0.001
Ratio of FA & B12 withFT4	r= -0.226 *p*=0.064	r = -0.319 p = 0.002	r= -0.020 *p*=0.857	r= -0.329 *p*=0.006	r = -0.336 p <0.001	r= -0.072 *p*=0.510	r= -0.449 *p <0.001*	r = -0.499 p <0.001	r= -0.126 *p*=0.251
Ratio of FA & B12 withTSH	r= 0.116 *p*=0.347	r= 0.225 *p*=0.015	r= 0.124 *p*=0.256	r= -0.119 *p*=0.333	r= 0.162 *p*=0.130	r= 0.190 *p*=0.081	r= -0.142 *p*=0.248	r= 0.155 *p*=0.150	r= 0.161 *p*=0.141

ROC analysis using the vitamin B12/folic acid ratio showed modest diagnostic utility for SCH and OH with low specificity and moderate sensitivity across trimesters (Figures 2.a; AUC range 0.535–0.632).ROC analysis of cord blood biomarkers showed low diagnostic accuracy for hypothyroidism (AUC ~0.5) (Figures 2.b).

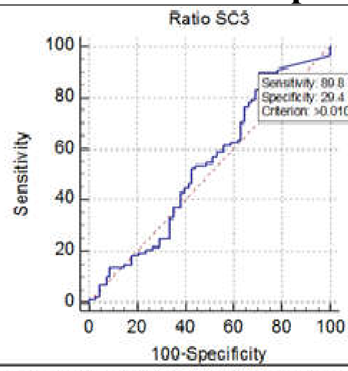
Figure 2.a: ROC Curve- Control with subclinical hypothyroid and control with overt Hypothyroid pregnant women in first, second and third trimesters respectively



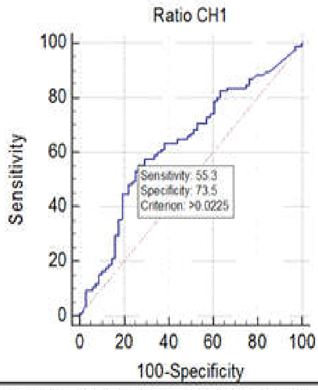
CS1- Control with subclinical hypothyroid pregnant women in first trimester. $p<0.05$ was considered significant



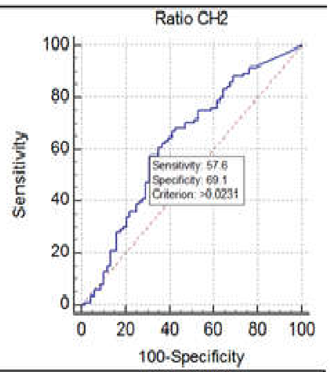
CS2- Control with subclinical hypothyroid pregnant women in second trimester. $p<0.05$ was considered significant



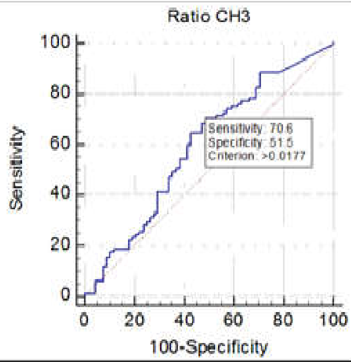
CS3- Control with subclinical hypothyroid pregnant women in third trimester. $p<0.05$ was considered significant



CH1- Control with overt hypothyroid pregnant women in first trimester . $p<0.05$ was considered significant

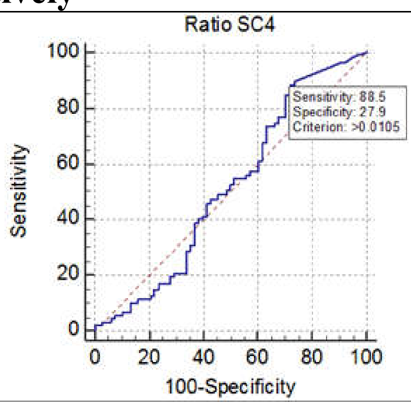


CH2- Control with overt hypothyroid pregnant women in second trimester. $p<0.05$ was considered significant

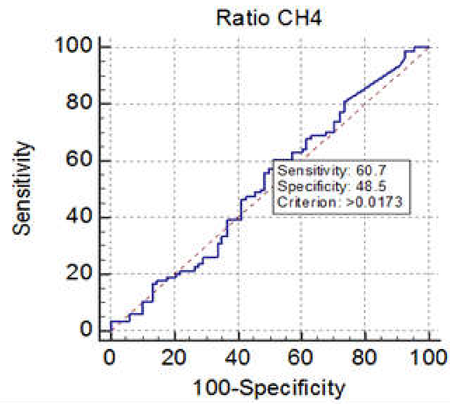


CH3- Control with overt hypothyroid pregnant women in third trimester. $p<0.05$ was considered significant

Figure 2.b: ROC curve in cord blood between control and subclinical and between control and overt hypothyroid pregnant women with ratio of folic acid and Vitamin B12, respectively



CS4- Control with subclinical hypothyroid pregnant women in cord blood. $p<0.05$ was considered significant



CH4- Control with overt hypothyroid pregnant women in cord blood. $p<0.05$ was considered significant

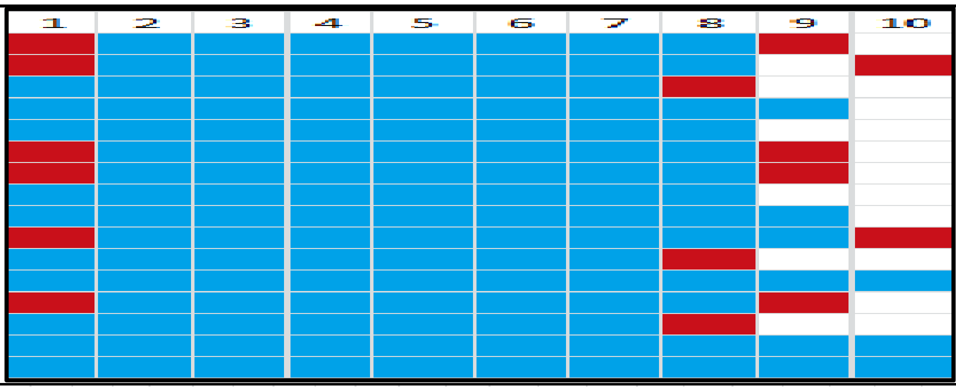
Cord blood analysis showed no correlation of folic acid with maternal thyroid hormones, while cord vitamin B12 significantly correlated with maternal FT3 in all groups and FT4 only in controls ($p < 0.001$; Table 3). The folic acid/B12 ratio in cord blood correlated negatively with FT3 and FT4 in control and SCH groups, and positively with TSH only in SCH ($p < 0.033$; Table 3) on Spearman correlation.

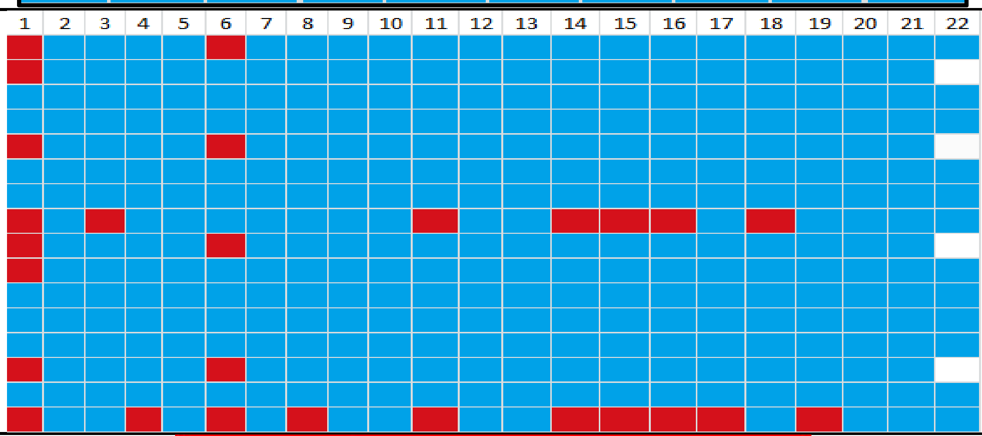
Table 3: Pearson Correlation and Spearman correlation of the cord blood ratio of folic acid and vitamin B_{12} with serum fT3, fT4 and TSH of III trimester of Control, Subclinical Hypothyroidism (SCH) and Overt hypothyroidism (OH)

Parameter	Cord Blood ratio of folic acid and vitamin B_{12}		
	Control (n=68)	SCH (n=88)	OH (n=85)
Pearson correlation			
III FT3	r= -0.410 p <0.001	r= -0.240 *p* =0.025	r= 0.092 *p*=0.403
III FT4	r= -0.433 p <0.001	r = -0.055 p = 0.614	r= -0.013 *p*=0.904
III TSH	r= -0.139 *p*=0.259	r= -0.069 *p*=0.526	r= -0.002 *p*=0.988
Spearman correlation			
III FT3	r= -0.464 p <0.001	r= -0.507 *p*<0.001	r= -0.562 *p*<0.001
III FT4	r= -0.455 p <0.001	r = -0.390 p <0.001	r= -0.096 *p*=0.386
III TSH	r= -0.181 *p*=0.140	r= 0.229 *p*=0.033	r= 0.043 *p*=0.699

DNA methylation analysis of placental *MTHFR* and *TCN2* genes revealed no significant difference in methylation levels ($p = 0.421$; Table 4), with low methylation at CpG sites (7.8% in *MTHFR*; 10.5% in *TCN2* (Tables 4), suggesting minimal epigenetic changes in these genes in hypothyroid pregnancies (Table 4). Out of 16 samples one sample showed methylation at promoter site.

Table 4: Association of MTHFR and TCN2 with respect to methylated and unmethylated CpG sites		
Unmethylated	321	128
Methylated	27	15
χ2 = 0.649; df = 1; *p* = 0.421. MTHFR- Methyltetrahydofolatereductase; TCN2- transcobalamin II		
Average methylation of all the sequences (MTHFR)		
DNA methylation summary overall sequences		
Unmethylated CpGs	92.20%	321 cases
Methylated CpGs	7.80%	27 cases
DNA methylation summary overall sequences		
CpGsanalysed	98.80%	348 cases
CpGs not analysed	1.20%	4 cases
Average methylation of all the sequences (TCN2)		
DNA methylation summary overall sequences		
Unmethylated CpGs	89.50%	128 cases
Methylated CpGs	10.50%	15 cases
DNA methylation summary overall sequences		
CpGsanalysed	89.40%	143 cases
CpGs not analysed	10.60%	17 cases
Methylation patterns and nucleotide positions TCN2, MTHFR		





Discussion:

Folate and vitamin B12 are critical micronutrients during pregnancy, involved in one-carbon metabolism and DNA methylation[10]. Although folic acid supplementation is widely acknowledged for its role in preventing neural tube defects (NTDs), concerns have emerged about its overuse, especially when accompanied by inadequate vitamin B12 intake[11]. This study investigated the relationship between folic acid, vitamin B12, and thyroid hormone fluctuations in pregnant women with subclinical (SCH) and overt hypothyroidism (OH), to assess whether imbalances in these micronutrients affect thyroid function and fetal development.The study analyzed serum levels of FT3, FT4, TSH, folic acid, and vitamin B12 across all three trimesters in three groups—control, SCH, and OH. A consistent decline in FT3 levels was observed from the first to third trimester in all groups. Significant differences were noted only in the first trimester between SCH and both OH and control, with intra-group variations also showing significance. FT4 levels showed a decreasing trend in controls, whereas in SCH and OH groups, an increasing trend was seen, attributed to levothyroxine (LT4) therapy. However, it is hypothesized that may be the inconsistent LT4 use in SCH led to fluctuating FT4 levels across trimesters. TSH levels remained elevated in SCH and OH, especially in the first trimester, but showed declines in later trimesters, again reflecting LT4 treatment efficacy. It was also observed that SCH participants had more irregular treatment patterns, affecting TSH trends.Folic acid levels increased progressively across trimesters in all groups, consistent with global prenatal supplementation practices[12]. The study observed no mutual significance,despite high intake of folic acidbetween groups, although differences were seen in control and SCH groups i.e between the groups. The growing awareness of folic acid's role in pregnancy, amplified by social media, has led many women to start supplementation even before conception. However, most do so without assessing other micronutrient levels, particularly vitamin B12, which is crucial for folate metabolism.

It is learnt that vitamin B12 levels showed a rising trend across trimesters but remained lower in SCH and OH compared to controls. Statistical significance was observed only in the second trimester between SCH and control. Within-group changes were significant in all three groups. The vitamin B12 deficiency persisted despite LT4 therapy and was associated with unresolved hypothyroid symptoms, which improved with B12 supplementation, similar results were seen in other studies too[13,14].The study revealed that folic acid alone showed no direct correlation with thyroid hormones, but the ratio of folic acid to vitamin B12 (FA/B12) did. A significant negative correlation was observed between this ratio and both FT3 and FT4 across all trimesters and groups, while TSH showed no consistent correlation. These findings suggest that excess folic acid relative to B12 may suppress thyroid hormone activity, potentially through interference in FT4-to-FT3 conversion, possibly affecting deiodinase enzyme activity. This was demonstrated by Sittig. et.al in 2012 in his study [15].Cord blood analysis found no correlation between folic acid levels and maternal thyroid hormones. However, a negative correlation emerged between cord blood FA/B12 ratio and maternal FT3 and FT4 in control and SCH groups. TSH was positively correlated only in the SCH group, possibly due to irregular treatment patterns as mentioned earlier. ROC curve analysis indicated that cord blood FA/B12 ratios could moderately predict SCH and OH in the third trimester, suggesting potential diagnostic utility. Given the fetus's reliance on maternal thyroid hormones until mid-gestation, imbalances in maternal micronutrients could impact fetal development.[16]However, this study did not observe a direct correlation between maternal folic acid and fetal thyroid hormones, but there was a correlation observed with the ratio of FA/VitB12 and the FA/B12 imbalance might indirectly influence fetal outcomes or contribute to epigenetic alterations. It was noticed that one sample out of 16 showed methylation at promoter site of TCN2, the subject was vegan and her serum level showed excess folic acid with low B12 suggesting that high folate with low or deficiency of Vitamin B12 may be the reason for this pattern.

The study also explored methylation patterns in placenta-specific genes—MTHFR and TCN2—involved in folate and vitamin B12 metabolism. Both genes showed low methylation (7.8% and 10.5%, respectively), indicating normal gene expression. No consistent pattern of hypermethylation or silencing was detected, though one MTHFR sample showed methylation at the promoter region. These findings suggest that excess folic acid does not universally cause hypermethylation in all folate-related genes.Other studies have reported that folate status can influence global DNA methylation depending on genotype, age, and exposure.[17,18,19,20] For instance, folic acid intake was shown to alter methylation patterns in the Insulin like Growth Factor-2 (IGF2) gene, correlating with birth weight and long-term health [21]. Similarly, high FA with low B12 levels was linked to placental DNA hypomethylation in both animal and human studies.[22,23,24]Excess folic acid, especially in the absence of adequate B12, may create a "peripheral hypothyroid" environment. This imbalance may not manifest immediately but could cause developmental or metabolic changes in the fetus or affect maternal health in future pregnancies. The observed negative impact on FT3 levels suggests a need to explore whether folate interferes with hormone conversion or enzyme function.Although this study did not perform global methylation analysis, literature indicates that high folic acid intake, especially without B12, can lead to genomic dysregulation. Studies have linked such imbalance to conditions like gestational diabetes[25] (Lindsey Eileen et al., 2024), cardiovascular disease (Herrmann et al., 2022), and even cancer[26]. In one animal study, maternal FA/B12 deficiency resulted in only 4% CpG island methylation and was associated with offspring metabolic dysfunction, immune alterations, and increased adiposity[27].

Conclusion: This study highlights the importance of maintaining a balanced intake of folic acid and vitamin B12 during pregnancy. While folic acid is essential, excessive intake without adequate B12 can alter thyroid hormone levels and possibly contribute to epigenetic changes. The FA/B12 ratio emerged as a key indicator of thyroid status and potential fetal impact. Routine monitoring of both nutrients during pregnancy is recommended to avoid silent complications. Future studies involving global methylation and thyroid-specific gene expression will be crucial to uncover the exact mechanisms and long-term effects of these micronutrient imbalances.

References

1. B Longo-Mbenza, B Kadima-Tshimanga, B Buassa-bu-Tsumbu, K M'Buyamba Jr. Diets rich in vegetables and physical activity are associated with a decreased risk of pregnancy induced hypertension among rural women from Kimpese. DR Congo. Niger J Med, 17 (1) (2008), pp. 45-49
2. Bonilla C, Lawlor DA, Taylor AE, Gunnell DJ, Ben–Shlomo Y, Ness AR, et al. (2012) Vitamin B-12 Status during Pregnancy and Child's IQ at Age 8: A Mendelian Randomization Study in the Avon Longitudinal Study of Parents and Children. PLoS ONE 7(12): e51084. https://doi.org/10.1371/journal.pone.0051084.
3. G. E. Krassas , K. Poppe , D. Glinoer. Thyroid Function and Human Reproductive Health. *Endocrine Reviews*, Volume 31, Issue 5, 1 October 2010, Pages 702–755, https://doi.org/10.1210/er.2009-0041.
4. L. J. Sittig, L. B. K. Herzing, H. Xie, K. K. Batra, P. K. Shukla, E. E. Redei. Excess folate during adolescence suppresses thyroid function with permanent deficits in motivation and spatial memory. Genes, brain and behaviour, 03 November 2011 https://doi.org/10.1111/j.1601-183X.2011.00749.x
5. SargoorR. Veena, GhattuV. Krishnaveni, Krishnamachari Srinivasan, Andrew K. Wills, Sumithra Muthayya, AnuraV. Kurpad , Chittaranjan S. Yajnik , Caroline H.D. Fall. Higher Maternal Plasma Folate but Not Vitamin B-12 Concentrations during Pregnancy Are Associated with Better Cognitive Function Scores in 9- to 10- Year-Old Children in South India. The Journal of Nutrition. Volume 140, Issue 5, May 2010, Pages 1014-1022
6. DelRíoGarcia C, TorresSánchez L, Chen J, Schnaas L, Hernández C, Osorio E, Portillo MG, López-Carrillo L. Maternal MTHFR 677C>T genotype and dietary intake of folate and vitamin B (12): their impact on child neurodevelopment. Nutr Neurosci, 12 (2009), pp. 13-20
7. Reynolds EH. The risks of folic acid to the nervous system in vitamin B12 deficiency: rediscovered in the era of folic acid fortification policies. J Neurol Neurosurg Psychiatry. 2017;88(12):1097–1098.
8. I-Maarri O, Buiting K, Peery EG, Kroisel PM, Balaban B, Wagner K, Urman B, Heyd J, Lich C, Branan CI, et al (2001). Maternal methylation imprints of human chromosome 15 are established during or after fertilization. Nat Genet. 27:341-4.
9. Watreland RA, Jirtle RL (2004). Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 20: 63-68.
10. Choi S.W., Friso S., Ghandour H., Bagley P.J., Selhub J., Mason J.B. Vitamin B-12 deficiency induces anomalies of base substitution and methylation in the DNA of rat colonic epithelium. J. Nutr. 2004;134:750–755. doi: 10.1093/jn/134.4.750.
11. R J Berry[1], Z Li, J D Erickson, S Li, C A Moore, H Wang, J Mulinare, P Zhao, L Y Wong, J Gindler, S X Hong, A CorreaPrevention of neural-tube defects with folic acid in China. China-U.S. Collaborative Project for Neural Tube Defect Prevention. N Engl J Med. 1999 Nov 11;341(20):1485-90. doi: 10.1056/NEJM199911113412001.
12. Oladapo A Ladipo. Nutrition in pregnancy: mineral and vitamin supplements[123]The American Journal of Clinical NutritionVolume 72, Issue 1, July 2000, Pages 280S-290S.
13. Abdul Jabbar, Aasma Yawar, Sabiha Waseem, Najmul Islam, Naeem Ul Haque, Lubna Zuberi, Ataullah Khan, Jaweed Akhter. Vitamin B12 deficiency common in primary hypothyroidism. J Pak Med Assoc.2008 May;58(5):258-61.
14. Fahd A Al-Khamis [1,2] Serum Vitamin B12 and thyroid hormone levels in Saudi patients with multiple sclerosis. J Family Community Med. 2016 Sep-Dec;23(3):151–154. doi: 10.4103/2230-8229.189126
15. Sittig, L. J., Herzing, L. B., Xie, H., Batra, K. K., Shukla, P. K., and Redei, E. E. (2012). Excess folate during adolescence suppresses thyroid function with permanent deficits in motivation and spatial memory. *Genes Brain Behav*. 11, 193–200.

16. Gerard N. Burrow, Delbert A. Fisher, and P. Reed Larsen. Maternal and Fetal Thyroid Function. Published October 20, 1994, N Engl J Med 1994;331:1072-1078 DOI: 10.1056/NEJM199410203311608 VOL. 331 NO. 16.
17. Jacob RA, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, Henning SM, Swendseid ME. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. *J Nutr.* 1998;128:1204–12.
18. Karla P Shelnutt [1], Gail P A Kauwell, Jesse F Gregory 3rd, David R Maneval, Eoin P Quinlivan, Douglas W Theriaque, George N Henderson, . Lynn B Bailey. Methylenetetrahydrofolate reductase 677C-->T polymorphism affects DNA methylation in response to controlled folate intake in young women. J Nutr Biochem 2004 Sep;15(9):554-60. doi: 10.1016/j.jnutbio.2004.04.003.
19. Simonetta Friso,[*†] Sang-Woon Choi,[*] Domenico Girelli,[†] Joel B. Mason,[*] Gregory G. Dolnikowski,[‡] Pamela J. Bagley,[*] Oliviero Olivieri,[†] Paul F. Jacques,[§] Irwin H. Rosenberg,[*] Roberto Corrocher,[†] and Jacob Selhub[*¶i]A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. 2002 Apr 16; 99(8): 5606–5611. Published online 2002 Apr 2. doi: 10.1073/pnas.062066299. PMCID: PMC122817 PMID: 11929966.
20. M Pufulete,[1] R Al-Ghnaniem,[2] A Khushal,[2] P Appleby,[3] N Harris,[4] S Gout,[4] P W Emery,[1] and T A B Sanders[1]. Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. 2005 May; 54(5): 648–653. doi: 10.1136/gut.2004.054718. PMCID: PMC1774481
21. Steegers-Theunissen RP, Obermann-Borst SA, Kremer D, Lindemans J, Siebel C, Steegers EA, Slagboom PE, and Heijmans BT (2009). Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child. PLos One 4:e7845.
22. McKay JA, Waltham KJ, Williams EA, Mathers JC (2011). Folate depletion during pregnancy and lactation reduces genomic DNA methylation in murine adult offspring. Genes Nutr 6: 189-196.
23. Kulkarni A, Dangat K, Kale A, Sable P, Chavan-Gautam P, Joshi S. effects of altered maternal folic acid, vitamin B12 and docosahexaenoic acid on placental global DNA methylation patterns in wistar rats. PLoS One 2011;6:e17706.
24. Kim JM, Hong K, Lee JH, Lee S, Chang N (2009). Effect of folate deficiency on placental DNA methylation in hypohomocysteinemic rats. J Nutr Biochem 20:172-176.
25. Lindsey Eileen and Maria Peterson. High-Fat Diets Fed during Pregnancy Cause Changes to Pancreatic Tissue DNA Methylation and Protein Expression in the Offspring: A Multi-Omics Approach . *Int. J. Mol. Sci.* 2024, *25*(13), 7317; https://doi.org/10.3390/ijms25137317
26. Wolfgang Herrmann[1], Markus Herrmann[2*]. The Controversial Role of HCY and Vitamin B Deficiency in Cardiovascular Diseases. Nutrients. 2022 Mar 28;14(7):1412. doi: 10.3390/nu14071412
27. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastin S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, McEvoy TG, and Young LE (2007). DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A 104:19351-19356.

Authors Key messages for the study:

Author 1: This study underscores the vital role of maintaining a balanced intake of folic acid and vitamin B12 during pregnancy, not only to support maternal thyroid function but also to potentially prevent adverse fetal outcomes. It draws attention to the lesser-known risk that excess folic acid combined with low vitamin

B12 may disrupt thyroid hormone levels and influence fetal development through subtle epigenetic modifications. By integrating biochemical, hormonal, and methylation data, the study offers valuable insights into how micronutrient imbalances may have long-term health implications for both mother and child.
Limitations: The sample size for the DNA methylation analysis was limited as the procedure was expensive, the researcher couldn't effort the cost, this caused reducing the power to detect subtle or gene-specific epigenetic changes.
Further scope: Long-term follow-up of the offspring should be taken-up to study the lasting effects of altered folate/B12 ratios on child health and development.

Author 2: Pregnancy is a crucial period in women's life and nutrition undoubtedly has a great impact on mother and her baby. Micro- nutrients play crucial role and as most of the research studies had highlighted the negative effects of excess folic acid supplementations in individuals not only during pregnancy but in various phases of life. Studies had suggested balancing the ratio of folic acid with vitamin B12 as imbalance in the ratio of these micro-nutrients may lead to many complications related to cognition, metabolic and even cause certain epigenetic changes which may lead to toxic effects/ health complications later in life. In this context the present study focused on the impact of micronutrients individually and as ration on thyroid hormones on pregnant women both in healthy and hypothyroid pregnant women.

Author 3: The study highlights the critical importance of maintaining a balanced intake of folic acid and vitamin B12 during pregnancy to support maternal thyroid health and optimal fetal development. To construct more information, future studies could explore lasting effects, especially if supported by increased funding and resources.

Author 4: It is a proven fact about the positive effects of folic acid during pregnancy. On the contrary there are fewer studies focused the adverse effect of increased folic acid supplementation. The present study focused to study the association of vitamin B_{12} and folic acid with thyroid profile to emphasize the importance of balancing the micronutrients during pregnancy, to observe the fluctuations in the patterns of thyroid hormones in pregnant women and its association with vitamin B_{12} and folic acid as the literature in this area is sparsely available.The study was taken up in public health interest, to assess the importance of balancing the various micronutrients in diet to prevent unbalanced nutritional complications and other health related disorders later in life.The prolong administration of folic acid alone may alter the ratio of folic acid and vitamin B_{12} further leading to the imbalance in these two micronutrients. It is recommended to go for gene expression related studies or DNA methylation studies may provide an extensive knowledge in this area of interest to explore more knowledge on the same.

Author 5: An important finding was the negative correlation between folic acid/B12 ratio and thyroid hormones in maternal and cord blood, indicating that excess folate with low B12 may adversely affect thyroid function.While minimal methylation changes were found in placental MTHFR and TCN2 genes, these results suggest the need to explore broader epigenetic impacts beyond these genes. Despite limitations, the study sets the stage for future research on global DNA methylation and gene expression changes due to micronutrient imbalances during pregnancy, with potential long-term implications for child health.