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Plasma 25-hydroxyvitamin D is positively associated with folate and vitamin B_{12} levels in adolescents

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ABSTRACT

Vitamin D affects the absorption of folate *in vitro*, and perhaps of vitamin B_{12} (B_{12}). However, epidemiological studies on the association of vitamin D with folate and B_{12} are inconclusive. We hypothesized a positive association of plasma 25-hydroxyvitamin D [25(OH)D] with folate and B_{12} levels in adolescents. This hypothesis was tested in a cross-sectional study of healthy adolescents (11–16 years old; n = 1416), selected from public middle schools from across Kuwait, using stratified multistage cluster random sampling. Plasma 25(OH)D was measured by LC–MS/MS. Serum B_{12} and total folate in hemolyzed whole blood were analyzed with commercial kits; RBC and plasma folate were calculated from total folate. Data on potential confounders were collected from the parents and adolescents. In a univariable model, 25(OH)D as a continuous variable was positively associated with each of total, RBC, and plasma folate ($P < .001$). After adjusting for potential confounders, this association remained significant with total folate ($\beta = 2.0$, $P < .001$) and red blood cell folate ($\beta = 1.8$, $P < .001$), but not with plasma folate ($\beta = 0.2$, $P = .34$). A similar pattern of association was evident when 25(OH)D was fitted as categorical variable. Correlation between B_{12} and 25(OH)D was weak but significant ($\rho = 0.1$, $P < .001$). 25(OH)D was positively associated with B_{12} in both univariable and multivariable models ($P < .001$) when fitted as a categorical variable only. Simultaneous quantile regression confirmed these results. We conclude that plasma 25(OH)D is positively associated with folate and B_{12} levels in adolescents. Properly designed large-scale randomized controlled trials are warranted to investigate the causal role of vitamin D in folate and B_{12} absorption.

Abbreviations: **25(OH)D**, 25-hydroxyvitamin D; **CAP**, College of American Pathologists; **LC–MS/MS**, liquid chromatography tandem mass spectrometry; **DBP**, vitamin D binding protein; **IF**, intrinsic factor; **RBC folate**, red cell folate; **RFC**, reduced folate carrier; **PCFT**, proton-coupled folate transporter; **B_{12}**, vitamin B_{12}; **VDR**, vitamin D receptor.

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1. Introduction

Folate and Vitamin B_{12} (B_{12}) are water-soluble vitamins, which play a major role in cellular one-carbon metabolism and, in particular, the remethylation of homocysteine. Folate is required for nucleotides synthesis, DNA synthesis and repair, methylation, and amino acid metabolism [1,2]. Because of its role in nucleic acid synthesis, it is essential for the integrity and maintenance of rapidly dividing tissues like bone marrow and intestinal epithelium [3]. Adequate dietary folate availability is especially important during periods of rapid cell division, such as during pregnancy and infancy [1]. Adolescence is also the time of rapid growth and requires adequate folate levels. Folate deficiency during early pregnancy, which is a time of the most rapid cell division, is associated with neural tube defects [4-6]. Folate deficiency is also a risk factor for cancer, cardiovascular, and cerebrovascular diseases [7]; and it is also associated with reduced erythropoiesis and megaloblastic anemia in adults and children [8].

Maintaining optimal body folate depends on adequate dietary intake and efficient absorption from the gastrointestinal system [9]. The absorption of dietary folate through the intestinal epithelial cells is mainly regulated by two types of folate transporters, namely the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT). Of these, RFC (gene symbol *SLC19A1*) has been shown to play a minor role in intestinal folate absorption [10,11]; whereas the PCFT (gene symbol *SLC46A1*) is the major folate transporter and is responsible for most of intestinal folate uptake [12,13]. PCFT is a high-affinity transporter and is localized to the apical side of enterocytes in the duodenum and jejunum in mice [14] and humans [15,16]. It is an electrogenic transporter and functions optimally at a low pH [14,15,17]. Loss-of-function mutations in PCFT gene result in the hereditary folate malabsorption disorder [15].

In vitro studies have shown that the PCFT gene expression is affected by vitamin D [1]. In Caco-2 cells, vitamin D_3 treatment significantly increased the expression of PCFT mRNA in a dose-dependent manner but had no effect on the expression of RFC gene. This vitamin D-dependent expression of the PCFT was mediated through the trans-activation of vitamin D receptor (VDR). The increased expression of PCFT mRNA was associated with an increase in PCFT protein and significantly increased uptake of folate across the apical membrane of Caco-2 cells, in a dose-dependent manner [1, 18]. Similar vitamin D-dependent expression of the rat duodenal PCFT was also reported *in vitro* [1]. However, these results have not been corroborated in animal studies [19]. The active form of vitamin D (1,25-dihydroxyvitamin D) has also been shown to increase the expression of RFC at the blood–brain barrier [9]. In pregnant mice, lipopolysaccharides reduced the expression of mRNA for PCFT and RFC and caused significant reduction in embryonic folate levels with concomitant increase in neural tube defect in embryos. Vitamin D_3 supplementation prevented this reduction in the mRNA for both folate transporters (PCFT and RFC) and restored the embryonic folate levels to normal [20].

Based on the potential role of vitamin D in the expression of PCFT and hence folate absorption, few studies have investigated the association between vitamin D status and blood folate levels [2,21,22] but the results are inconclusive. A recent study reported positive association between calcitriol (the active form of vitamin D) and serum folate levels in women undergoing *in vitro* fertilization [21]. In this study, a positive association between calcitriol and B_{12} was also reported. Similar to this, another small study (n = 80) also reported a positive association between serum 25-hydroxyvitamin D [25(OH)D] and B_{12} in obese women [23]. Mao et al reported significant correlation between folate and plasma 25(OH)D levels in patients with diabetes aged >50 years but not in patients aged <50 [2]. On the contrary, Lucock et al reported a negative association between red blood cell folate (RBC folate) and 25(OH)D_3 in summer and winter but not in other seasons [22]. Studies on the effect of vitamin D supplementation on blood folate (plasma and/or RBC folate) did not show a significant increase in folate levels parallel to the increase in 25(OH)D levels in response to supplementation [19,24]. To our knowledge, large scale community-based epidemiological studies on the association between plasma 25(OH)D levels and blood folate (plasma and/or RBC folate) and B_{12} levels have not been conducted.

Based on the potential influence of vitamin D on folate absorption, and the importance of folate during a period of rapid growth like adolescence, we hypothesized a positive association of plasma 25(OH)D with various parameters of body folate status and plasma B_{12} levels. This hypothesis was tested in a cross-sectional study on large sample of adolescents. The objectives were to investigate the association of plasma 25(OH)D with total blood folate, RBC folate, plasma folate and plasma B_{12} levels in a nationally representative sample of adolescents. If the levels of folate and B_{12} are affected by vitamin D status, it will have significant public health implications, as vitamin D deficiency is widespread across the globe regardless of gender, age groups or ethnicity. This is particularly relevant to our setting, where vitamin D deficiency and insufficiency is highly prevalent in adolescents [25].

2. Methods and materials

2.1. Study population and participants

A cross-sectional study was conducted in which 11–16 years old students were recruited from 12 public middle schools in all the governorates (provinces) of Kuwait. The sample was selected using stratified multistage cluster random sampling, with probability proportional to size in each governorate. Details of the study have been described previously [25,26]. Fig. 1 shows the number of the participants who were invited, agreed and participated, and were finally included in the analysis. The study strictly adhered to the ethical standards of the Code of Ethics of the World Medical Association (Declaration of Helsinki); and ethical approval was granted by The Ethics Committee at Ministry of Health, Kuwait (No: 2015/248), as well as The Ethics Committee of the Health Sciences Centre, Kuwait University (No: DR/EC/2338). Written informed consent from the parents or the child's guardian and verbal assent from the adolescent was obtained for each participant included in the study.

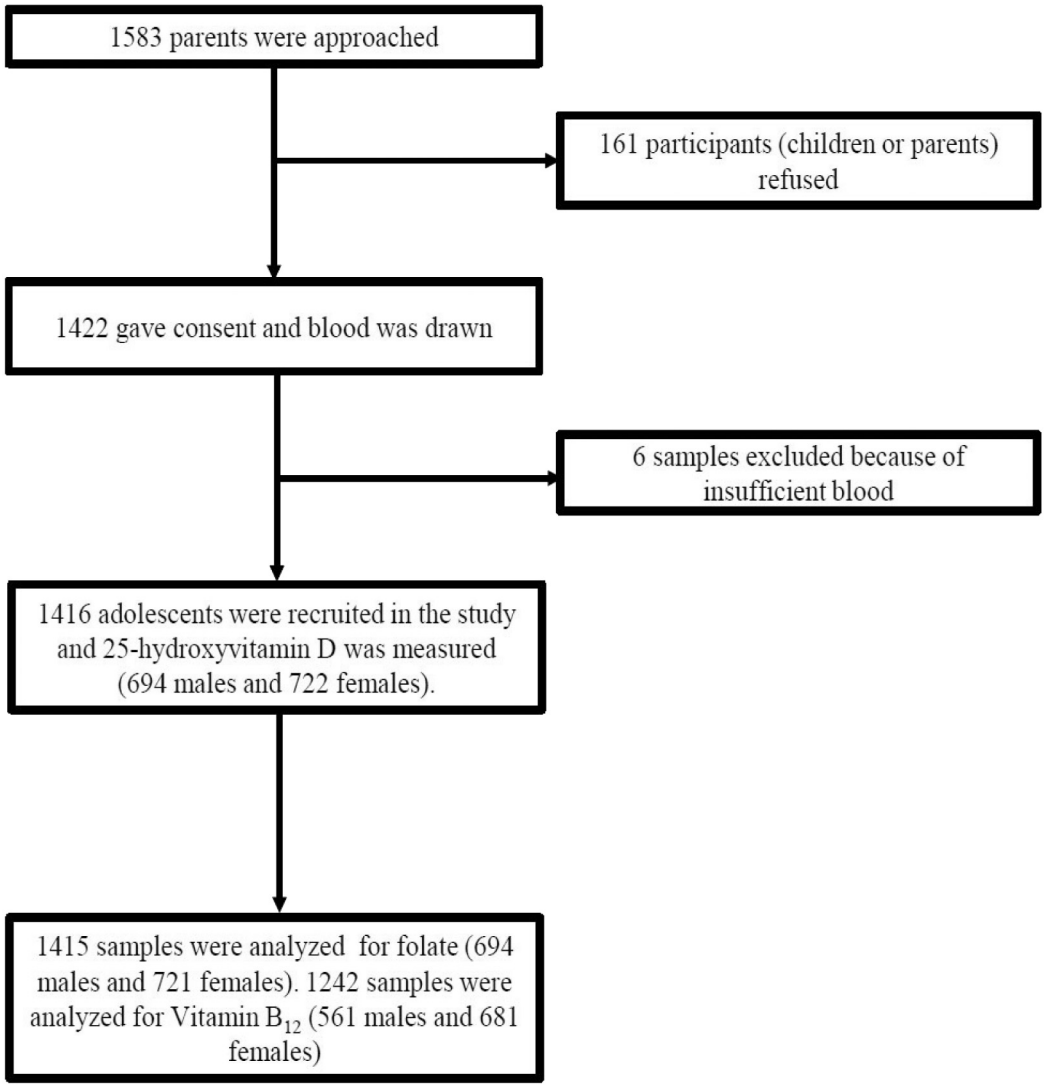

Fig. 1 – Subject recruitment and selection.

2.2. *Blood collection and plasma 25(OH)D analysis*

Five milliliters of venous blood was collected from each child by a specialized pediatric nurse with experience in drawing blood from children. Samples for 25(OH)D analysis were protected from light throughout handling and processing. Plasma was separated and stored at –80 °C until analysis. Plasma 25(OH)D was analyzed in a CAP-accredited laboratory by liquid chromatography–tandem mass spectrometry (LC–MS/MS), as described previously [27], using the commercially available kits from Chromsystems (Cat. #2000/1000/F; Chromsystems Instruments & Chemicals GmbH, Grafelfing, Germany). Precision and accuracy of analysis were monitored by including control samples (MassCheck controls). The assay's lower limit of detection is 5 nmol/L for 25(OH)D_3. The Intra-assay coefficient of variation (CV) is 3.7%, and the inter-assay CV is 5.3% to 6.0%.

2.3. *Folate and vitamin B_{12} analysis*

All other biochemical analyses, including folate, B_{12}, complete blood count, ferritin and other laboratory measurements were conducted in a major teaching hospital, where these tests are routinely performed under strict quality control. Folate and B_{12} were analyzed with the Elecsys and Cobas E analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Serum B_{12} was analyzed with the Roche commercial kit (Cat. # 04745736 190). The lower limit of detection of this assay is 22 pmol/L, with the intra-assay CV of 2.5% and inter-assay CV of 2.7% to 3.2%. Folate in hemolyzed whole blood was analyzed with the Roche commercial kits (Cat# 03253678 122). RBC folate was calculated from the total folate as RBC folate = (Total folate × 3100)/% hct. Plasma folate was calculated by subtracting RBC folate from the total folate. The lower limit of detection of this assay is 105 nmol/L, with the intra-assay CV of 5.0% to 8.5% and the inter-assay CV of 6.2% to 9.7%.

2.4. *Data collection on potential confounders*

Data on potential confounding factors were collected through a self-administered questionnaire from the parents and through a face-to-face interview with the adolescents using a structured questionnaire. Details of the questionnaire

development have been previously published [25,26]. The self-administered questionnaire from parents included questions on various demographic variables and dietary habits, whereas information on habitual sun exposure, smoking habits, dietary intake and physical activity were collected through the face-to-face interview with the adolescents. Standing height and body weight of the study participants were assessed using digital weight and height scale (Detecto®) in a standardized manner. BMI was calculated and converted into the BMI Z-scores using the WHO growth charts for classifying the subjects as normal weight, overweight or obese.

2.5. *Statistical analyses*

Data were doubled entered into specifically designed database using Epidata and were analyzed using Stata 14 (Stata Corp LP, College Station, TX, USA). Spearman correlation coefficient was calculated between 25(OH)D and folate levels as well as between B_{12} and 25(OH)D. This relationship was depicted graphically using scatter plot in Figs. 2 and 4. As folate level was normally distributed, we used multiple linear regression to investigate the association between 25(OH)D level and each of total, RBC and plasma folate while adjusting for potential confounders. First, we calculated the crude association and then the adjusted association for both sex and age. Finally, we adjusted for any other variables that showed association with folate level at <0.20 level of significance. In addition to age and sex, the final model included nationality (Kuwaiti or non-Kuwaiti), how often the child has meal before going to school (everyday, 4 days per week, 3 days per week, 2 days per week, 1 day per week, never), having health condition that limits physical activity diagnosed by a doctor (yes, no), smoking shisha, also known as water pipe, hookah or hubble-bubble, smoking cigarette (yes, no), number of times walking to/from school, habitual exposure to sun light during weekdays, using sunscreen (never, rarely, sometimes and often/always), wearing a shirt with sleeves that cover the shoulder (none/rarely/sometimes, often, always), time for first meal during weekdays and during weekends, number of times of consumption of sugary drinks, BMI categories and anemia. In separate models, 25(OH)D was fitted as a continuous or categorical variable using quartiles or acceptable cutoff points for 25(OH)D [28,29]. The assumptions for this analysis were checked including the residual, multicollinearity and homoscedasticity. To investigate the association between B_{12} and 25(OH)D, we had to log-transform B_{12} because it was not normally distributed. The final model included all the potential confounding variables that showed association with B_{12} level at <0.20 level of significance. The

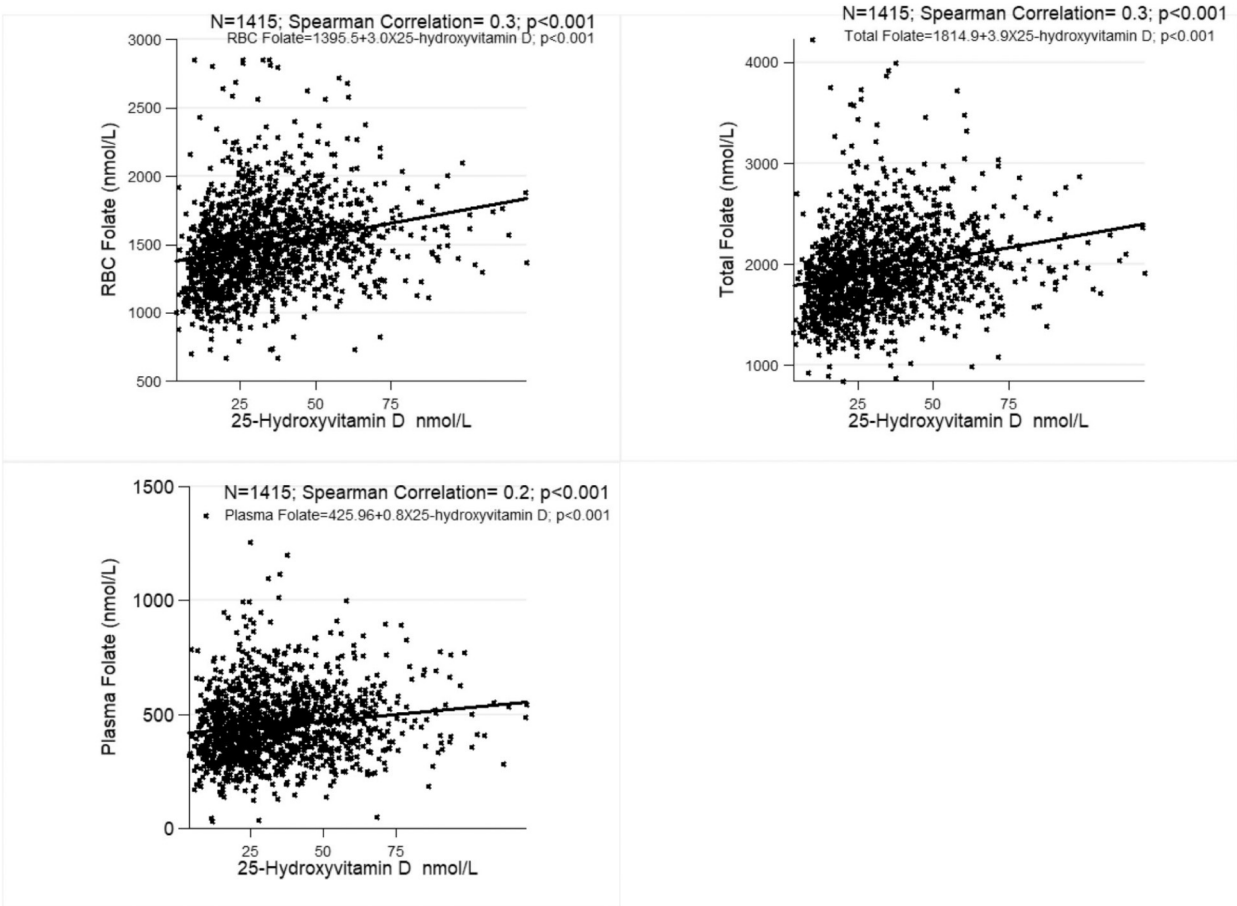

Fig. 2 – Association of 25-hydroxyvitamin D with total, RBC and plasma folate.

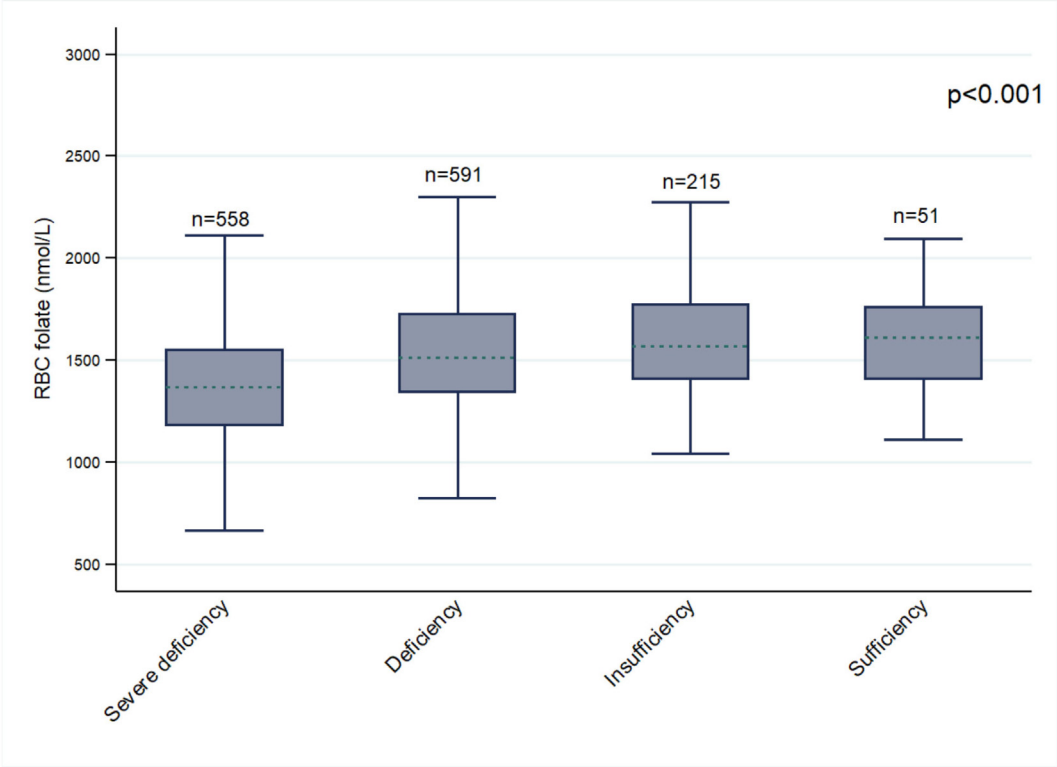

Fig. 3 – Red blood cell (RBC) folate level by 25-hydroxyvitamin D status.

analysis above was repeated using simultaneous-quantile regression with quantile 0.5 obtaining standard errors *via* bootstrapping after 1000 replicates. With the number we recruited in the study, we have more than 80% power to detect a difference of 50 nmol/L in RBC folate between adolescents with and without vitamin D deficiency using the mean (SD) we found in our study.

3. Results

3.1. *Association between folate and 25(OH)D*

Measurement of folate was available for 1415 study participants, of whom 721 (51%) were females. The means (SD) of total, RBC, and plasma folate were 1950 (420), 1500 (320), and 550 (160) nmol/L, respectively. The median (IQR) for 25(OH)D was 30 (19–44) nmol/L. Of the overall sample, 217 (15%) had RBC folate levels below the reference range (1187–2854 nmol/L). The association between 25(OH)D and RBC folate, plasma folate and total folate is shown in Fig. 2. As shown in Fig. 3, the means (SD) of RBC folate was 1400 (300) nmol/L, 1550 (320) nmol/L, 1600 (310) nmol/L and 1620 (280) nmol/L among those with severe vitamin D deficiency, deficiency, insufficiency and sufficiency, respectively ($P < .001$). Also, deficiency of RBC folate (<1187 nmol/L) tended to get lower per quartile of 25(OH)D (Chi-square (χ^2) for trends, $P < .001$).

Table 1 shows the association between RBC folate and 25(OH)D before and after adjusting for potential confounders. There was highly significant positive association between RBC folate and 25(OH)D before and after adjusting for potential confounders. This was evident whether 25(OH)D was fitted as a continuous variable or as categorical variable. Similarly, Tables 2 and 3 show the association of 25(OH)D with total folate and plasma folate, respectively. Significant association was found between 25(OH)D and total folate in all models while the association between 25(OH)D and plasma folate was significant in univariable analysis but not in multivariable analysis. We used backward and forward stepwise selection of the variables to verify whether 25(OH)D will be selected as a predictor for folate levels. 25(OH)D (as a continuous and categorical variable) was selected as a significant predictor for RBC folate and total folate in both backward and forward selection confirming the results in Tables 1 and 2. Also, using the same approach, 25(OH)D was a significant predictor for plasma folate whether it was fitted as a continuous or categorical variable, which is slightly different from the results shown in Table 3. Finally, vitamin D binding protein (DBP) was measured for 746 participants, hence we were able to calculate free 25(OH)D as describe by Tsuprykov et al [30]. There was highly significant association between free 25(OH)D (categorized into tertiles) and folate in all models ($P < .001$).

3.2. *Association between vitamin B_{12} and 25(OH)D*

Measurements of B_{12} were available for 1242 participants. The geometric mean (SD) of plasma B_{12} was 272 (1.4) pmol/L. Only 48 (4.8%) participants had deficient B_{12} levels (below 156 pmol/L). There was weak but significant correlation between B_{12}

Table 1 – Association between 25-hydroxyvitamin D and red blood cell folate before and after adjusting for potential confounders

Vitamin D status*/ level	Model 1 β [95% CI]	Model 2 β [95% CI]	Model 3 β [95% CI]
25(OH)D levels nmol/L	3.0 [2.3, 3.7]	1.8 [1.0, 2.5]	1.8 [1.1, 2.5]
P	<.001	<.001	<.001
Q1 (25(OH)D < 19.2 nmol/L) (n = 349)	[Reference]	[Reference]	[Reference]
Q2 (25(OH)D ≥ 19.2 to <29.7 nmol/L) (n = 356)	120 [70, 160]	80 [40, 130]	90 [40, 140]
Q3 (25(OH)D from 29.7 to <44.1 nmol/L) (n = 355)	170 [130, 220]	100 [50, 150]	90 [40, 140]
Q4 (25(OH)D ≥ 44.1 nmol/L) (n = 355)	240 [190, 280]	150 [90, 200]	150 [100, 200]
P	<.001	<.001	<.001
Severe deficiency (25(OH)D < 25 nmol/L) (n = 558)	[Reference]	[Reference]	[Reference]
Deficiency (25(OH) D ≥ 25 to <50 nmol/L) (n = 591)	150 [110, 180]	90 [50, 120]	80 [40, 120]
Insufficiency (25(OH) D ≥ 50 to <75 nmol/L) (n = 215)	200 [150, 250]	110 [60, 170]	120 [70, 170]
Sufficiency (25(OH) D ≥ 75 nmol/L) (n = 51)	220 [130, 310]	140 [60, 230]	140 [50, 240]
P	<.001	<.001	<.001

Values are regression coefficients and their 95% confidence intervals from the total sample of 1415 adolescents. 25(OH)D: 25-hydroxyvitamin D; CI: Confidence interval; Q1 to Q4: Quartile one to Quartile four; **Model 1**: unadjusted; **Model 2**: adjusted for gender and age. **Model 3**: adjusted for gender and age in addition to other variables, which showed significant association with folate at <0.2 level of significance including nationality, having a meal before going to school, currently smoking shisha (water pipe), having a disease condition diagnosed by a doctor, number of times walking to/from school, habitual exposure to sun light during weekdays, using sunscreen, wearing a shirt with sleeves that cover the shoulder, time for first meal during weekdays and during weekends, number of times of consumption of sugary drinks, Body Mass Index categories, and anemia. *Vitamin D status was defined based on the cutoffs provided by the Endocrine Society and the Society for Adolescent Health and Medicine [28,29].

Table 2 – Association between 25-hydroxyvitamin D and total folate before and after adjusting for potential confounders

Vitamin D status*/level	Model 1 β [95% CI]	Model 2 β [95% CI]	Model 3 β [95% CI]
25(OH)D levels nmol/L	3.9	2.0	2.0
	[3.0, 4.8]	[1.0, 2.9]	[1.05, 3.0]
P	<0.001	<0.001	<0.001
Q1 (25(OH)D < 19.2 nmol/L) (n = 349)	[Reference]	[Reference]	[Reference]
Q2 (25(OH)D ≥ 19.2 to <29.7 nmol/L) (n = 356)	160	110	110
	[100, 220]	[50, 170]	[50, 170]
Q3 (25(OH)D from 29.7 to <44.1 nmol/L) (n = 355)	230	110	110
	[170, 290]	[50, 180]	[40, 170]
Q4 (25(OH)D ≥ 44.1 nmol/L) (n = 355)	310	160	170
	[250, 370]	[100, 230]	[100, 240]
P	<0.001	<0.001	<0.001
Severe deficiency (25(OH)D < 25 nmol/L) (n = 558)	[Reference]	[Reference]	[Reference]
Deficiency (25(OH)D ≥ 25 to <50 nmol/L) (n = 591)	190	90	90
	[150, 240]	[40, 140]	[40, 140]
Insufficiency (25(OH)D ≥ 50 to <75 nmol/L) (n = 215)	250	120	130
	[190, 310]	[50, 190]	[60, 200]
Sufficiency (25(OH)D ≥ 75 nmol/L) (n = 51)	310	190	190
	[200, 430]	[80, 310]	[70, 310]
P	<.001	<.001	<.001

Values are regression coefficients and their 95% confidence intervals from the total sample of 1415 adolescents. 25(OH)D: 25-hydroxyvitamin D; CI: Confidence interval; Q1 to Q4: Quartile one to Quartile four; **Model 1**: unadjusted; **Model 2**: adjusted for gender and age. **Model 3**: adjusted for gender and age in addition to other variables, which showed significant association with folate at <0.2 level of significance including nationality, having a meal before going to school, currently smoking shisha (water pipe), having a disease condition diagnosed by a doctor, number of times walking to/from school, habitual exposure to sun light during weekdays, using sunscreen, wearing a shirt with sleeves that cover the shoulder, time for first meal during weekdays and during weekends, number of times of consumption of sugary drinks, Body Mass Index categories, and anemia. * Vitamin D status was defined based on the cutoffs provided by the Endocrine Society and the Society for Adolescent Health and Medicine [28,29].

Table 3 – Association between 25-hydroxyvitamin D and plasma folate before and after adjusting for potential confounders

Vitamin D status*/level	Model 1 β [95% CI]	Model 2 β [95% CI]	Model 3 β [95% CI]
25(OH)D levels nmol/L	0.8	0.1	0.2
	[0.4, 1.1]	[−0.2, 0.5]	[−0.2, 0.5]
P	<.001	.46	.34
Q1 (25(OH)D < 19.2 nmol/L) (n = 349)	[Reference]	[Reference]	[Reference]
Q2 (25(OH)D ≥ 19.2 to <29.7 nmol/L) (n = 356)	41	22	17
	[19, 63]	[0.6, 44]	[−4, 37]
Q3 (25(OH)D from 29.7 to <44.1 nmol/L) (n = 355)	58	15	14
	[36, 80]	[−8, 39]	[−8, 36]
Q4 (25(OH)D ≥ 44.1 nmol/L) (n = 355)	68	14	16
	[46, 91]	[−10, 39]	[−7, 40]
P	<.001	.25	.40
Severe deficiency (25(OH)D < 25 nmol/L) (n = 558)	[Reference]	[Reference]	[Reference]
Deficiency (25(OH)D ≥ 25 to <50 nmol/L) (n = 591)	42	6	8
	[24, 59]	[−12, 24]	[−10, 25]
Insufficiency (25(OH)D ≥ 50 to <75 nmol/L) (n = 215)	48	0.3	8
	[24, 72]	[−24, 25]	[−15, 32]
Sufficiency (25(OH)D ≥ 75 nmol/L) (n = 51)	86	42	42
	[42, 128]	[−0.4, 84]	[0.8, 83]
P	<.001	.26	.25

Values are regression coefficients and their 95% confidence intervals from the total sample of 1415 adolescents. 25(OH)D: 25-hydroxyvitamin D; CI: Confidence interval; Q1 to Q4: Quartile one to Quartile four; **Model 1**: unadjusted; **Model 2**: adjusted for gender and age. **Model 3**: adjusted for gender and age in addition to other variables, which showed significant association with folate at <0.2 level of significance including nationality, having a meal before going to school, currently smoking shisha (hookah or water pipe), having a disease condition diagnosed by a doctor, number of times walking to/from school, habitual exposure to sun light during weekdays, using sunscreen, wearing a shirt with sleeves that cover the shoulder, time for first meal during weekdays and during weekends, number of times of consumption of sugary drinks, Body Mass Index categories, and anemia. * Vitamin D status was defined based on the cutoffs provided by the Endocrine Society and the Society for Adolescent Health and Medicine [28,29].

and 25(OH)D (Spearman correlation = 0.13, $P < .001$). This crude association is presented in Fig. 4. Table 4 shows the association between B_{12} (log-transformed) and 25(OH)D before and after adjustment. There was significant association between B_{12} and 25(OH)D in all models in which 25(OH)D was fitted as a categorical variable. Stepwise selection methods confirmed the analysis reported in Table 4. We also repeated this analysis using simultaneous-quantile regression to confirm these results. There was no difference between the results of this analysis and the previous analysis. Finally, we examined the association between free 25(OH)D and B_{12}. There was no evidence for association between free 25(OH)D and B_{12} in univariate analysis ($P = .13$) but the association was significant after adjusting for age and gender ($P = .03$). In final model, there was no evidence for association between free 25(OH)D and B_{12} ($P = .19$).

4. Discussion

The expression of the major transporter for dietary folate (PCFT) is genetically controlled by vitamin D through VDR [1,18,19]. Thus, it is theoretically plausible that vitamin D deficiency may reduce the absorption of dietary folate. In this study we evaluated the association of plasma 25(OH)D levels with three markers of folate status, namely total folate, RBC folate and plasma folate. In a large epidemiological study, we have demonstrated a robust association between 25(OH)D levels and folate levels using various statistical approaches. We also demonstrated a clear association between 25(OH)D levels and B_{12}. As such our data supported the hypothesis of positive association of plasma 25(OH)D with folate, particularly the RBC folate, and plasma B_{12} levels.

Our data showed a significant association between 25(OH)D levels and RBC folate in univariable and multivariable analysis. Unlike plasma folate, RBC folate is accumulated in RBC only during erythropoiesis, and is mostly resistant to changes in day-to-day variation in folate intake [31]. Although 25(OH)D was associated with both plasma folate and RBC folate, the association was stronger with RBC folate, suggesting that vitamin D has a long-term influence on the folate status. Furthermore, the association was stronger between free 25(OH)D and RBC folate than the total 25(OH)D, further supporting the hypothesis of vitamin D-dependent folate absorption. Similar positive association between 25(OH)D and folate has been reported in diabetic patients who were above 50 years of age [2], and in pregnant women who were undergoing *in vitro* fertilization [21].

Other studies involving vitamin D supplementation and its subsequent effect on plasma folate status failed to show a positive association [19,24]. There are, however, several limitations with these studies. Both studies lacked statistical power as the sample size in both studies was very small. The first study was originally designed as a randomized controlled trial to study bioavailability of vitamin D_3 and D_2 on plasma concentrations of 25(OH)D in healthy adults (n = 107) [19]. However, the investigators at later stage compared 19 randomly selected individuals from the vitamin D_3 group with 19 individuals from the placebo group at baseline and after treatment and concluded that there was no evidence

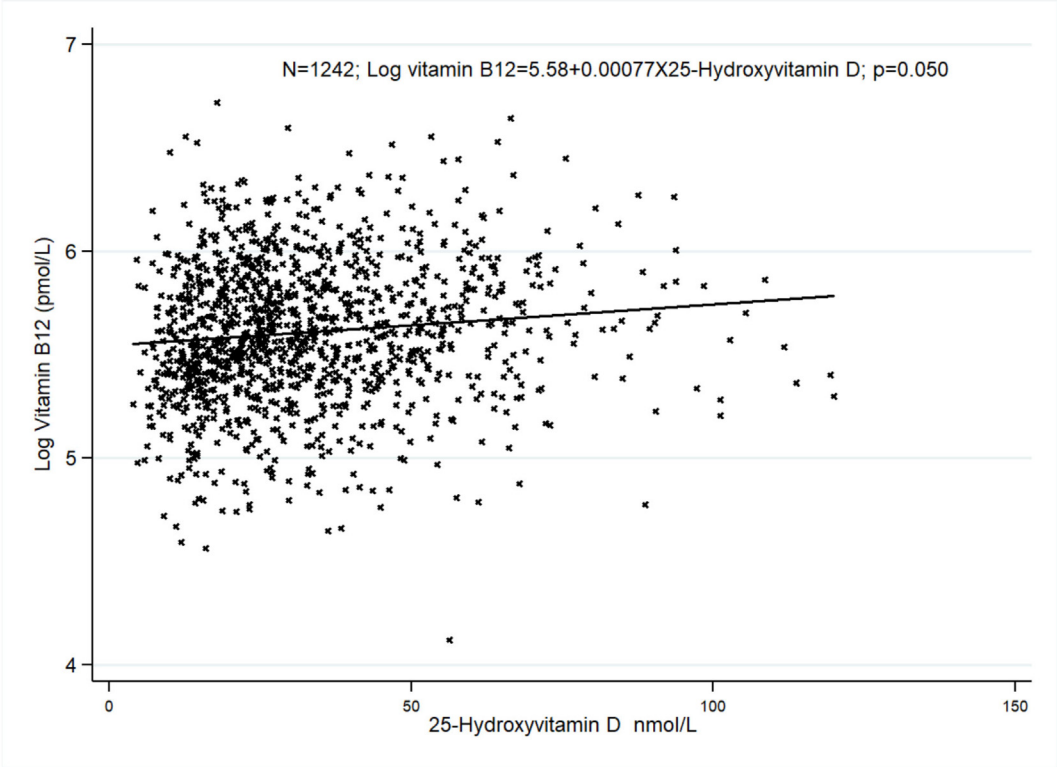

Fig. 4 – Association between 25-hydroxyvitamin D and vitamin B_{12}.

Table 4 – Association between 25-hydroxyvitamin D and log vitamin B_{12} before and after adjusting for potential confounders

Vitamin D status/level	Model 1 β [95% CI]	Model 2 β [95% CI]	Model 3 β [95% CI]
25(OH)D levels nmol/L	1.00 [1.00–1.00]	1.00 [1.00–1.00]	1.00 [1.00–1.00]
P	.050	.007	.19
Q1 (25(OH)D < 19.2 nmol/L) (n = 337)	[Reference]	[Reference]	[Reference]
Q2 (25(OH)D ≥ 19.2 to <29.7 nmol/L) (n = 346)	1.09 [1.03, 1.14]	1.24 [1.10, 1.41]	1.21 [1.07, 1.36]
Q3 (25(OH)D from 29.7 to <44.1 nmol/L) (n = 344)	1.07 [1.02, 1.13]	1.30 [1.14, 1.48]	1.25 [1.10, 1.43]
Q4 (25(OH)D ≥ 44.1 nmol/L) (n = 343)	1.12 [1.06, 1.18]	1.47 [1.28, 1.69]	1.35 [1.17, 1.56]
P	<.001	<.001	<.001
Severe deficiency (25(OH)D < 25 nmol/L) (n = 544)	[Reference]	[Reference]	[Reference]
Deficiency (25(OH)D ≥ 25 to <50 nmol/L) (n = 572)	1.06 [1.02, 1.10]	1.24 [1.12, 1.38]	1.21 [1.09, 1.35]
Insufficiency (25(OH)D ≥ 50 to <75 nmol/L) (n = 205)	1.12 [1.06, 1.18]	1.46 [1.26, 1.68]	1.36 [1.18, 1.57]
Sufficiency (25(OH)D ≥ 75 nmol/L) (n = 49)	1.07 [0.97, 1.19]	1.31 [1.03, 1.67]	1.14 [0.88, 1.47]
P	<.001	<.001	<.001

Values are regression coefficients and their 95% confidence intervals from the total sample of 1242 adolescents. 25(OH)D: 25-hydroxyvitamin D; CI: Confidence interval; Q1 to Q4: quartile one to Quartile four; **Model 1**: unadjusted; **Model 2**: adjusted for gender and age. **Model 3**: adjusted for gender and age in addition to other variables, which significant association with vitamin B12 at <0.2 level of significance including nationality, how often the adolescent stays in shade or uses umbrella during the last 3 months, number of times walking to/from school, having a disease condition diagnosed by a doctor, currently smoking shisha (water pipe), time for first meal during weekdays, number of times of consumption of sugary drinks, number of times eating breakfast prepared outside home, and Body Mass Index categories. β [95%CI] were back transformed to arithmetic scale. * Vitamin D status was defined based on the cutoffs provided by the Endocrine Society and the Society for Adolescent Health and Medicine [28,29].

that vitamin D affects the intestinal uptake and status of folate. The other study included 10 volunteers (four men and six women) in a single arm study in which the authors reported no significant change in folic acid after 1,25-$(OH)_2$-D_3 supplementation for 10 days [24]. The level of 1,25-$(OH)_2$-D in the blood is very tightly regulated and is unlikely to change with supplementation, unless there is severe deficiency of vitamin D. As the expression of the PCFT is affected by 1,25-$(OH)_2$-D, the lack of effect on folate absorption with vitamin D supplementation is thus expected. Furthermore, the duration of supplementation was short, 8 weeks in one study and 10 days in the other. The lack of association in these studies could be due to the relatively shorter duration of vitamin D supplementation, which may not be long enough to achieve the steady state folate levels. Achieving the steady state folate levels require a much longer duration of supplementation with folate [32], and perhaps with vitamin D. Investigating the link between folate absorption and vitamin D status through properly designed large randomized controlled trials is warranted given the public health implications of this issue.

Our data not only demonstrated that 25(OH)D level is associated with folate level but also showed that this association is linear, which means the association exists at all levels of 25(OH)D including extremely low concentrations. RBC folate levels also tended to be associated with 25(OH)D quartiles in a linear manner (χ^2 for trend $P < .001$). Based on such association, one would expect a very high prevalence of folate deficiency in our population, as the prevalence of vitamin D deficiency and insufficiency in this population is very high [25]. However, in our sample, only 15% of the study subjects had RBC folate levels below the reference range (1187–2854 nmol/L). This apparent discord between the association of 25(OH)D with folate levels, and between the prevalence of vitamin D deficiency and folate deficiency could be explained by the fact that multiple absorption mechanisms exist for dietary folate utilizing different transporters. Under physiological intakes of folate, most of the absorption is carried out by the PCFT, the expression of which is affected by vitamin D. However, other transporters like RFC and the organic anion–transporting polypeptide 2B1 (OATP2B1, gene code *SLCO2B1*) also take part in folate absorption, particularly during high intake (supplementation) and during folate deficiency [13,33]. These are low affinity transporters which are not affected by vitamin D and may compensate for the reduction of folate absorption by PCFT during vitamin D deficiency. However, these mechanisms are not as efficient as folate absorption through PCFT [13]. As such overt folate deficiency may be averted but low levels of folate in the presence of vitamin D deficiency may still exist, as is seen in this study.

Another potential link between vitamin D and folate is that both vitamins are affected by ultra-violet radiation (UVR), albeit in opposing directions. Vitamin D is synthesized when its precursor (7-dehydrcholesterol) in the skin is exposed to UVR in the range of 280–315 nm [34], whereas this wavelength has been reported to degrade folate both in *ex vivo* experiments and *in vivo* [35-37]. Both plasma and RBC folate have been shown to be degraded by UVR. The synthetic form of folate (folic acid) is known to be the most sensitive to UVR degradation [35,38]. The reduced metabolites, methylene tetrahydrofolate and methyl tetrahydrofolate, are also known to be degraded but are less sensitive than folic acid [35,37]. This has led to the development the folate-vitamin D-hypothesis, which proposes that the skin pigmentation has evolved to maintain a balance between these two essential vitamins. The dark-pigmented skin is evolved to protect folate relative to vitamin D synthesis, whereas, the light-pigmented skin favors vitamin D synthesis to maintain the balance [38,39]. Longer wavelength UVR (315-400 nm) and prolong exposure can degrade vitamin D as well as folate [34,38]. In this study, we collected data on the time spent outdoor between 10 am 4 pm on weekdays and on weekends. Adjusting for this proxy variable for UVR exposure did not affect the significant positive association between RBC folate and 25(OH)D. This suggests that the positive influence of vitamin D status on folate absorption surpass the contrasting effects of the UVR exposure on the two vitamins.

We also found a significant positive association between 25(OH)D and B_{12}. Similar association has been reported in the literature by others [21,23]. The mechanism by which vitamin D might affect B_{12}, or the other way around, is not clear. It has been suggested that vitamin D may be indirectly linked to B_{12} absorption through calcium, which is affected by vitamin D. The absorption of B_{12} requires binding with the intrinsic factor (IF) which is secreted by the parietal cells in the stomach. The B_{12}-IF complex binds with specific receptors on the ileal mucosa, where the IF is dissociated and B_{12} is absorbed. This process is reported to be calcium-dependent [40]; and vitamin D is a major regulator of plasma calcium level by affecting its intestinal absorption and its mobilization from the bones. Alternatively, receptors involved in B_{12} absorption and its reuptake through the kidney tubules may influence the serum level of vitamin D metabolites. It has been suggested that the cubulin/megalin receptors in the renal tubules are involved in the reuptake of DBP from the renal filtrate, which may in turn affect the plasma levels of vitamin D metabolites, the major one of which is 25(OH)D [41].

Our study has several strengths; first we employed a large nationally representative sample to investigate the association between 25(OH)D level and folate or B_{12}. To our knowledge, none of the previous studies involved such a large and nationally representative sample. Second, our study comprised a very homogenous group of adolescents with a narrow age range, thus eliminating any age-dependent effects. Other studies are based on a sample with a much wider age range. For example the study by Mao et al had an age range of 31–104 years and have reported age-dependent effects on the association of 25(OH)D with folate and B_{12} [2]. Furthermore, the presence of the disease (diabetes) might also have influenced the outcome. Our study is based on normal healthy subjects. Third, we used markers for both short-term and long-term folate status (plasma and RBC folate) and showed that 25(OH)D is associated with both short-term and long-term folate status. Fourth, we controlled for a large number of covariates ranging from physical activity to sun exposure and demographic and anthropometric factors. Fifth, to our knowledge, this is the first study that investigated the association of free 25(OH)D with folate and B_{12} levels. Using both total 25(OH)D and free 25(OH)D to investigate the association of vitamin D with folate and B_{12} made our results more robust.

A limitation of our study is that we did not control for dietary intake for folate and B_{12}. This study was originally designed for the association between 25(OH)D and cognitive function. The major sources of folate are green leafy vegetables and folate fortified wheat products. The consumption of green leafy vegetables in this age group is minimal, particularly in Kuwait, and thus it is unlikely to be different in individuals with different 25(OH)D levels. As a part of this study, we obtained dietary data, on a smaller subsample, for calcium and vitamin D related foods, which included most foods with wheat products. In Kuwait, wheat products are the staple foods, and these are folate-fortified by the Government policy. All the wheat products are supplied by a central agency (Kuwait flour mills) which are consumed by almost the entire population. In our food frequency questionnaire, we had data available on all the different types of bread. On this limited sample (~200) we calculated the dietary folate intake which was not significantly different based on vitamin D status categories (data not shown). Therefore, it is unlikely that adjusting for dietary intake of folate would have affected our results. Similarly, none of the study subjects reported to be vegetarians or vegans, and thus it is unlikely that the observed association between 25(OH)D and B_{12} be affected by differences in the dietary intake of B_{12}, which comes mostly from animal sources. Finally, although the narrow age range of our study participants reduced the age-dependent differences in folate status, it limited the generalizability of our findings to other age groups.

In conclusion, the data from this study support our hypothesis of a positive association between plasma 25(OH)D levels and total and RBC folate levels. The data also support a positive association between 25(OH)D levels and B_{12} levels. Because of the public health implications of this association, properly designed large-scale randomized controlled trials are warranted to investigate the link between folate absorption and vitamin D.

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