

ASSESSMENTS OF VITAMIN B12 AND FOLIC ACID STATUS AMONG DISPLACED PREGNANT WOMEN ATTENDING ANTENATAL CARE CLINIC IN DOMIZ CAMP OF A SYRIAN REFUGEES /KURDISTAN REGION, IRAQ

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ABSTRACT

Background: Vitamin B12, folic acid and their relevant biomarkers are essential micronutrients for healthy fetal growth and development. Thus, deficiency in the levels of these vitamins and relevant biomarkers during pregnancy leads to many adverse outcomes such as neural tube and neurological problems for both mother and fetus.

Objective: The current research project was undertaken to detect the status of vitamin B12, Folic acid and relevant biomarkers such as Homocysteine, Methylmalonic acid and Holotranscobalamin among displaced pregnant women attending antenatal care in Domiz Camps Clinic of Syrian refugees.

Methods and Subjects: This cross-sectional research was performed at Syrian refugee's camps in Domiz- Duhok City, Kurdistan Region, Iraq, from February 2019 until June 2019. This study included 540 pregnant women whose ages ranged between 15-44 years. Participants were subdivided into three groups based on their trimesters. Each group included 180 pregnant women. Vitamin B12 and folic acid were measured by Automatic Clinical Chemistry Analyzer COBAS 6000 while Methylmalonic acid (MMA), Homocysteine (Hcy), and Holotranscobalamin (HoloTc) were measured by ELISA.

Results: The results of the current study revealed that the prevalence of vitamin B12 insufficiency (<200 pg/mL) was high across all three trimesters, which constitutes 73% in the total cohort. The mean vitamin B12 (pg/mL) during 1st semester was within the normal range (226.5± 6.95), whereas during the 2nd and 3rd semesters it was highly significantly (P<0.0001) low (179.25± 4.52 and 151.60±4.32, respectively). Prevalence of folic acid (ng/ml) insufficiency (<3.8 ng/mL) was 11.5% in total studied subjects and means of folic acid during the 3 semesters were 11.2± 0.41, 11.0± 0.41 and 9.2±0.41, respectively. Methylmalonic acid levels (umol/L) also showed a highly significant (P < 0.001) differences between the three semesters. The mean MMA levels during the three semesters were 0.130±0.003, 0.142±0.002, and 0.154± 0.003, respectively. Whereas homocysteine (umol/L) showed non-significant (P=0.2085) differences between all trimesters; and their levels were 7.6± 0.45, 8.0± 0.39, and 8.6± 0.42. respectively. Furthermore, Holotranscobalamin also showed a non-significant (P=0.883) difference between studied trimesters; and its level during the three trimesters was 137.4± 2.86, 135.1± 3.25 and 137.16± 4.20 pmol/l, respectively. From the total sum, 22.2% of the total pregnant women showed an increased MMA level (more than 0.280 umol/l), and 18.5% of the total participant showed an elevated level of Hcy (more than 15 umol/l). Whereas only 7.9% of 540 pregnant women showed low level of HoloTc less than 40 pmol/l. From the total cohort, only 7.03% showed combining lowered HoloTc level plus elevated MMA and Hcy level which indicates the manifestation of vitamin B12 deficiency during pregnancy.

Conclusion: From the results of the current study, it can be concluded that the levels of maternal Vit B12 and folic acid significantly decrease at the last trimesters. Vitamin B12 deficiency was prevalent in the studied subjects, while folic acid insufficiency was relatively uncommon. Furthermore, levels of HoloTc plus MMA and Hcy can be considered as an accurate diagnostic indicator during pregnancy.

1. Introduction

Pregnancy (gestational period) is a period of fetal growth and development, which requires increased nutrients (Landon *et al.*, 2018). A pregnant woman necessitates additional doses of almost all essential

vitamins, including vitamin D, iron, folic acid, and vitamin B₁₂, to maintain maternal metabolism and outcome (Kominiarek *et al.*, 2016; Mousa *et al.*, 2019). Vitamin B₁₂ also known as (cobalamin) is a micronutrient critical for the growth, differentiation, and maintenance of cells. In addition, it is essential for the synthesis of DNA, RNA, red blood cells production, neural myelination, lipids, and protein metabolism along with folic acid (Mahmood, 2014). Vitamin B₁₂ and folate are cofactors required to transform homocysteine to methionine (Saravanan *et al.* 2010, Lee *et al.*, 2016; Sukumar *et al.*, 2016). These two micronutrient deficiencies is caused due to malnutrition, malabsorption, alcoholism, and some medications (Barnabé *et al.*, 2015). Shortage in the concentration of folate and B₁₂ has been related with pregnancy complication such as spontaneous abortion (Sayyah-Melli *et al.*, 2016), preterm birth (Rogne *et al.*, 2017), preeclampsia (Mujawar *et al.*, 2011), neural tube defect (Imbard *et al.*, 2013), and hyper-homocysteinemia which is a risk factor for cardiovascular disease (Ganguly *et al.*, 2015). Additionally, deficiency of vitamin B₁₂ increase offspring insulin resistance and excess adiposity (Achebe *et al.*, 2017).

Insufficiency of folate and B₁₂ vitamins enhance public health problem worldwide. Many studies reported prevalence of vitamin B₁₂ deficiency for pregnant women in their countries such as 61% in Venezuela (Garcia-Casal *et al.*, 2005), 46% in South Korea (Park *et al.*, 2004), 51.1% in India (Samuel *et al.*, 2013), 32% in Nigeria (Vander Jagt *et al.*, 2011), 22.6% in USA (Finkelstein *et al.*, 2019), 73% in Turkey (Özdemir *et al.*, 2018), and 40% in the UK (Adaikalakoteswari *et al.*, 2015). Recently, Sukumar *et al.*, (2016) reported the pooled calculation of vitamin B₁₂ insufficiency (<200 pg./mL) was 21%, 19%, and 29%, respectively, in all three pregnancy trimesters.

Thus, the current study aimed to determine the status of vitamin B₁₂ and folic acid, and some relevant

biomarkers such as homocysteine, methylmalonic acid and Holotranscobalamin among displaced pregnant women attending antenatal care in Domiz camp clinic of Syrian refugees in Kurdistan Region, Iraq.

2. Subjects and Methods

2.1 Study Design and Data Collection

This cross-sectional research was performed at a Syrian refugees' camp in Domiz from February until June 2019. It comprised 540 pregnant women whose ages ranged from 15-44 years. Participants were subdivided into three groups, each of 180 pregnant women based on their trimesters. Methylmalonic acid (MMA), Homocysteine (Hcy), and Holotranscobalamin (HoloTc) were measured by ELISA, while Vitamin B₁₂ and folic acid were measured by Automatic Clinical Chemistry Analyzer (COBAS 6000). Complete blood counts (CBC) were measured by an Automated Coulter Counter (Swelab Alfa plus standard).

2.2 Biographic Measurement

Two types of questionnaire forms were prepared for Socio-demographic and specific information of pregnant women. The socio-demographic information includes the economic situation, educational level, and occupation of pregnant women, whereas specific information includes women's age, gestational age, women's parity, gravidity, abortion, and stillbirths' number.

2.3 Biochemical Measurements

About 5ml of venous blood was withdrawn from pregnant women by vena puncture after an overnight fasting, then the collected blood was transferred into vacutainer-tube. Blood was divided into two parts, 2ml of blood sample placed in the AYSET-EDTA tube to perform CBC, while the remaining 3ml blood placed into AYSET-GEL, (clot activator tube) for serum. The gel tube was left to clot at room temperature for 30-40 minutes. Then serum was separated by centrifugation using KOKUSAN (model H-19F JAPAN) at 2500 rpm

for 10 minutes. Later, the separated serum was stored in deep freeze (at -20 °C) using a 1ml BIO-RAD Eppendorf tube labelled numerically for later analysis. Finally, the stored serum for each woman could allowed to thaw at room temperature, and used for the determination of vitamin B₁₂, and folic acid levels using Automatic Clinical Chemistry Analyzer (COBAS 6000). Homocysteine, Methylmalonic acid (MMA), and Holotranscobalamin (HoloTc) were determined using ELISA kit (from Abbexa, Cambridge, UK.).

Serum vitamin B₁₂ and folic acid were analyzed using an auto-analyzer biochemical machine called COBAS e6000 (Roche, Germany) at Azadi teaching hospital / Duhok city.

Homocysteine (Hcy) level was determined in serum by Homocysteine ELISA Kit (Abbexa - United Kingdom), with the normal references range according to Abbexa kit is <15 umol/l. Methylmalonic acid by Methylmalonic acid ELISA Kit (Abbexa - United Kingdom), with the normal references ranged according to Abbexa kit from 0.0026 to 0.169 umol/l. Human serum Holotranscobalamin was determined by Holotranscobalamin ELISA Kit (Abbexa - United Kingdom), with references range according to Abbexa kit ranged 40-255 pmol/l. The references converted to pmol/l using the formula from Victor Herbert (1994) gold stander model.

The concentrations of the above parameters were determined according to the protocol supplied with each kit. At the end of assay procedure protocol, the sample optical density was measured at 450 nm using BioTek 800TS microplate reader (Winooski, VT, USA). The concentration of target protein in the sample was calculated via creating the best fit curve in Microsoft Excel, in which normal absorbance (X-axis) value can be plotted against the regular (Y-axis) concentration log as an XY scatter plot. The R-squared value must be higher than 0.95, and as close to 1 as possible. ELISA tests for MMA, Hcy, and HoloTc were done at Directorate of preventive health affairs /Duhok city.

3. Statistical Analysis

The data were analyzed using SPSS software for windows version 25 (SPSS INC, Chicago, IL, USA). Descriptive statistics were used to describe the basic features of the study. ANOVA was used to compare the means among more than two groups, also, to determine the statistically significant difference between groups, a P value of ≤ 0.05 was considered statistically significant. Regression analysis is used to determine the relationship between the set of independent and dependent variables. Correlation analysis was used to determine the relationship between the studied parameters. Two-way ANOVA was used for statistical analysis of changes during pregnancy. Finally, covariance was used for the measurement of the relationship between two random variables.

4. Results

The results of the comparison of B₁₂ and folic acid levels among participants from three different groups. Included. The biographic and biochemical characteristics of the total sample which are as follow:

4.1 Biographic Parameters

The results of biographic parameters analysis are shown in Table 4.1. Since the results of the educational levels are homogenous (3.3 ± 0.1 , 3.4 ± 0.1 , 3.5 ± 0.1 , respectively), it showed no difference between them. The same thing is true for the results of occupation for the three trimesters, with no differences between them since the results are very close (1.1 ± 0.04 , 1.1 ± 0.04 , and 1.1 ± 0.04 , respectively) to each other's. The socioeconomic status means also showed no significant differences between them ($P=0.336$), since most of the participants were not a full-time employee and graduated from primary or intermediate schools. The participants of the age groups for all trimesters were very close (26.1 ± 0.4 , 26.1 ± 0.4 , and 26.5 ± 0.4 , respectively) with no significant differences between them ($P=0.732$). Moreover, in the current study, level

of smokers was low.

(2.5%), while the level of alcohol addict was extremely low (only 0.1%). Likewise, 1.5% of the pregnant women have a history of congenital abnormality born baby with NTDs. Furthermore, 47.7% of the pregnant women were taking folic acid. In sum, 20.3% (110/540)

Table 4.1: Represents the biographic parameters of pregnant women included in the current study during the three trimesters.

Variables	Trimester 1 (N= 180)	Trimester 2 (N= 180)	Trimester 3 (N= 180)	P-value
Age Mean± SEM	26.1 ±0.42	26.1 ±0.45	26.5 ±0.44	0.732 ^a
Gestational age Mean± SEM	8.81 ±0.22	19.76 ±0.27	34.72 ±0.25	<0.0002 *
Parity Mean± SEM	1.70 ±0.10	1.72 ±0.11	1.66 ±0.09	0.900
Gravidity Mean± SEM	3.21 ±0.13	3.25 ±0.14	3.28 ±0.14	0.943
Abortion Mean± SEM	0.41 ±0.05	0.43 ±0.06	0.53 ±0.07	0.365
Stillbirth Mean± SEM	0.09 ±0.03	0.08 ±0.027	0.08 ±0.02	0.960
Educational level Mean± SEM	3.39 ±0.11	3.43 ±0.10	3.56 ±0.10	0.492
Occupation Mean± SEM	1.11 ±0.03	1.11 ±0.04	1.10 ±0.04	0.982
Socio economic status Mean± SEM	1.65 ±0.03	1.68 ±0.03	1.72 ±0.03	0.336
Smoking	12/180 (6.6%)	2/180 (1.1%)	0/180(0.0%)	<0.0001*
Alcohol ingestion	1/80 (0.5%)	0/180 (0.0%)	0/180 (0.0%)	0.358 ^a
Congenital abnormality	3/180 (1.7%)	3/180 (1.7%)	2/180 (1.1%)	0.881
Taking folic acid Supplementation	127/180(70.6%)	70/180(38.8%)	61/180(33.8%)	<0.0002*
Vegetarian diet	11/180 (6.1%)	5/180(2.7%)	2/180(1.1%)	<0.0002 *

* values less than 0.05 are considered as significant.

of the pregnant women had a history of abortion, whereas only 5% of total pregnant women had a history of stillbirth. From the overall participants, Primigravida, multigravida and grand multigravida are shown by 17.4%, 61.1% and 21.5%, respectively. While 30.3% of pregnant women had primipara, 45.0% (243) multipara, and only (3.7%) grand multipara. Besides, only 3.3% of the total study cohort were vegetarian.

4.2 The Biochemical Characteristics

The status of vitamin B₁₂, folic acid and relevant biomarkers in pregnant women during the three

trimesters

The results of table 4.2 represent the prevalence of Vit. B₁₂ insufficiency (<200 pg/ml) which is as high as 73% of the total studied participants. The mean vitamin B₁₂ level (pg/mL) was low at a highly significantly (P<0.0001) level during the second and third trimester, while during the 1st trimester, the level was normal (226.5± 6.95). While, during the 2nd and 3rd trimesters, the levels were 179.25± 4.52 and 151.60± 4.32, respectively. All women have normal mean level of HoloTc during the three trimesters which ranged between 135.1±3.25 and 137.4± 2.86 with no significant differences between them (P=0.883). In sum, among 540 pregnant women studied, 43 of the respondents demonstrated to have a low level of Holotranscobalamin and the level below the lower limit found in 7.96 % (<40 pmol/L) in total participants. The consequences of women during the three trimesters revealed normal mean levels of MMA (0.130±0.003, 0.142±0.002, and 0.154±0.003, respectively), with the presence of a highly significant (P = 0.0001) difference between them. In the total subjects, 22.2% of the pregnant women showed a high level of Methylmalonic acid. The measurement of Homocysteine level is very essential due to its close association with Vit B₁₂ and folic acid deficiency. As the results show, most women demonstrated low levels of homocysteine which ranged from 7.6± 0.45 and 8.6± 0.42 with no significant differences between them (P=0.2085). From sum, of 540 pregnant women, 18.5% of the respondents demonstrated a high level of Homocysteine. Pregnant women have normal mean levels of folate during the three trimesters which were 11.2± 0.41, 11± 0.41), and 9.2± 0.41), respectively.

Table 4.2: The status of vitamin B₁₂, folic acid and relevant biomarkers in pregnant women included in the current study during the three trimesters

Study Parameters	1 st : Trimester (N=180)	2 nd : Trimester (N=180)	3 rd : Trimester (N=180)	P-Value
B12 -pg/ml (M±SEM)	227±6.95	179±4.52	146±4.32	<0.0001*

HoloTc- pmol/l (M±SEM)	137.4±2.86	135±3.25	137.1±4.20	0.8830
MMA- umol/l (M±SEM)	0.130±0.003	0.142±0.002	0.154±0.003	<0.0001*
Hcy-umol/l (M±SEM)	7.6±0.45	8.0±0.39	8.6±0.42	0.2085
Folic acid- ng/ml (M±SEM)	11.2±0.41	11.0±0.41	9.2±0.41	<0.0001*

*(P<0.05) is considered as significant.

5. Discussion

It was hypothesized that B₁₂ and folic acid levels in pregnant women might be low due to pregnancy and socio-economic status. Nutritional deficiencies of micronutrients are prevalent among pregnant women in most developing countries (Youssry *et al.*, 2017). Physiologically, the level of vitamin B₁₂ was significantly reduced during pregnancy due to hormonal changes, increased blood hemodilution, active vitamin transport to the fetus, a decline in albumin, increased glomerular filtration, and changes in vitamin B₁₂-binding protein capacity and saturation (Greibe *et al.*, 2011; Khan *et al.*, 2018; Morkbak *et al.*, 2007; Obeid *et al.*, 2017). The prevalence of vitamin B₁₂ insufficiency (<200 pmol/L) was high across all three trimesters in the present study which was 73% of the total subjects.

The reduction of gestational maternal vitamin B₁₂ level in the current study is like those reported for pregnant women in other countries such as 73% in Turkey (Özdemir *et al.*), 61% in Venezuela (Garcia-Casal *et al.*, 2005), 51.1% in India (Samuel *et al.*, 2013) and 46% in South Korea (Park *et al.*, 2004). Whereas a much lower levels had been recorded in other countries such as 32% in Nigeria (VanderJagt *et al.*, 2011), 22.6% in USA (Finkelstein *et al.*, 2019), and 40% in the UK (Adaikalakoteswari *et al.*, 2015). Recently Sukumar *et al.*, (2016) reported that the pooled calculation of vitamin B₁₂ insufficiencies (<200 pg/mL) were 21%, 19%, and 29%, respectively, during the three trimesters. Since it was pointed out in previous studies that the measurement of B₁₂ concentration in pregnancy might give false-positive results. Therefore,

in the current study it was decided to analyze the levels of Homocysteine (Hcy), Methylmalonic acid (MMA), and Holotranscobalamin (HoloTc). Combining elevated levels of both biomarkers MMA and Hcy plus lowered HoloTc level confirms vitamin B₁₂ deficiency during pregnancy (Herrmann *et al.*, 2012; Obeid, 2017).

Holotranscobalamin (HoloTc), also known as an active B₁₂, represents the earliest laboratory parameter for vitamin B₁₂ deficiency during pregnancy. Circulating level of HoloTc remain stable during pregnancy, and for this reason, HoloTc is regarded as a better marker than direct total vitamin B₁₂ measurement for monitoring and screening status of cobalamin during pregnancy (Greibe *et al.*, 2011; Morkbak *et al.*, 2007; Murphy *et al.*, 2007). The findings of the present study confirm the same since normal means were reported for all three trimesters (137.4± 2.86, 135.1± 3.25 and 137.16± 4.20, respectively) with no significant differences between the studied groups ((P=0.883). While, among 540 pregnant women included in the current study, 7.9% of respondents demonstrated a low level of Holotranscobalamin (below the lower limit of the reference levels (<40 pmol/L). Also, the results showed a significant positive correlation with vitamin B₁₂ (P<0.05 to 0.0001). Whereas regression results showed no significant difference between the measurements of B₁₂, and HoloTc (P= 0.209; P > 0.05). This means that the independent variables do not reliably predict the dependent variable. Thus, it fails to reject the null hypothesis and supports the idea of false-positive results of direct vit. B₁₂ testing for pregnant women.

Consequently, the pregnancy-related decline in direct cobalamin measurement has resulted from changes in vitamin B₁₂-binding protein via alternation in cobalamin attached to haptocorrin (HC) rather than alternation in Holotranscobalamin (HoloTc) level, and with changing their saturation during pregnancy with decreased HC saturation and increased HoloTc

(Greibe *et al.*, 2011; Morkbak *et al.*, 2007; Murphy *et al.*, 2007). The metabolic biomarkers Methylmalonic acid and Homocysteine are sensitive, and functional B₁₂ marker will rise when the B₁₂ stores depleted (Green *et al.*, 2017; Herrmann *et al.*, 2012). Since vitamin B₁₂ is transported into cells and used as a cofactor for two enzymes: Methylcobalamin (CH₃-B₁₂) is a cofactor for the cytosolic enzyme methionine synthase that convert Homocysteine to methionine. Adenosylcobalamin (Ado-B₁₂) which is the cofactor for the mitochondrial enzyme methylmalonyl-CoA mutase that convert methylmalonyl-CoA to succinyl-CoA. Cobalamin insufficiency will impair these enzymes' proper function and lead to the accumulation of the biomarkers in the blood (Herrmann *et al.*, 2012; Obeid, 2017; Sharma *et al.*, 2019; Wolffenbittel *et al.*, 2019). Therefore, both metabolic biomarkers must be measured due to their close relationship with B₁₂ deficiency in pregnancy for further confirmation. Together with MMA and Hcy also showed normal mean for all the three trimesters in the present study Methylmalonic acid level ranged from 0.130±0.003 to 0.154±0.003 during the three trimesters. Homocysteine level ranged from 7.6±0.45 to 8.6±0.42). Both normal means of biomarker levels showed that it is more likely the direct reading of cobalamin concentration in pregnancy gave a false-positive result. The reduction of cobalamin is due to changes in vitamin B₁₂-binding protein (Haptocorrin and Holotranscobalamin) and its saturation. Whereas regression results showed that the presence of significance differences between the levels of B₁₂, MMA, and Hcy (P <0.000 to P <0.05). The findings of the present study showed increased levels of both biomarkers MMA, and Hcy through the latter phases of pregnancy compared with the first trimesters. These results agree with those of other scholars (Choi *et al.*, 2016; Green *et al.*, 2017; Greibe *et al.*, 2011; Obeid *et al.*, 2017). This may be an indicator for the depletion of maternal intracellular cobalamin stores (negative B₁₂ balance), possibly because of

increased metabolic rate, superior transfer to the fetus, an increase in the serum creatinine, and do not take folic acid since Hcy level also raised in clinical folate deficiency. Finally, among 540 pregnant women, 7.03% of the participant displays combining elevated levels of both biomarkers MMA (>0.70 umol/L), and Hcy (>15 umol/L), plus a lowered level of Holotranscobalamin (<40 pmol/L), which are diagnostic indicator for cobalamin deficiency that confirms vitamin B₁₂ deficiency in pregnancy (Hvas *et al.*, 2005).

Socioeconomic factors such as maternal education, poverty, and job status might be related to nutrient adequacy. Conversely, no significant differences were observed between maternal education, occupation status, and the deficiency of folic acid and B₁₂ levels in this study. According to socioeconomic status, participants from a non-sufficient group the normal levels of B₁₂ and folic acid were observed 29.7% and 32.3%, while in the sufficient group their normal levels were observed in 70.3% and 68.7%, respectively. These results agree to a large extent with those of Vilamor *et al.*, (2008) who reported that vitamin B₁₂ was positively associated with amount of money spent on food per day. This is also further supported by the results of Shahab-Fordows *et al.*, (2015) who found that vit B₁₂ deficiency is more common among poor and rural regions. Furthermore, participants demonstrated normal mean levels of folic acid across all three trimesters which ranged from 9.2±0.41 to 11.2±0.41. This was also correlated to their intake of folate supplements. Thus only 11.50% (<3.8 ng/ml) of the pregnant women show decreased levels of folic acid. Although most respondents were from low socioeconomic status, in general, there were no deficiencies in folic acid, which could be attributed to the intake of folic acid supplements. Consequently, the hypothesis claiming that females with low SES usually suffer from B₁₂ and folic acid deficiency was partially rejected. In addition, since most respondents were belonging to

SES group and taking folic acid supplements, their B₁₂ and folic acid levels remained normal.

6. Conclusion

It can be concluded from the results of the current study that the levels of maternal cobalamin and folic acid significantly decrease at the latter stage of pregnancy. Vitamin B₁₂ insufficiency was prevalent in the studied population, while folic acid insufficiency was relatively uncommon. Furthermore, measurement of HoloTc plus MMA and Hcy showed superior diagnostic accuracy compared to direct vitamin B₁₂ measurement in detecting B₁₂ deficiency in pregnancy. Thus, vitamin B₁₂ supplementation, together with folic acid, should be reconsidered.

7. References

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